Acta Medica Alanya

RESEARCH ARTICLE

ARAŞTIRMA

Acta Medica Alanya

2021;5(2): 164-170

DOI:10.30565/medalanya.939161

Ramelteon used to treat insomnia can reduce the occurrence of osteoporosis

Uykusuzluğu tedavi etmek için kullanılan Ramelteon, osteoporoz oluşumunu azaltabilir

Duygu Kose[1*], Ahmet Kose[2], Zekai Halici[1], Muhammet Ali Gurbuz[3], Adem Maman[4], Muhammed Yayla[5]

1.Department of Pharmacology, Ataturk University, Faculty of Medicine, 25240 Erzurum-Turkey
2.Department of Orthopedics and Traumatology, Health Science University, Erzurum-Turkey
3.Departments of Histology and Embryology, Ataturk University, Faculty of Medicine, Erzurum-Turkey
4.Department of Nuclear Medicine, Ataturk University, Faculty of Medicine, Erzurum-Turkey
5.Department of Pharmacology, Kafkas University, Faculty of Medicine, Kars-Turkey

ABSTRACT

Aim: Melatonin promoted osteoblast differentiation and causes an increase in levels of markers of bone differentiation and proliferation. Ramelteon (RAMEL) activates melatonin receptors and binds to these receptors as non-selective. In this study, we investigated the preventive effects of the melatonin agonist RAMEL on osteoporosis by radiological, histological, and molecularly.
Methods: Groups 1: Control, Group 2: Osteoporosis: Overectomized group (OP), Group 3: OP + ramelteon 2 mg/kg, Group 4: OP + ramelteon 4 mg/kg. 24 animals underwent bilateral ovariectomy. RAMEL was administered orally once a day in the prophylactic treatment mode for 8 weeks, 6 weeks after ovariectomy.
Results: Fourteen weeks after ovariectomy, there was a significant reduction in femoral bone mineral density (BMD) (g/cm2) in the OP group compared to the control group. Compared to the OP group, RAMEL treatment significantly increased the BMD level ($p<0.05$). Bone matrix protein 2 (BMP2) and runt-related transcription factor 2 (RUNX2) mRNA levels were significantly lower in the OP group than in the control group ($p<0.05$). RUNX2 and BMP2 mRNA levels were significantly higher in the RAMEL treatment groups than in the OP group ($p<0.05$).
Conclusion: To take advantage of the peripheral effects of melatonin, RAMEL, a peripheral melatonin agonist, can be used to prevent osteoporosis.

Keywords: Ramelteon, melatonin, osteoporosis, bone

ÖZ

Amaç: Melatonin osteoblast farklılaşmasını teşvik eder ve kemik farklılaşması ve proliferasyon belirteçlerinin düzeylerinde artışa neden olur. Ramelteon (RAMEL) melatonin reseptörlerini aktive eder ve bu reseptörlere seçici olmayan şekilde bağlanır. Bu çalışmada melatonin agonisti RAMEL' in osteoporoz üzerindeki önleyici etkileri radyolojik, histolojik ve moleküler olarak araştırıldı.
Yöntem: Grup 1: Kontrol, Grup 2: Osteoporoz: Over eksizyonu yapılan grup (OP), Grup 3: OP + ramelteon 2 mg/kg, Grup 4: OP + ramelteon 4 mg/kg. 24 hayvana, bilateral ovariektomi uygulandı. RAMEL, ovariektomiden 6 hafta sonra, 8 hafta boyunca profilaktik tedavi modunda günde bir kez oral yoldan uygulandı.
Bulgular: Ovariektomiden on dört hafta sonra, kontrol grubuna kıyasla OP grubunda femoral kemik mineral yoğunluğunda (g/cm2) anlamlı bir azalma oldu. OP grubu ile karşılaştırıldığında, RAMEL tedavisi BMD seviyesini önemli ölçüde artırdı ($p<0.05$). Bone matrix protein 2 (BMP2) ve runt-related transcription factor 2 (RUNX2) mRNA seviyeleri OP grubunda kontrol grubuna göre anlamlı derecede düşüktü ($p<0.05$). RUNX2 ve BMP2 mRNA seviyeleri, RAMEL tedavi gruplarında OP grubuna göre anlamlı olarak daha yüksekti ($p<0.05$).
Sonuç: Melatoninin periferik etkilerinden yararlanmak için, bir periferik melatonin agonisti olan RAMEL, osteoporozu önlemek için kullanılabilir.

Anahtar Kelimeler: Ramelteon, melatonin, osteoporoz, kemik

Received: 19.05.2021 Accepted: 15.06.2021 Published (Online): 30.08.2021

*Corresponding Author: Duygu Kose, MD, Department of Pharmacology, Faculty of Medicine, Atatürk University 25240 Erzurum-Turkey; Tel: + 90 442 2318738, E-mail: duygum.46@hotmail.com

ORCID: 0000-0002-3468-1567

To cited: Köse D, Köse A, Halıcı Z, Gürbüz MA, Maman A, Yayla M. Ramelteon used to treat insomnia can reduce the occurrence of osteoporosis. Acta Med. Alanya 2021;5(2): 164-170 doi:10.30565/medalanya.939161

INTRODUCTION

Melatonin secretion during the night from the pineal gland has positive effects on bone in various aspects [1]. Melatonin promoted osteoblast differentiation and causes an increase in levels of markers of bone differentiation and proliferation, including increased synthesis of osteopontin, bone alkaline phosphatase, osteocalcin, a bone matrix protein 2 (BMP2) and runt-related transcription factor 2 (RUNX2) [2-5]. It has been shown that melatonin has a beneficial effect on osteoporosis after ovariectomy in rats [6] and promotes fracture healing [7].

Melatonin receptors (MT) have been identified in three subgroups. These receptors are MT1, MT2 and the recent MT3 [8]. MT1 and MT2 are membrane receptors and most effects of melatonin is mediated by these receptors [9,10] MT1 and MT2 expressions were seen in peripheral tissues and cells and contribute to several immune and vasomotor effects [11]. MT1 mainly mediates vasoconstriction, whereas MT2 mainly causes vasodilatation. However, tissue distributions and the roles of MT3 receptors are not yet fully understood. Several studies have shown that MT2 receptors are responsible for the effects of melatonin on the bone [2,12].

Ramelteon (RAMEL) activates both MT1 and MT2 melatonin receptors. In the United States, FDA approved it in 2005 for the treatment of insomnia and for individuals who have difficulty falling asleep [13]. RAMEL has a higher affinity for both receptor subtypes [13].

Many experimental studies in the current literature have shown several positive effects of melatonin in human bone proliferation and differentiation [2,14,15,16,17]. But long-term use of melatonin does not seem to be possible in the treatment of osteoporosis because of side effects resulting from hormonal stress on the chronological rhythm. Therefore, the current study investigated preventing osteoporosis using RAMEL, which was administered orally once daily in a preventive treatment mode for 8 weeks, 6 weeks after ovariectomy operation. To test this therapeutic strategy, improvement was assessed by Dual-energy X-ray absorptiometry (DEXA) and radiological analysis; in histopathology, hematoxylin-eosin (H&E) staining was evaluated, and molecularly, expression BMP2 and RUNX2 genes were measured with real-time PCR (Real-Time PCR).

MATERIALS AND METHODS

Animals

32 female albino Sprague-Dawley (10-12 weeks old) rats were provided from Atatürk University (ATADEM) Medical Experimental Research Center. The animals weighed were between 240 and 260 g and were divided into four equal groups. Experiments were performed according to the standard experimental procedures to keep normal temperature conditions (22 °C). Animal experiments were performed according to International Guidelines. This experiment was approved by the Institutional Animal Care and Use Committee of Ataturk University (2019/E.1900179336). During the experiment, the animals were kept under controlled light conditions (12 h light/dark cycle) and air-conditioned room conditions at 22 °C. Standard rat chow and tap water were provided ad libitum.

Chemicals

Ramelteon (Ramelda©), Ketamine (Ketalar 500 mg/10 ml), xylazine (Basilazin %2), and Metamizole sodium (500 mg/ml) were purchased from Abdi Ibrahim, Pfizer, Biotek, Sanofi-Aventis, respectively.

Surgical Procedures

The rats were given a diet of standard commercial rat pellets. Twenty-four animals underwent bilateral ovariectomy [18]. For this procedure, animals were anesthetized intraperitoneally with 80 mg/kg ketamine + 8 mg/kg xylazine. After making a longitudinal incision (0.5-1 cm) in the midline region of the lower abdomen, the ovaries were removed with a small peritoneal incision. Metamizole sodium was administered for postoperative pain control.

Drug administration

After surgical procedures, the ovariectomized rats were randomly divided into the following three groups:

Group 1: Control: Sham-operated group

Group 2: OSTEOPOROSIS (OP); Ovariectomized control group

Group 3: Ovariectomized and 2 mg / kg ramelteon administered (in 1 ml dH2O) (RAMEL2)

Group 4: Ovariectomized and 4 mg / kg ramelteon administered (in 1 ml dH2O) (RAMEL4)

Drug administration was started 6 weeks after ovariectomy surgery. All the drugs were given orally once per day and for eight weeks.

Dual-energy X-ray absorptiometry (DEXA) estimations

Bone mineral density (BMD) was analyzed by DEXA using Discovery Wi (Hologic Inc., Bedford, MA, USA). The same investigator performed each measurement and all analysis were performed using the same GY region (ROI) window size.

Histopathological Analysis

Decalcification and Tissue Processing Procedure

Following the surgical procedure, femoral samples were kept in 10% formalin solution for fixation. The bones were kept for 7-10 days in 8% hydrochloric acid - 8% formic acid solution (mixed in equal volume) with a total volume of 10 times the tissue for decalcification. The decalcification solution was renewed every day and the distal end of the femur bones was perforated with a needle to check whether the decalcification process was over. To neutralize the decalcified bone tissues, soaking processes were applied in tapping water (30 minutes) - ammonium solution (5 drops / 100 ml - 30 minutes) and tapping water (12 hours), respectively. The proximal end of the femoral bones was cut with a lancet and placed in tissue cassettes for tissue processing. The tissue processing procedure was performed by passing the tissues through increasing alcohol (70% -80% -90%-absolute), xylol, and paraffin series, respectively, using the Leica TP 1050 device. Manually, using molten paraffin, tissues were formed into blocks and five sections were taken from each block onto positively charged slides with the aid of the Leica RM 2145 microtome device. Two of the sections were used for Hematoxylin Eosin (H&E) staining.

H&E Staining Procedure: Slides were kept in an EN 055 model incubator for 5 minutes at 60o and 30 minutes in xylene for fixation and deparaffinization. To hydrate the tissues, the sections were kept for three minutes in two series of xylol, decreasing alcohol series (96% -90% -80% -70% -50%) and under tapping water. The tissues were colored by hematoxylin staining (5 minutes), soaking in tapping water (5 minutes), eosin staining (2 minutes), and then the mounting process was applied with xylene, entellan and coverslip.

Monitoring and evaluation: H&E preparations were analyzed and recorded using an Olympus BH 40 light microscope and integrated camera. In the analysis stage of H&E stained preparations under a light microscope, the following criteria were taken into account: old bone mass; trabecula/ spicule thickness; alveolar volume, and new bone formation [20,21].

Molecular Studies

RNA extraction: 20 mg bone tissues were homogenized with Tissue Lyser II (Qiagen) using liquid nitrogen. Total RNA extracted according to the RNAeasy mini kit directions in QIAcube RNA isolation system. Total mRNA amounts were determined by using nanodrop spectrophotometry (EPOCH Take 3 Plate, Biotek) at 260/280 nm and then was stored at -80 ° C until the experiment.

cDNA Synthesis: High Capacity cDNA Reverse Transcription Kit was used for cDNA synthesis. 10µl RNA and 10 ul master mix were used in each reaction. After the reaction, the amount of cDNA was determined by nanodrop spectrophotometry (EPOCH Take3 Plate, Biotek) and stored at -20 °C until the experiment.

Reaction volume

10 µl total RNA 2 µl 10 X RT Random Primers

2 µl 10 X RT Buffer 1 Multil MultiScribe Reverse Transcriptase

0.8 µl 25 X dNTPs mix 4.2 µl diethylpyrocarbonate H2O.

Quantitative Determination of RUNX2 and BMP2mRNA Expressions

RUNX2 and BMP2 mRNA expression was performed by Real-Time PCR. Bactin was used as the reference gene. All procedures were performed using the Step One Plus Real-Time PCR System (Applied Biosystems). The following TaqMan® Gene Expression Assays for 200ng cDNA were continued by pipetting as described below for 40 cycles. Obtained Ct values were automatically converted into ΔΔCt [19].

Reaction Volume; 9 µl cDNA (200ng); 10 µl TaqMan Master Mix; 1 µl Assay

Statistical methods: One-way (ANOVA) post hoc Duncan's tests were performed to comparing between the groups in IBM SPSS 25.00 packet program. Means±standard deviation was used for analyses and P<0.05 was evaluated as statistically significant.

RESULTS

BMD analyses: Forty-two days after the ovariectomy, femoral BMD (g/cm2) levels were significantly decreased in the OP group when compared to the control group (Fig. 1 and 2). When compared to the OP group, RAMEL administration significantly improved BMD levels in 4 mg/kg dose (p<0,05).

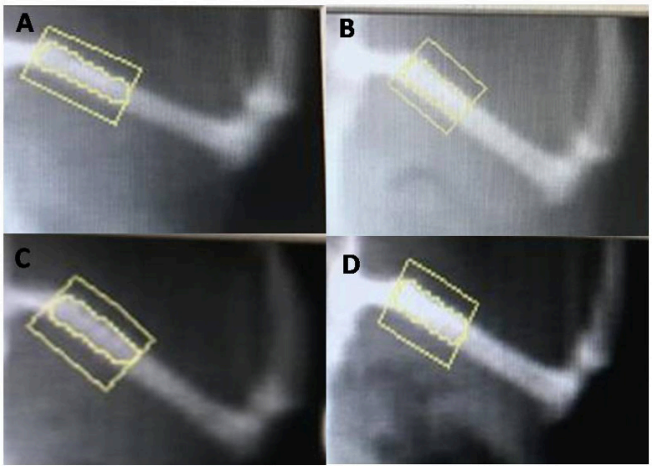

Figure 1: Left femoral bone DEXA images of SHAM, OSTEOPOROSIS, and RAMEL groups. A; SHAM, B; OSTEOPOROSIS, C; OP+RAMEL2, D; OP+RAMEL4. (Abbreviations: RAMEL: Ramelteon, OP: Osteoporosis, DEXA: Dual-energy X-ray absorptiometry)

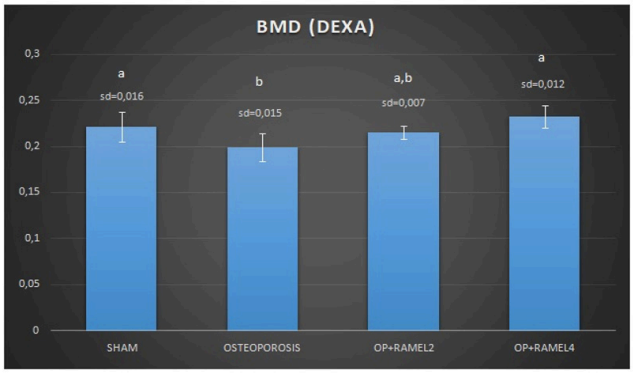

Figure 2: BMD measured with DEXA results of SHAM, OSTEOPOROSIS, and RAMEL groups. This means which have the same letter are not significantly different to the test of Duncan (p=0.05). Results are expressed as mean±SD. This means which have the same letter are not significantly different to the test of Duncan (p=0.05). Abbreviations: RAMEL: Ramelteon, OP: Osteoporosis, DEXA= Dual-energy X-ray absorptiometry, BMD=Bone mineral density, SD= Standard deviation

Real-Time PCR results

RUNX2 and BMP2 mRNA levels: Using Real-Time PCR, we investigated RUNX2 and BMP2mRNA expressions in the rats' femur bone tissue. RUNX2 and BMP2 mRNA levels were significantly lower in the OSTEOPOROSIS group when compared to the SHAM group (p<0.05) (Fig. 3 and 4). In contrast, RUNX2 and BMP2 mRNA levels were significantly higher in the RAMEL treatment groups when compared to the OSTEOPOROSIS group (p<0.05).

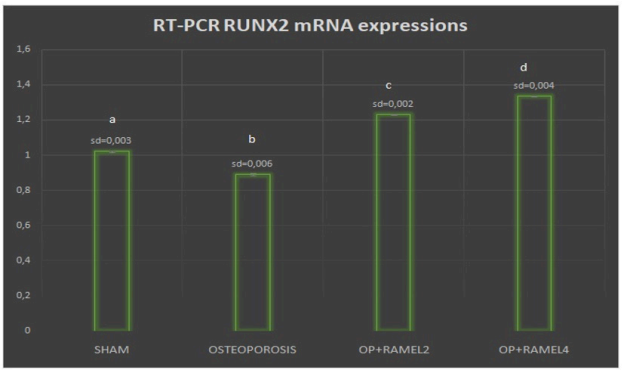

Figure 3: Relative mRNA expression levels of RUNX 2 in femur bone tissue of SHAM, OSTEOPOROSIS, and RAMEL groups. Expression of RUNX 2 was detected by quantitative Real-Time PCR analysis. The relative expression levels were calculated by the 2 (-ΔΔCT) method. β-Actin was used as the reference gene. Results are expressed as mean±SD. This means which have the same letter are not significantly different to the test of Duncan (p=0.05). (Abbreviations: RAMEL: Ramelteon, OP: Osteoporosis, SD= Standard deviation.)

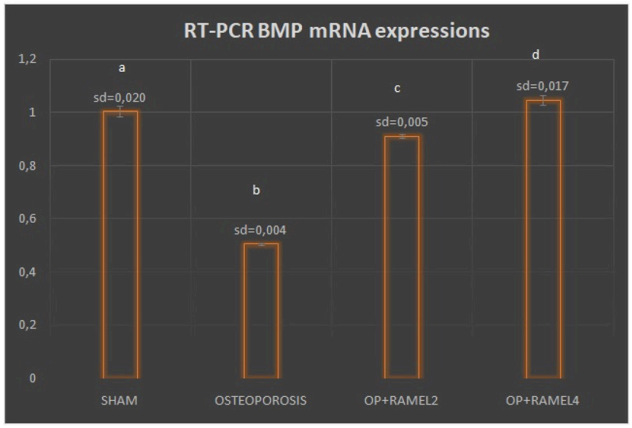

Figure 4: Relative mRNA expression levels of BMP in femur bone tissue of SHAM, OSTEOPOROSIS, and RAMEL groups. Expression of BMP was detected by quantitative Real-Time PCR analysis. The relative expression levels were calculated by the 2(−ΔΔCT) method. β-Actin was used as the reference gene. Results are expressed as mean±SD. This means which have the same letter are not significantly different to the test of Duncan (p=0.05). Abbreviations: RAMEL: Ramelteon, OP: Osteoporosis, SD= Standard deviation.

Histopathology Results

H&E staining results: For evaluation, the frontal sections of the rat femur bones, the head and neck parts that are expected to be most affected by osteoporosis were analyzed.

SHAM: The general appearance of the compact (CB) and trabecular bone (TB) lines and bone marrow (BM) is normal. The thickness of the trabecular bone spicules, the dimensions of the alveolar spaces, and the integrity of the compact bone are similar to those of healthy animals (Fig. 5a).

OP: More observed total bone loss in trabecular bone compared with the sham group. Loss of bone tissue continuity has been observed in some parts of the trabecular bone due to extensive damage (triangles). There is increased osteoclast activity (curved arrow). (Fig. 5b)

OP + RAMEL2: New ossification areas draw attention in the cortical and trabecular bone line (arrowheads). The more intense eosinophilic staining of the bone tissue adjacent to the bone marrow, compared to the other parts, indicates that the osteoid accumulation in these areas is high. Callus tissues associated with the cortical bone have been identified. Compared to the OP + RAMEL4 group, it was evaluated that a low drug dose increased the ossification rate more (Fig. 5c).

OP + RAMEL4: Pale eosinophilic appearance indicates a low level of osteoid accumulation in new bone formation areas. It can be stated that the bone development rate is slower compared to the OP + RAMEL2 group. Although there are new ossification zones in the cortical and trabecular bone line (arrowheads), it was observed that bone damage due to osteopenia continued (triangles) (Fig. 5d).

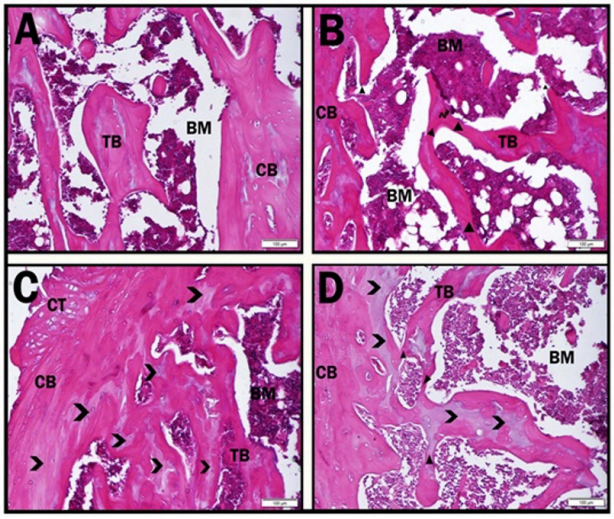

Figure 5. Effects of ramelteon in rats exposed to osteoporosis through ovariectomy. H&E staining findings in osteoporosis in femur bone tissue of SHAM, OSTEOPOROSIS, and RAMEL groups. A; SHAM, B; OSTEOPOROSIS, C; OP+RAMEL2, D; OP+RAMEL4. (Abbreviations: CB: Cortical bone, TB: Trabecular Bone, BM: Bone Marrow CT: Callus Tissue, Triangle: Possible bone resorption areas, Curved Arrow: Osteoclastic activity areas, Arrow Head: New bone formation areas, RAMEL: Ramelteon, OP: Osteoporosis)

To better understand the H&E staining findings, the above findings are summarized in the table below (tab 1) [20,21]. Osteoporosis leads to a decrease in old bone mass, consequently it should be taken into account that it causes a decrease in trabecular / spicule thickness and an increase in alveolar volume.

Table 1: H&E staining scores in femur bone tissue of SHAM, OSTEOPOROSIS, and BOSE groups.

GROUPS	old bone mass	trabecula/ spicule thickness	alveolar volume	new bone formation
SHAM	+++	++	++	+
OSTEOPOROSIS	++	+	+++	+
OP+RAMEL2	+	+++	+	+++
OP+RAMEL4	++	++	++	++

Grade 0: - (0% negative), Grade 1: +(0–33% mild positive), Grade 2: ++

(33–66% moderate positive), Grade 3: +++(66–100% severe positive). H&E: Hematoxylin-Eosin Staining, OP: Osteoporosis, RAMEL: Ramelteon

DISCUSSION

Melatonin promotes osteogenesis by activating MT2, increasing bone alkaline phosphatase, osteocalcin, RUNX2, and BMP2 [5]. Melatonin has a beneficial effect on bone physiology at therapeutic doses and increased bone alkaline phosphatase and osteocalcin levels [7]. Bone alkaline phosphatase, osteocalcin and osteopontin are produced from osteoblasts and have critical roles in osteoblastic bone formation and play a role in the balance of calcium ions and bone mineralization. Melatonin has osteoblast-inducing effects by inducing RUNX2, inhibiting osteoclast formation and osteoclast differentiation [2]. Melatonin supports osteoblastic differentiation through the BMP signaling pathway [3]. Our study supports these studies in the literature.

We examined RUNX2 and BMP2 expressions [2,3]. We observed that the expressions of RUNX2 and BMP2 decreased in the OSTEOPOROSIS group compared to the SHAM group and increased with RAMEL treatment. We found that RAMEL, a peripheral melatonin agonist, increased RUNX2, and BMP2 in the RAMEL treatment groups compared to the OSTEOPOROSIS group, just like melatonin.

In histopathologic analyses, decreased bone mass and trabecula/spicule thickness and increased alveolar volume with OSTEOPOROSIS improved with RAMEL treatment. Old bone mass and new bone formation increased significantly in the RAMEL treatment groups compared to the OSTEOPOROSIS group. Especially new bone formation is evident in OP + RAMEL4 group.

Although the constructive effect of exogenous melatonin against osteoporosis is known, it is not possible to use it for a long period of time [6,15]. To take advantage of the peripheral effects of melatonin, RAMEL, a peripheral melatonin agonist, can be used to prevent osteoporosis. RAMEL may be the first-choice drug in patients with both insomnia and osteoporosis risk.

Limitations of the study: We had only two RAMEL treatment groups due to the small number of animals.

Conclusion: As a result of our study, the protective effect of RAMEL on osteoporosis formation was demonstrated by radiographic, histopathological and molecular analyzes, in the experimental osteoporosis model in rats. The protective effects of RAMEL in osteoporosis were enhanced by molecular analysis of RUNX2 and BMP2, and radiologically by DEXA (BMD). In conclusion, we suggest that RAMEL, a routinely approved drug, reduces the occurrence of osteoporosis. However, more clinical and experimental studies are needed to clarify the effects of RAMEL on osteoporosis in more detail.

Conflict of Interest: The author has no conflict of interest related to this article.

Funding sources: The author declared that this study has received no financial support.

Ethics Committee Approval: the Institutional Animal Care and Use Committee of Ataturk University 2019/E.1900179336

Peer-review: Externally and internally peer-reviewed.

REFERENCES

1. Yildirimturk S, Batu S, Alatli C, Olgac V, Firat D, Sirin Y. The effects of supplemental melatonin administration on the healing of bone defects in streptozotocin-induced diabetic rats. J Appl Oral Sci. 2016; 24:239-49. DOI: 10.1590/1678-775720150570
2. Maria S, Samsonraj RM, Munmun F, Glas J, Silvestros M, Kotlarczyk MP et al. Biological effects of melatonin on osteoblast/osteoclast cocultures, bone, and quality of life: Implications of a role for MT2 melatonin receptors, MEK1/2, and MEK5 in melatonin-mediated osteoblastogenesis. J Pineal Res. 2018; 64. DOI: 10.1111/jpi.12465
3. Park KH, Kang JW, Lee EM, Kim JS, Rhee YH, Kim M, Jeong SJ, Park YG, Kim SH. Melatonin promotes osteoblastic differentiation through the BMP/ERK/Wnt signaling pathways. J Pineal Res. 2011; 51:187-94. DOI: 10.1111/j.1600-079X.2011.00875.x
4. Hakanson DO, Bergstrom WH. Pineal and adrenal effects on calcium homeostasis in the rat. Pediatr Res. 1990; 27:571-3. DOI: 10.1203/00006450-199006000-00006
5. Li T, Jiang S, Lu C, Yang W, Yang Z, Hu W, Xin Z, Yang Y. Melatonin: Another avenue for treating osteoporosis? J Pineal Res. 2019; 66:e12548. DOI: 10.1111/jpi.12548
6. Uslu S, Uysal A, Oktem G, Yurtseven M, Tanyalcin T, Basdemir G. Constructive effect of exogenous melatonin against osteoporosis after ovariectomy in rats. Anal Quant Cytol Histol. 2007; 29:317-25. PMID: 17987812
7. Halici M, Oner M, Guney A, Canoz O, Narin F, Halici C. Melatonin promotes fracture healing in the rat model. Eklem Hastalik Cerrahisi. 2010; 21:172-7. DOI: 10.1159/000492576
8. Conway S, Canning SJ, Howell HE, Mowat ES, Barrett P, Drew JE, Delagrange P, Lesieur D, Morgan PJ. Characterization of human melatonin mt(1) and MT(2) receptors by CRE-luciferase reporter assay. Eur J Pharmacol. 2000; 390:15-24. DOI: 10.1016/s0014-2999(99)00914-0
9. Reppert SM, Weaver DR, Ebisawa T. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron. 1994; 13:1177-85. DOI: 10.1016/0896-6273(94)90055-8
10. Dubocovich ML, Rivera-Bermudez MA, Gerdin MJ, Masana MI. Molecular pharmacology, regulation, and function of mammalian melatonin receptors. Front Biosci. 2003; 8:d1093-108. DOI: 10.2741/1089
11. Dubocovich ML, Markowska M. Functional MT1 and MT2 melatonin receptors in mammals. Endocrine. 2005; 27:101-10. DOI: 10.1385/ENDO:27:2:101
12. Sharan K, Lewis K, Furukawa T, Yadav VK. Regulation of bone mass through pineal-derived melatonin-MT2 receptor pathway. J Pineal Res. 2017; 63. DOI: 10.1111/jpi.12423
13. McGechan A, Wellington K. Ramelteon. CNS Drugs. 2005; 19:1057-65; discussion 66-7. DOI: 10.2165/00023210-200519120-00007

14. Hardeland R. Melatonin in aging and disease -multiple consequences of reduced secretion, options, and limits of treatment. Aging Dis. 2012; 3:194-225. PMID: 22724080

15. Chen W, Chen X, Chen AC, Shi Q, Pan G, Pei M, Yang H, Liu T, He F. Melatonin restores the osteoporosis-impaired osteogenic potential of bone marrow mesenchymal stem cells by preserving SIRT1-mediated intracellular antioxidant properties. Free Radic Biol Med. 2019. DOI: 10.1016/j.freeradbiomed.2019.10.412

16. Amstrup AK, Sikjaer T, Heickendorff L, Mosekilde L, Rejnmark L. Melatonin improves bone mineral density at the femoral neck in postmenopausal women with osteopenia: a randomized controlled trial. J Pineal Res. 2015; 59:221-9. DOI: 10.1111/jpi.12252

17. Kotlarczyk MP, Lassila HC, O'Neil CK, D'Amico F, Enderby LT, Witt-Enderby PA, Balk JL. Melatonin osteoporosis prevention study (MOPS): a randomized, double-blind, placebo-controlled study examining the effects of melatonin on bone health and quality of life in perimenopausal women. J Pineal Res. 2012; 52:414-26. DOI: 10.1111/j.1600-079X.2011.00956.x

18. Aydin A, Halici Z, Albayrak A, Polat B, Karakus E, Yildirim OS et al. Treatment with Carnitine Enhances Bone Fracture Healing under Osteoporotic and/or Inflammatory Conditions. Basic Clin Pharmacol Toxicol. 2015; 117:173-9. DOI: 10.1111/bcpt.12384

19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T) Method. Methods. 2001; 25:402-8. DOI: 10.1006/meth.2001.1262

20. Nian H, Ma MH, Nian SS, Xu LL. Antiosteoporotic activity of icariin in ovariectomized rats. Phytomedicine. 2009; 16:320-6. DOI: 10.1016/j.phymed.2008.12.006

21. Oliver RA, Yu Y, Yee G, Low AK, Diwan AD, Walsh WR. Poor histological healing of a femoral fracture following 12 months of estrogen deficiency in rats. Osteoporos Int. 2013; 24:2581-9. DOI: 10.1590/1678-775720150570

Author/ORCID	Authorship Contrubition
Duygu Köse **0000-0002-3468-1567**	**Consept, design, materials, data collection, interpretatiton, literature search, manuscript writing,final approval, critical review.**
Ahmet Köse **0000-0002-7744-1029**	**Consept, design, materials, interpretatiton, literature search, manuscript writing,final approval, critical review.**
Zekai Halıcı **0000-0001-6854-6059**	**Consept, design, materials, data collection, manuscript writing,final approval, critical review.**
Muhammet Ali Gürbüz **0000-0001-8750-2393**	**Consept, design, materials, data collection, interpretatiton, manuscript writing, final approval, critical review.**
Adem Maman **0000-0002-7742-1028**	**Consept, design, materials, data collection, interpretatiton, literature search, manuscript writing, critical review.**
Muhammed Yayla **0000-0002-0659-3084**	**Consept, design, materials, data collection, interpretatiton, literature search, manuscript writing,final approval, critical review.**