Int. J. Morphol.,
*34(2):*763-769, 2016.

Effects of Melatonin on Tibia Bone Defects in Rats

Efectos de la Melatonina sobre Defectos Oseos en la Tibia de Ratas

Koparal, M.[*]; Irtegün, S.[**]; Alan, H.[***]; Deveci, E.[****]; Gülsün, B.[*****] & Pektanç, G.[****]

KOPARAL, M.; IRTEGÜN, S.; ALAN, H.; DEVECI, E.; GÜLSÜN, B. & PEKTANÇ, G. Effects of melatonin on tibia bone defects in rats. *Int. J. Morphol., 34(2)*:763-769, 2016.

SUMMARY: The aim of this study was to evaluate the effects of melatonin healing in a tibial bone defect model in rats by means of histopathological and immunohistochemistry analysis. Twenty one male Wistar albino rats were used in this study. In each animal, bone defects (6 mm length) were created in the tibias. The animals were divided into three groups. In group 1 control group (rats which tibial defects). Group 2 melatonin (10 mg/kg) + 14 days in the tibial defect group) was administered intraperitoneally to rats. Group 3 melatonin (10 mg/kg) + 28 days in the tibial defect group) was administered intraperitoneally to rats. Histopathological analysis of samples was performed to evaluate the process of osteoblastic activity, matrix formation, trabecular bone formation and myeloid tissue in bone defects. Immunohistochemical and immunoblot analysis demonstrated non-collagenous proteins (osteopontin and osteonectin) differences in tibial bone defects. The expression of osteopontin on tibia was increased by 14 days melatonin treatment. The expression of osteonectin on tibia was dramatically increased by 14 days melatonin treatment.

KEY WORDS: Tibial defects; Melatonin; Osteonectin; Osteopontin.

INTRODUCTION

Bone repair is one of the challenges faced by reconstructive surgery. Bone regeneration is required for repair of defects and fractures. Multiple factors can impair fracture consolidation, including bone loss caused by diseases, trauma, or tumor resection. However, there remains a need to learn more about the biology of fracture healing as well as to develop strategies for ensuring normal repair of the skeleton (Gautier & Sommer, 2003).

Melatonin, is an neuroendocrine hormone it also stimulates osteoblasts and fibroblast proliferations. Melatonin plays an important role in bone ossification and remodeling by promoting the production of Type I Collagen by osteoblasts and increasing osteoblastic proliferation (Nakade *et al*., 1999). *In vitro* studies have shown melatonin to have a positive effect on osteoblastic differentiation and bone healing (Roth *et al*., 1999). In a study using rat tibia, Halici *et al*. (2010) showed melatonin to have an accelerating effect on bone healing. The investigation and applications of melatonin in the hard tissues, bone and tooth, have received great attention. Melatonin has been investigated relative to bone remolding osteoporosis, osseointegration of dental implants and dentine formation (Cardinali *et al*., 2003; Ladizesky *et al*., 2003; Cutando *et al*., 2007, 2008; Kotlarczyk *et al*., 2012; Liu *et al*., 2013). Osteopontin is one of the major non-collagen proteins in extracellular bone matrix, it has a role in osteoclast-mediated bone resorption. The aim of this study was to investigate the effects of melatonin during tibial bone defect repair by means of histopathologic and immunohistochemical methods.

MATERIAL AND METHOD

The study protocol was approved by the Animal Research Committee (DUHADEK) of Dicle University, Turkey. Twenty one adult Sprague-Dawley rats, each weighing 250–300 g (±10 g) were used as experimental animals. The animals were fed *ad libitum* with water and

[*] Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Adiyaman University, Adiyaman, Turkey.
[**] Department of Medical Biology, Faculty of Medicine, University of Dicle, Diyarbakir, Turkey.
[***] Department of Oral andMaxillofacial Surgery, Faculty of Dentistry, Inonu University, Malatya, Turkey.
[****] Department of Histology and Embryology, Faculty of Medicine, University of Dicle, Diyarbakir, Turkey.
[*****]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Dicle University, Diyarbakir, Turkey.

standard laboratory animal diet, under the care of trained wardens. The rats were divided into 3 groups as follows: The tibial bone defect group served as the control group. In the experimental group, Melatonin + tibial defect group 14 days and Melatonin + tibial defect group 28 days was accepted. Melatonin (10 mg/kg) was dissolved in 1 % ethanol just before use. Melatonin was injected intraperitoneally. After exposing the right proximal tibia of each animal, a standardized 6.0 mm diameter non-critical bone defect was created by using a motorized drill under irrigation with saline solution (Oliveira *et al*., 2010; Granito *et al*., 2011; Pinto *et al*., 2013).

Tissue Preparation for Light Microscopy. At the end of the study, animals were sacrified by decapitation. The skin, as well as all the soft tissues surrounding the tibia bone was removed. The samples were fixed with neutral buffered 10% formalin solution and decalcified with 5 % EDTA. After preservation, nasal samples were directly dehydrated in a graded series of ethanol and embedded into paraffin wax. Five mm sections were cut with microtome (Rotatory Microtome, Leica, RM 2265, Germany) and mounted on the coated slides. The sections were stained with Haematoxylin and Eosin (H-E) in order to be observed under light microscope (Eclipse 80i, Nikon, Japan).

Immunohistochemical staining. Antigen retrieval process was performed in citrate buffer solution (pH 6.0) two times first 7 min, later 5 min boiled in microwave oven at 700 W. They were allowed to cool to room temperature for 30 min and washed in distilled water for 5 min two times. Endogenous peroxidase activity was blocked in 0.1 % Hydrogen peroxide for 15 min. Ultra V block (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 10 min prior to the application of primary antibodies Osteonectin (mouse monoclonal, 1/200, Santa Cruz Biotech.) and Osteopontin (mouse monoclonal, 1/200, Santa Cruz Biotech) for overnight. Secondary antibody (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 20 min. Slides then were exposed to streptavidin-peroxidase for 20 min. Diaminobenzidine (DAB, Invitrogen, Carlsbad) was used as a chromogen. Control slides were prepared as mentioned above but omitting the primary antibodies. After counterstaining with Hematoxylin, washing in tap water for 5 min and in distilled water for 2 x 5 min, the slides were mounted.

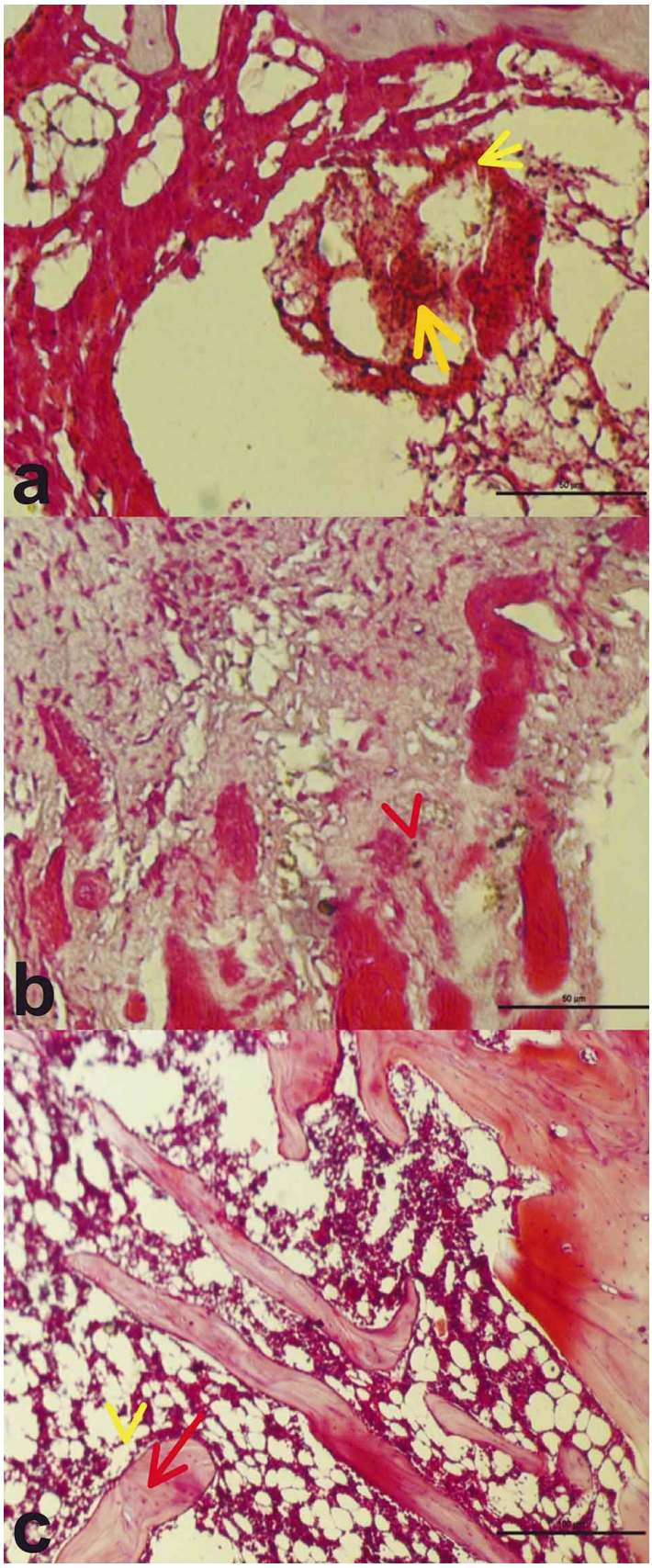
Western Blotting. Cell lysis and protein quantification. The snap frozen tibia was grinded to a fine powder in a chilled mortar in the presence of liquid nitrogen. Immediately after grinding, 50 mg tibia powder was transferred into a microcentrifuge tube containing 250 μl RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 0.2 % (w/v) sodium fluoride, 0.2 % (w/v) sodium orthovanadate and 1x protease inhibitor mixture [CompleteTM EDTA free; Roche Diagnostics]) and incubated in ice for 1 h. After incubation, aliquots were snap-frozen in liquid nitrogen and stored -86 °C. All these steps were performed on ice to minimize protein degradation. Total cellular protein concentration was determined in triplicate using a BCA protein assay according to manufacturer's instructions (Pierce, Thermo scientific). The BCA assay was performed in a 96-well plate using Multiscan™ GO mikroplate from Thermo Scientific.

SDS-PAGE. All protein samples were resolved by 10 % (v/v) polyacrylamide gel using a Mini Protean Tetra Cell apparatus system (Bio-Rad). The protein samples were prepared in 1x SDS loading buffer (2 % (w/v) SDS, 5 % (v/v) glycerol, 0.01 % (w/v) bromophenol blue, 8 % (w/v) DTT, which was added just before heating) and heated at 100 °C for 5 min. The protein samples (20 μg) were then loaded on to the 10 % (v/v) polyacrylamide gel and electrophoresed at 200 V for 1 h in a SDS running buffer (2.4 mM Tris, 19.2 mM glycine, 0.01 % (w/v) SDS).

Membrane transfer and antibody staining. Separated proteins from the SDS-PAGE were transferred onto the PVDF membrane at 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, pH 8.3) using a Criterion Blotter Transfer System (Bio Rad). The membrane was then blocked with 5 % (w/v) skim milk powder in PBS-T (PBS+ 0.1 % (v/v) Tween-20) for 1 h at room temperature. After blocking, the membrane was probed with primary antibodies (anti-osteopontin 1:200 dilution from Santa Cruz Biotech, anti-osteonectin 1:200 dilution from Santa Cruz Biotech and ant-β-actin 1:1000 from Abcam) for 2 h at room temperature. The membrane was then washed four times over 30 min with PBS-T before probing with horseradish peroxidase-conjugated secondary antibodies in 1:10000 dilutions for 1 h at room temperature. The membrane was again washed four times over 30 min with PBS-T. The protein bands were visualized using ECL (Bio-Rad) according to manufacturer's instruction. The images were taken using ChemiDoc™ MP (Bio-Rad).

RESULTS

The histological characteristics of each tibia bone cavity within each group are described. After tibial defects, inflammatory cell infiltration, and began to take shape osteoclastic activity increased fibrous structures. At the end of the 14th day, collagen fibers began to appear with small



lamellar structures (Fig. 1a). Melatonin + bone defect (14th day) group, an increase in bone matrix, now little is seen of trabecular bone (Fig. 1b).

Melatonin + bone defect (28 days) group, The samples in treated melatonin group showed increased bone lamellae formation in the trabecular bone and osteoblastic activity in the lacunar area. Osteoblasts were lining as polygonal between spongeous and trabecular bone tissues (Fig. 1c).

In the tibial defect group treated (Control group) osteoblast cells formed thin trabeculae performed on the collagen fibers. osteoblasts showed a positive reaction with osteonectin (Fig. 2a). In the group treated Melatonin + tibial defect (14 day) osteoblasts showed integrity of collagen fibers with matrix release. Taking place at the periphery of the trabecular bone, it showed positive expression of osteonectin (Fig. 2b).

In the group treated Melatonin + tibial defect (28 day) significantly shaped osteocytes osteon structures, thick bone tabekül have increased expression of osteonectin. Osteoblasts increased matrix development, the cells are arranged in the periphery of trabecular bone (Fig. 2c).

In the tibial defect group treated (Control group); defect regions, which prevents the formation of bone matrix, in inflammatory cell infiltration and vascular hemorrhagic areas were observed. Steopontin expression was observed between fibrous structures positively (Fig. 2d). Trabecular bone and tissue specific myeloid showed increased matrix development (Fig. 2e). Trabecular bone has started to increase the development matrix and osteocytes. Increasing myeloid tissue, new bone formation was accelerated (Fig. 2f). The expression of osteopontin and osteonectin on tibia were increased by 14 days melatonin treatment (Figs. 3 and 4).

Fig. 1. a) Tibial defect group (14th day). An increase in inflamatory cells (arrow), small trabecular bone fragments (arrow) (H-E staining, Bar= 100 µm). b) Melatonin + Tibial defects (14th day), collagen fibers and bone matrix, an increase in small trabecular bone fragments (H-E staining, Bar= 100 μm). c) Melatonin+Tibial defects (28 days). The extension and expansion of trabecular bone, osteocytes and osteoblasts in bone lamella (arrow). A significant increase in myeloid tissue (H-E staining, Bar= 100 μm).

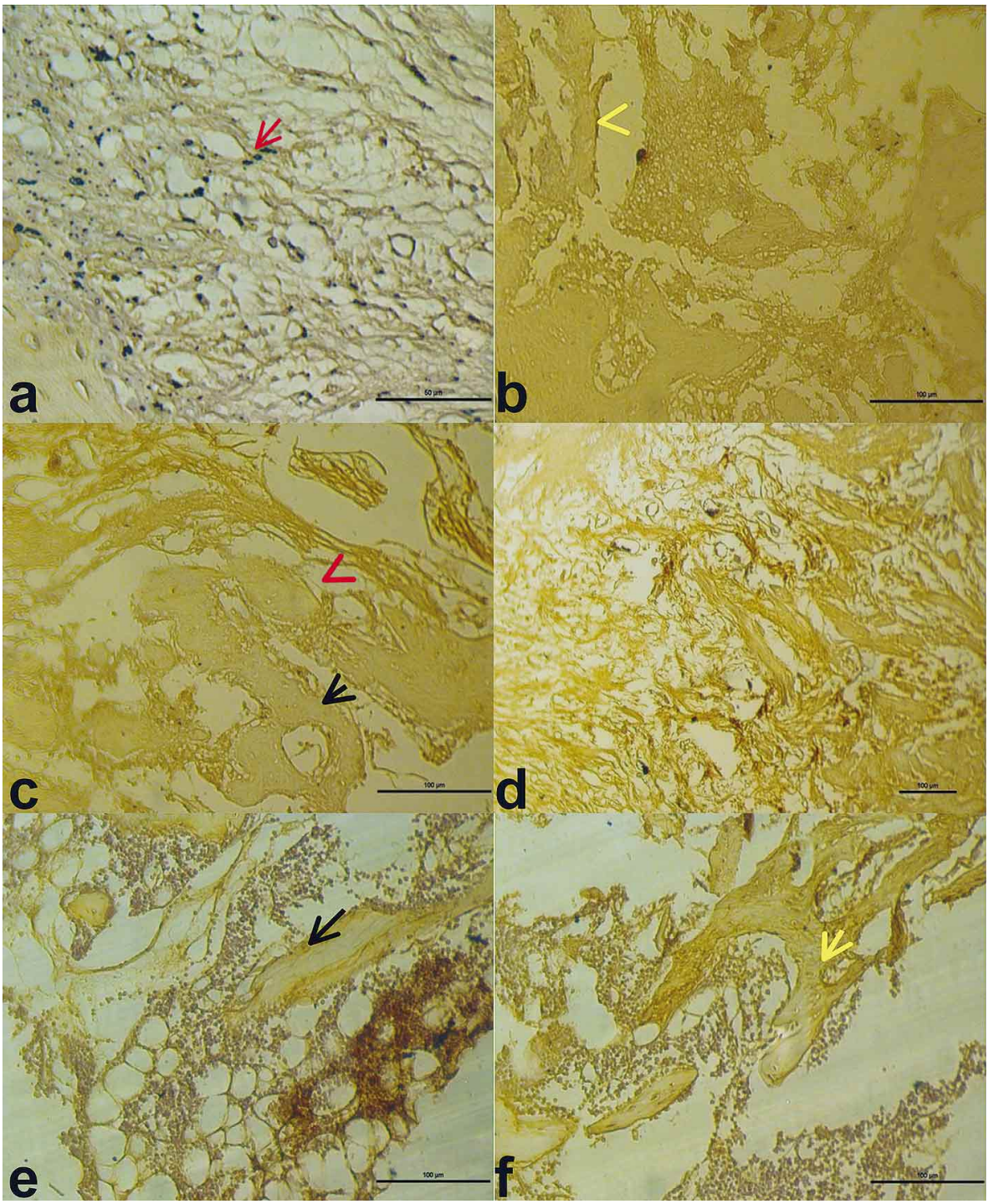


Fig. 2. a) Tibial defect group (14th day). Osteonectin positive reaction in osteblast cells (arrow) (Osteonectin immunostaining, Bar= 100 μm). b) Melatonin + Tibial defects 14th day, matrix density increase, bone trabeculae in the periphery of osteoblast cells, positive expression of osteonektin (Osteonectin immunostaining, Bar= 100 μm). c) Melatonin + Tibial defects 28 day, an increase in bone matrix, trabecular bone and osteon structure markedly, (Osteonectin immunostaining, Bar= 100 μm). d) Tibial defect group (14th day). The positive expression of osteopontin fibrous structures and in the inflammation area (arrow) (Osteopontin immunostaining, Bar= 100 μm). e) Melatonin + Tibial defects 14th day, matrix and osteocyte development in trabecular bone (arrow) (Osteopontin immunostaining, Bar= 100 μm). f) Melatonin+Tibial defects 28 day, An increase in the matrix and osteocytes (arrow). Myeloid tissue dense, distinct new bone formation (Osteopontin immunostaining, Bar= 100 μm).

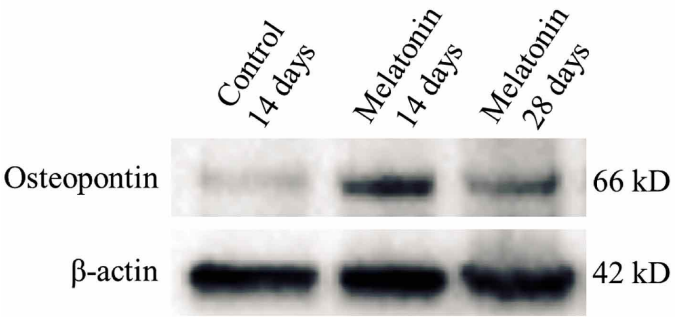


Fig. 3. The expression of osteopontin on tibia was increased by 14 days melatonin treatment. Equal amounts of total proteins were run on the gel and analysed by Western blotting using anti-osteopontin and anti-β-actin antibodies. β-actin was used as a loading control.

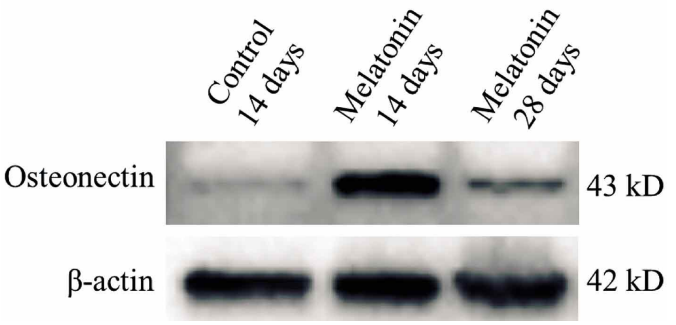


Fig. 4. The expression of osteonectin on tibia was dramatically increased by 14 days melatonin treatment. Equal amounts of total proteins were run on the gel and analysed by Western blotting using anti-osteonectin and anti-β-actin antibodies. β-actin was used as a loading control.

DISCUSSION

Monofoulet *et al*. (2010) compared the kinetics and healing pattern of bone lesions in mice using two protocols that consisted of making holes in the femoral shaft and distal epiphysis. Epiphyseal defect healing occurred only after 13 weeks. In our study, the improvement did not occur in defects in the 14th day of the group, while at 28 days, the beginning of trabecular bone formation was observed. However the osteblas osteoid matrix and activity not yet fully developed. Various drugs and antioxidants may be effective in the treatment of bone defects. Melatonin has previously been shown to stimulate osteoblastic proliferation, melatonin could increase the rate of bone ossification. Melatonin also inhibits osteoclast formation and activation (Koyama *et al*., 2002; Park *et al*., 2011).

Cutando *et al*. (2008) revealed that after a two-week treatment period, melatonin significantly increased the perimeter of bone that was in direct contact with the treated implants, bone density, new bone formation and inter-thread bone in comparison with control implants. In a study, cortical width and length, the early stages (15 and 30 days) increased in new bone formation a greater number of cells in the cortical regions of impressions that promote the melatonin produced. At the same time, significant osteoid matrix synthesis and mineralization accelerated early cell differentiation. It was observed to increase (Calvo-Guirado *et al*., 2015).

Bone remodeling is a constant and dynamic process in which osteoclasts resorb old bone and osteoblasts form new bone (Cardinali *et al*., 2003; Ostrowska, 2009). Melatonin influences the release of growth hormone and promotes bone formation (Roth *et al*.; Ladizesky *et al*., 2006) by suppressing osteoclast activity; stimulating the formation and proliferation of a mineralized matrix and Type I collagen; increasing osteoblast alkaline phosphatase activity through the increased genetic expression of Type I collagen, osteopontin, bone sialoprotein and osteocalcin. Previous studies suggested that osteonectin can stimulate angiogenesis. Several mousemodels have demonstrated that disruption of angiogenesis in bone results in perturbation of the growth plate; however, the growth plate is normal in osteonectin-null mice (Vu *et al*., 1998; Gerber *et al*., 1999). In this study, melatonin brought in osteoblast proliferation defect has accelerated the increase in osteoid matrix synthesis and mineralization.

Osteonectin is a protein known to be involved in cell-matrix interactions and angiogenesis. The new vessel formation by affecting angiogenesis osteonectin and it is important for normal ossification. Osteopontin (OPN) is non-collagen proteins in extracellular bone matrix. 14th day in groups administered melatonin has been shown to increase the osteonectin and osteopontin protein molecule.

Guardia *et al*. (2011) found that after five- and eight-week treatment periods, melatonin significantly increased the inter-thread bone and new bone formation in comparison to control implants in both periods. It was shown that melatonin could increase the osteogenic effect by increasing the osteoblast cell proliferation and stimulating matrix activity. During the formation of the tibial defects, as differentiation towards active new bone formation were significantly higher on the 14th day. Our study demonstrated that melatonin helped reduction in this critical tibia defect.

KOPARAL, M.; IRTEGÜN, S.; ALAN, H.; DEVECI, E.; GÜLSÜN, B. & PEKTANÇ, G. Efectos de la melatonina sobre defectos oseos en la tibia de ratas. *Int. J. Morphol., 34(2)*:763-769, 2016.

RESUMEN: El objetivo fue evaluar por medio de análisis histopatológico e inmunohistoquímico los efectos cicatrizantes de la melatonina en un modelo de defecto óseo tibial en ratas. Se utilizaron 21 ratas albinas Wistar macho. En cada animal, se crearon defectos óseos en las tibias de 6 mm de longitud. Los animales se dividieron en tres grupos. El Grupo 1 correspondió al grupo control (defectos tibiales sin tratamiento). Al Grupo 2 se administró melatonina por vía intraperitoneal (10 mg/kg) 14 días posteriores al defecto tibial. Al Grupo 3 se administró melatonina por vía intraperitoneal (10 mg/kg) 28 días posteriores al defecto tibial. Se realizó un análisis histopatológico para evaluar los procesos de actividad osteoblástica, formación de matriz, formación de hueso trabecular y tejido mieloide en los defectos óseos. Los análisis inmunohistoquímicos y de inmunotransferencia mostraron diferencias de proteínas no colágenas (osteopontina y osteonectina). La expresión de osteopontina en defectos óseos tibiales se incrementó en el Grupo 2. La expresión de osteonectina en la tibia se incrementó fuertemente bajo el tratamiento con melatonina por 14 días.

PALABRAS CLAVE: Defectos tibiales; Melatonina; Osteonectina; Osteopontina.

REFERENCES

Calvo-Guirado, J. L.; Gómez-Moreno, G.; Maté-Sánchez, J. E.; López-Marí, L.; Delgado-Ruiz, R. & Romanos, G. E. New bone formation in bone defects after melatonin and porcine bone grafts: experimental study in rabbits. *Clin. Oral Implants Res., 26(4)*:399-406, 2015.

Cardinali, D. P.; Ladizesky, M. G.; Boggio, V.; Cutrera, R. A. & Mautalen, C. Melatonin effects on bone: experimental facts and clinical perspectives. *J. Pineal Res., 34(2)*:81-7, 2003.

Cardinali, D. P.; Ladizesky, M. G.; Boggio, V.; Cutrera, R. A. & Mautalen, C. Melatonin effects on bone: experimental facts and clinical perspectives. *J. Pineal Res., 34(2)*:81-7, 2003.

Cutando, A.; Gómez-Moreno, G.; Arana, C.; Acuña-Castroviejo, D. & Reiter, R. J. Melatonin: potential functions in the oral cavity. *J. Periodontol., 78(6)*:1094-102, 2007.

Cutando, A.; Gómez-Moreno, G.; Arana, C.; Muñoz, F.; Lopez-Peña, M.; Stephenson, J. & Reiter, R. J. Melatonin stimulates osteointegration of dental implants. *J. Pineal Res., 45(2)*:174-9, 2008.

Gautier, E. & Sommer, C. Guidelines for the clinical application of the LCP. *Injury, 34 Suppl. 2*:B63-76, 2003.

Gerber, H. P.; Vu, T. H.; Ryan, A. M.; Kowalski, J.; Werb, Z. & Ferrara, N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat. Med., 5(6)*:623-8, 1999.

Guardia, J.; Gómez-Moreno, G.; Ferrera, M. J. & Cutando, A. Evaluation of effects of topic melatonin on implant surface at 5 and 8 weeks in Beagle dogs. *Clin. Implant. Dent. Relat. Res., 13(4)*:262-8, 2011.

Granito, R. N.; Rennó, A. C.; Ravagnani, C.; Bossini, P. S.; Mochiuti, D.; Jorgetti, V.; Driusso, P.; Peitl, O.; Zanotto, E. D.; Parizotto, N. A. & Oishi, J. *In vivo* biological performance of a novel highly bioactive glass-ceramic (Biosilicate®): A biomechanical and histomorphometric study in rat tibial defects. *J. Biomed. Mater. Res. B Appl. Biomater., 97(1)*:139-47, 2011.

Halici, M.; Öner, M.; Güney, A.; Canöz, Ö.; Narin, F. & Halici, C. Melatonin promotes fracture healing in the rat model. *Eklem Hastalik Cerrahisi, 21(3)*:172-7, 2010.

Kotlarczyk, M. P.; Lassila, H. C.; O'Neil, C. K.; D'Amico, F.; Enderby, L. T.; Witt-Enderby, P. A. & Balk, J. L. 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., 52(4)*:414-26, 2012.

Koyama, H.; Nakade, O.; Takada, Y.; Kaku, T. & Lau, K. H. 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(7)*:1219-29, 2002.

Ladizesky, M. G.; Boggio, V.; Albornoz, L. E.; Castrillón, P. O.; Mautalen, C. & Cardinali, D. P. Melatonin increases oestradiol-induced bone formation in ovariectomized rats. *J. Pineal Res., 34(2)*:143-51, 2003.

Ladizesky, M. G.; Boggio, V.; Cutrera, R. A.; Mondelo, N.; Mastaglia, S.; Somoza, J. & Cardinali, D. P. Melatonin effect on bone metabolism in rats treated with methylprednisolone. *J. Pineal Res., 40(4)*:297-304, 2006.

Liu, J.; Zhou, H.; Fan, W.; Dong, W.; Fu, S.; He, H. & Huang, F. Melatonin influences proliferation and differentiation of rat dental papilla cells *in vitro* and dentine formation *in vivo* by altering mitochondrial activity. *J. Pineal Res., 54(2)*:170-8, 2013.

Monfoulet, L.; Rabier, B.; Chassande, O. & Fricain, J. C. Drilled hole defects in mouse femur as models of intramembranous cortical and cancellous bone regeneration. *Calcif. Tissue Int., 86(1)*:72-81, 2010.

Nakade, O.; Koyoma, H.; Ariji, H.; Yajima, A. & Kaku, T. Melatonin stimulates proliferation and type I collagen synthesis in human bone cells *in vitro*. *J. Pineal Res., 27(2)*:106-10, 1999.

Oliveira, P.; Ribeiro, D. A.; Pipi, E. F.; Driusso, P.; Parizotto, N. A. & Renno, A. C. Low level laser therapy does not modulate the outcomes of a highly bioactive glass-ceramic (Biosilicate) on bone consolidation in rats. *J. Mater. Sci. Mater. Med., 21(4)*:1379-84, 2010.

Ostrowska, Z. Menopause, obesity, and bone status. *Postepy. Hig. Med. Dosw. (Online), 63*:39-46, 2009.

Park, K. H.; Kang, J. W.; Lee, E. M.; Kim, J. S.; Rhee, Y. H.; Kim, M.; Jeong, S. J.; Park, Y. G. & Kim, S. H. Melatonin promotes osteoblastic differentiation through the BMP/ERK/Wnt signaling pathways. *J. Pineal Res., 51(2)*:187-94, 2011.

Pinto, K. N.; Tim, C. R.; Crovace, M. C.; Matsumoto, M. A.; Parizotto, N. A.; Zanotto, E. D.; Peitl, O. & Rennó, A. C. Effects of biosilicate(®) scaffolds and low-level laser therapy on the process of bone healing. *Photomed. Laser Surg., 31(6)*:252-60, 2013.

Roth, J. A.; Kim, B. G.; Lin, W. L. & Cho, M. I. Melatonin promotes osteoblast differentiation and bone formation. *J. Biol. Chem., 274(31)*:22041-7, 1999.

Vu, T. H.; Shipley, J. M.; Bergers, G.; Berger, J. E.; Helms, J. A.; Hanahan, D.; Shapiro, S. D.; Senior, R. M., & Werb, Z. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell, 93(3)*:411-22, 1998.

Correspondence to:
Dr. Engin Deveci
Department of Histology and Embryology
Faculty of Medicine
University of Dicle
Diyarbakir
TURKEY

Email: engindeveci64@gmail.com

Received: 05-11-2015
Accepted: 07-03-2016