Journal of Surgical Research **173,** 83–90 (2012)
doi:10.1016/j.jss.2010.08.036

Melatonin Impairs Fracture Healing by Suppressing RANKL-Mediated Bone Remodeling

Tina Histing, M.D.,*,‡,[1] Christina Anton,*,‡ Claudia Scheuer, M.D.,†,‡ Patric Garcia, M.D.,*,‡ Joerg H. Holstein, M.D.,*,‡ Moritz Klein, M.D.,*,‡ Romano Matthys,§ Tim Pohlemann, M.D.,*,‡ and Michael D. Menger, M.D.†,‡

*Department of Trauma, Hand, and Reconstructive Surgery; †Institute for Clinical and Experimental Surgery, University of Saarland, Homburg/Saar, Germany; ‡CRC Homburg (Collaborative Research Center, AO Foundation, Switzerland); and §AO Development Institute, Davos, Switzerland

Originally submitted July 11, 2010; accepted for publication August 19, 2010

Background. **Melatonin, the major pineal hormone, is known to regulate distinct physiologic processes. Previous studies have suggested that it supports skeletal growth and bone formation, most probably by inhibiting bone resorption. There is no information, however, whether melatonin affects fracture healing. We therefore studied in a mouse femur fracture model the influence of melatonin on callus formation and biomechanics during fracture healing.**

Methods and Materials. **Thirty CD-1 mice received 50 mg/kg body weight melatonin i.p. daily during the entire 2-wk or 5-wk observation period. Controls (n = 30) received equivalent amounts of vehicle. Bone healing was studied by radiological, biomechanical, histomorphometrical, and protein biochemical analyses at 2 and 5 wk after fracture.**

Results. **Biomechanical analysis at 2 wk after fracture healing showed a significantly lower bending stiffness in melatonin-treated animals compared with controls. A slightly higher amount of cartilage tissue and a significantly larger callus size indicated a delayed remodeling process after melatonin treatment. Western blot analysis showed a significantly reduced expression of receptor activator of nuclear factor-κB ligand (RANKL) and collagen I after melatonin treatment. The reduced expression of RANKL was associated with a diminished number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts within the callus of the newly formed bone.**

Conclusions. **Because bone resorption is an essential requirement for adequate remodeling during fracture healing, we conclude that melatonin impairs fracture healing by suppressing bone resorption through down-regulation of RANKL-mediated osteoclast activation.**

Key Words: **melatonin; mice; fracture healing; bone formation; osteoclasts; RANKL; biomechanics.**

INTRODUCTION

The secretion of melatonin, the major pineal hormone, plays a critical role in the control of the circadian rhythm [1], the reproductive function [2], the body temperature [3], sexual activity [4], immunomodulation [5], and aging [6]. Some of these actions are closely related to sleep initiation and maintenance [7]. Besides, it has been indicated that melatonin also influences bone metabolism through regulation of osteoblast and osteclast activity [8]. The ability of melatonin to promote osteoblast maturation was first demonstrated in mouse preosteoblasts and rat osteoblast-like osteosarcoma cells, where low concentrations of melatonin increased the mRNA levels of several genes expressed in osteoblasts, including alkaline phosphatase, osteopontin, and osteocalcin [9]. Another line of evidence that melatonin affects bone metabolism is derived from osteoporosis studies. Menopause is associated with a substantial decline of melatonin secretion [10], and this fall of melatonin plasma levels is thought to be an important factor in the development of osteoporosis. In line with this, melatonin treatment has been shown to increase bone mineral density in ovariectomized rats [11].

[1] To whom correspondence and reprint requests should be addressed at Department of Trauma, Hand, and Reconstructive Surgery, University of Saarland, D-66421 Homburg/Saar, Germany. E-mail: tina.histing@uks.eu.

0022-4804/$36.00

The effects of melatonin on bone metabolism are presumably a result of inhibition of osteoclast activity through changing the balance between receptor activator of nuclear factor-κB ligand (RANKL)/receptor activator of nuclear factor-κB (RANK) and osteoprotegerin (OPG). RANKL is a potent stimulator of bone resorption by binding RANK in the cell membrane of osteoclasts. On the other hand, OPG is a soluble decoy receptor for RANKL that interferes with RANKL/RANK binding [12].

Although there may be substantial interest how these actions of melatonin influence bone regeneration, there is complete lack of information, whether and how it acts during the process of fracture healing. Under normal conditions, bone remodeling during fracture healing proceeds in cycles in which osteoclasts remove the old bone before osteoblasts can invade the fracture zone and form new bone by secreting osteoid. Because of the ability of melatonin to inhibit bone resorption through down-regulation of RANKL, melatonin may indeed affect the process of remodeling during fracture healing. To test this hypothesis, we herein studied the effect of melatonin on callus formation, bone remodeling, and RANKL and OPG expression in a stably fixed femur fracture model in mice.

MATERIALS AND METHODS

Animals and Specimens

CD-1 mice were bred at the Institute for Clinical and Experimental Surgery, University of Saarland. They were kept at a 12-h light and dark cycle and were fed a standard diet with water *ad libitum*. All animal procedures were performed according to the National Institute of Health guidelines for the use of experimental animals and were approved by the German legislation on the protection of animals. For the present study, a total of sixty 12- to 14-wk-old animals were used. Evaluation was done after 2 and 5 wk observation period to assess the influence of melatonin during the early and late phase of fracture healing.

Thirty mice were treated daily with 50 mg/kg body weight (BW) melatonin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) i.p. for the entire 2-wk and 5-wk observation period. This high dose of melatonin was chosen because others have shown that such high doses of melatonin are capable of increasing bone mass and promoting cortical bone formation *in vivo* [8, 13]. After 2 wk, 10 of these mice were killed for radiological, biomechanical, and histomorphometrical analysis, and another five mice were killed for protein biochemical analysis. In the remaining 15 animals, daily melatonin treatment was continued for a total of 5 wk. Ten of these mice were then killed for radiological, biomechanical, and histomorphometrical analysis, and the remaining five mice were killed for protein biochemical analysis.

Thirty vehicle-treated mice served as controls. After 2 wk 10 of these mice were killed for radiological, biomechanical, and histomorphometrical analysis, and another five mice were killed for protein biochemical analysis. The remaining 15 animals were kept for a total of 5 wk. Ten of these mice were then killed for radiological, biomechanical, and histomorphometrical analysis, and the remaining five mice were killed for protein biochemical analysis.

Surgical Procedure

Mice were anesthetized by an intraperitoneal injection of xylazine (25 mg/kg BW) and ketamine (75 mg/kg BW). Under sterile conditions a 4 mm medial parapatellar incision was performed at the right knee to dislocate the patella laterally. After drilling a hole (0.5 mm in diameter) into the intracondylar notch, an injection needle with a diameter of 0.4 mm was drilled into the intramedullary canal. Subsequently, a tungsten guide wire (0.2 mm in diameter) was inserted through the needle into the intramedullary canal. After removal of the needle, the femur was fractured by a 3-point bending device and an intramedullary titanium screw (18 mm length, 0.5 mm in diameter) was implanted over the guide wire to stabilize the fracture [14]. The screw consisted of a distal cone-shaped head (diameter 0.8 mm) and a proximal thread (M 0.5 mm, length 4 mm) (AO Foundation, Research Implants Systems (RIS), Davos, Switzerland). After fracture fixation, the wound was closed using 6-0 synthetic sutures. Fracture and implant position were confirmed by radiography (MX-20; Faxitron X-ray Corporation, Wheeling, IL).

Radiological Analysis

At the end of the 2 and 5 wk observation period, the animals were re-anesthetized and ventro-dorsal X-rays (MX-20 Faxitron X-ray Corporation) of the healing femora were performed. Fracture healing was analyzed according to the classification of Goldberg with stage 0 indicating radiological non-union, stage 1 indicating possible union, and stage 2 indicating radiological union [15].

Biomechanical Analysis

For biomechanical analysis, the right and the left femora were resected at 2 and 5 wk and freed from soft tissue. After removing the implants, callus stiffness was measured with a nondestructive bending test using a 3-point bending device with a 20 N load cell (Mini-Zwick Z 2.5; Zwick GmbH, Ulm, Germany). Due to the different states of healing, the loads that had to be applied varied markedly between the individual animals. Loading was stopped individually in every case when the actual load-displacement curve deviated more than 1% from linearity [16]. Control that the load was not destructive was performed macroscopically and microscopically (histology). To account for differences in bone stiffness of the individual animals, the unfractured left femora were also analyzed, serving as an internal control. All values of the fractured femora are given in percent of the corresponding unfractured femora. To guarantee standardized measuring conditions, femora were mounted always with the ventral aspect upwards. A working gauge length of 6 mm was used. Applying a gradually increasing bending force with 1 mm/min, the bending stiffness (N/mm) was calculated from the linear elastic part of the load-displacement diagram [17].

Histomorphometric and Immunohistochemical Analysis

For histology, bones were fixed in IHC zinc fixative (BM Pharmingen, San Jose, CA, USA) for 24 h, decalcified in 10% EDTA solution for 2 wk and then embedded in paraffin. Longitudinal sections of 5 μm thickness were stained according to the trichrome method. At a magnification of 1.25× (Olympus BX60 Microscope; Olympus, Tokyo, Japan; Zeiss Axio Cam and Axio Vision 3.1; Carl Zeiss, Oberkochen, Germany; ImageJ Analysis System; NIH, Bethesda, MD) structural indices were calculated according to the suggestions provided by Gerstenfeld *et al.* [18]. These included total callus area (bone, cartilaginous and fibrous callus area)/femoral bone diameter (cortical width plus marrow diameter) at the fracture gap [CAr/BDm (mm)], callus diameter/femoral bone diameter [CDm/BDm], bone (total osseous tissue) callus area/total callus area [TOTAr/CAr (%)], cartilaginous callus area/total callus area [CgAr/CAr (%)], and fibrous tissue callus area/total callus area [FTAr/CAr (%)].

Additionally, we used a score system to evaluate the quality of fracture bridging [19]. Both cortices were analyzed for bone bridging (two points), cartilage bridging (one point), or bridging with fibrous tissue (0 point). This score system results in a maximum of four points for each specimen, indicating complete bone bridging.

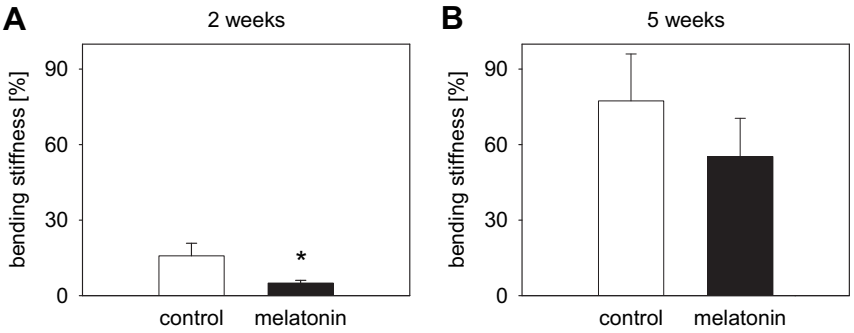

FIG. 1. Biomechanical analysis of the bending stiffness at 2 wk (A) and 5 wk (B) of fracture healing in controls (*white bars*) and melatonin-treated animals (*black bars*). Means ± SEM; *$P < 0.05$ *versus* control.

For determination of tartrate-resistant acid phosphatase (TRAP) activity, bones were fixed in IHC zinc fixative (BM Pharmingen) for 24 h, decalcified in 10% EDTA solution for 2 wk and then embedded in paraffin. Longitudinal sections of 5 μm thickness were stained according to the trichrome method. After deparaffinizing again, the sections were incubated in a mixture of 5 mg naphotol AS-MX phosphate and 11 mg fast red TR salt in 10 mL0.2 M sodium acetate puffer (pH 5.0) for 1 h at 37°C. Sections were counterstained with methyl green and covered with glycerin gelatine.

For immunohistochemical detection of the melatonin receptor in the callus tissue, zinc-fixed paraffin-embedded specimens, which were decalcified for 2 wk in 10% EDTA solution, were cut in sections of 5 μm thickness and deparaffinized with x-Tra (Medite Medizintechnik, Burgdorf, Germany). After rehydration by a descending ethanol line and antigen demasking with 0.05% saponin (Sigma-Aldrich, Deisenhofen, Germany) for 30 min at room temperature, endogenous peroxidases and unspecific binding sites were blocked with 1% H_2O_2 or 4% donkey normal serum (Dianova, Hamburg, Germany). The primary polyclonal rabbit anti-melatonin receptor antibody Mel-1A-R (1:50; R-18, Santa Cruz, Heidelberg, Germany) was incubated at 4°C overnight. As secondary antibody a peroxidase-conjugated donkey anti-rabbit-IgG antibody (1:100; GE Healthcare, Freiburg, Germany) was incubated for 30 min at room temperature. 3,3′Diaminobenzidine (DAB, Sigma-Aldrich) was used as chromogen for the enzyme reaction. Cell nuclei were counterstained with Mayer's hemalaun. After dehydration with ethanol and x-Tra, the slices were covered with x-Tra-Kit (Medite Medizintechnik).

Western Blot Analysis

The callus tissue was frozen and stored at –80°C until further processing. For whole protein extracts and Western blot analysis of OPG, collagen I, PCNA, cleaved caspase-3, and RANKL, the callus tissue

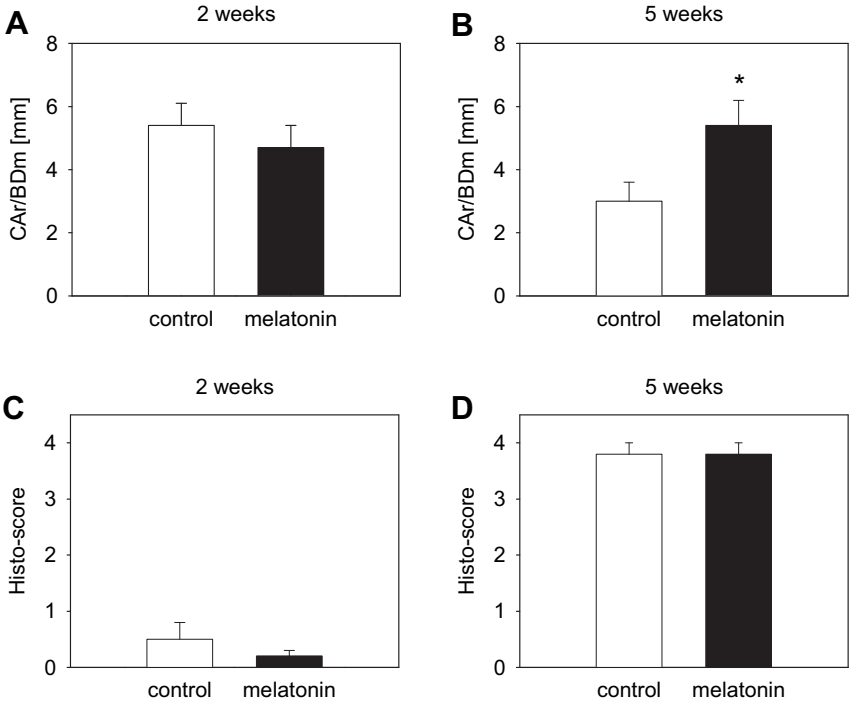

FIG. 2. Histomorphometric analysis of the total callus area (CAr) in relation to the diameter of the femur (BDm) (A) and (B), and the healing score (C) and (D) after 2 wk (A) and (C), and 5 wk (B and D) of fracture healing in controls (*white bars*) and melatonin-treated animals (*black bars*). For histologic scoring, both cortices were analyzed for bone bridging (2 points), cartilage bridging (1 point) or bridging with fibrous tissue (0 point). Means ± SEM; *$P < 0.05$ *versus* control.

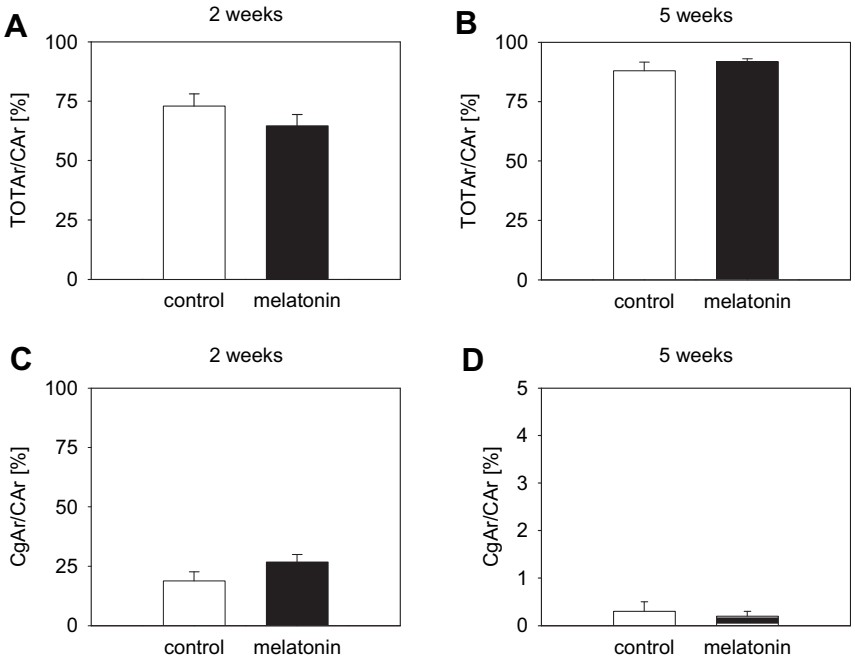

FIG. 3. Histomorphometric analysis of the tissue distribution within the callus area. Total osseous tissue callus area/total callus area (TOTAr/CAr %) (A) and (B), and cartilaginous callus area/total callus area (CgAr/CAr %) (C) and (D), after 2 wk (A) and (C), and 5 wk (B) and (D), of fracture healing in controls (*white bars*) and melatonin-treated animals (*black bars*). Means ± SEM.

was homogenized in lysis buffer [10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, 0.02% NaN3, 0.2 mM PMSF, and protease inhibitor cocktail (1:100 vol/vol; Sigma-Aldrich, Taufkirchen, Germany)], incubated for 30 min on ice and centrifuged for 30 min at 16,000 × *g*. Protein concentrations were determined using the Lowry assay. The whole protein extracts (10 μg protein per lane) were separated discontinuously on sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylendifluoride membranes. After blockade of nonspecific binding sites, membranes were incubated for 4 h with the following antibodies: mouse anti-mouse PCNA (1:500; DAKO Cytomation, Hamburg, Germany), rabbit anti-mouse OPG (1:100; Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-mouse cleaved caspase-3 (1:400; Cell Signaling, Frankfurt, Germany), mouse anti-mouse RANKL (1:300; Abcam, Cambridge, UK), and rabbit anti-mouse collagen I (1:75; Santa Cruz Biotechnology, Heidelberg, Germany). This was followed by corresponding horseradish peroxidase conjugated secondary antibodies (1.5 h, 1:5000; GE Healthcare Amersham, Freiburg, Germany). Protein expression was visualized using luminol-enhanced chemiluminescence (ECL, GE Healthcare Amersham). Signals were densitometrically assessed (Quantity One, Geldoc; BioRad, München, Germany) and normalized to β-actin signals (1:20,000, anti-β-actin, Sigma-Aldrich) to correct for unequal loading.

Statistical Analysis

All data are given as means ± SEM. After proving the assumption for normal distribution (Kolmogorov-Smirnov test) and equal variance (F-test), comparison between the experimental groups was performed by Student's *t*-test or one way ANOVA and Student Newman-Keuls *post-hoc* test. For nonparametrical data Mann-Whitney test was used. Statistics were performed using the GraphPad Prism 4.0 software package (Graphpad, San Diego, CA). A P value < 0.05 was considered to indicate significant differences.

RESULTS

Radiological Analysis

Radiological analyses 2 and 5 wk after fracture could not demonstrate significant differences between melatonin-treated animals and controls ($P > 0.05$).

Biomechanical Analysis

Biomechanical analysis at 2 wk after fracture healing showed a significantly lower bending stiffness in melatonin-treated animals compared with controls (Fig. 1A). After 5 wk, the melatonin-treated animals still showed a lower bending stiffness compared with controls, however, the difference did not prove statistically significant (Fig. 1B).

Histologic Analysis

All samples demonstrated a typical pattern of secondary fracture healing with callus formation, including intramembranous and endochondral ossification. At 2 wk after fracture healing, the size of the total callus of the melatonin-treated animals was almost the same as that of controls (Fig. 2A). After 5 wk, however, the callus size was significantly larger in melatonin-treated animals compared with controls (Fig. 2B). This indicates a delay in bone remodeling after melatonin treatment.

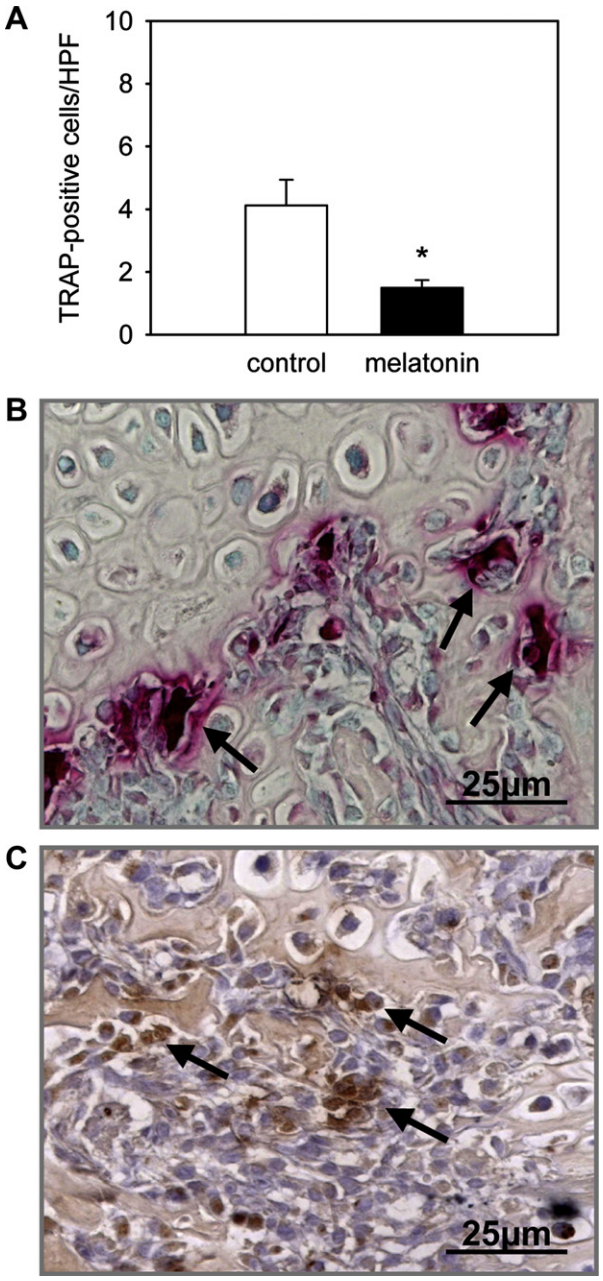

FIG. 4. TRAP-positive cells at 2 wk of bone healing (A) in controls (*white bars*) and melatonin-treated animals (*black bars*). Means ± SEM; $*P < 0.05$ *versus* control. Immunohistochemical staining of TRAP-positive cells (B), arrows, and Mel 1aR expression (C), arrows, within the callus of a control animal after 2 wk of fracture healing. (Color version of figure is available online.)

At 2 wk after fracture, melatonin-treated animals showed a slightly but not significantly lower healing score compared with controls (Fig. 2C). After 5 wk, the histologic scores did not differ between the two groups (Fig. 2D).

Analysis of callus composition revealed that at 2 wk bone formation was slightly reduced and cartilage formation was increased in melatonin-treated animals compared with controls (Fig. 3A and C). After 5 wk, all animals showed a comparable amount of bone tissue, i.e., ~90% and cartilaginous tissue could not anymore be detected (Fig. 3B and D).

Analysis of tartrate-resistant acid phosphatase (TRAP) activity demonstrated a significantly reduced number of TRAP-positive cells after melatonin treatment compared with controls (Fig. 4A). TRAP activity was detected predominately in multinuclear osteoclasts within the central region of the callus (Fig. 4B). Of interest, TRAP-positive cells could neither be found in the periosteum nor in the endosteal region of the callus. Immunostaining with a monoclonal antibody against Mel 1aR demonstrated that osteoblasts within the callus are capable of expressing melatonin receptors (Fig. 4C). The negative control showed no positively stained cells.

Protein Expression Analysis

At 2 wk, Western blot analysis demonstrated that expression of OPG, which is an inhibitor of osteoclastogenesis, was not affected by melatonin treatment (Fig. 5A and B). However, the expression of RANKL, which is an essential factor for osteoclast formation, activation and survival, promoting bone resorption and bone loss, was significantly reduced compared with controls (Fig. 5A and C). The expression of collagen I, a marker of osteoblastogenesis, was also significantly reduced after melatonin treatment (Fig. 5A and D). The expression of PCNA, which serves as an indicator of cell proliferation, was slightly but not significantly lower in the fracture callus of melatonin-treated animals compared with controls (Fig. 5A and E). Cleaved caspase-3, a marker for apoptotic cell death, was not detectable in the callus tissue of either of the groups (data not shown).

DISCUSSION

In the present study, we tested the hypothesis that melatonin affects the remodeling process during fracture healing. The data of our experiments confirmed our hypothesis. We herein demonstrate for the first time that melatonin induces a marked delay of fracture repair, as indicated by a significantly lower bending stiffness compared with non-treated controls during the early time period of healing. The action of melatonin involves most probably an inhibition of bone resorption through down-regulation of RANKL, an essential factor for osteoclast activity. This view is supported by the significantly reduced expression of RANKL and the diminished number of TRAP-positive cells within the fracture callus.

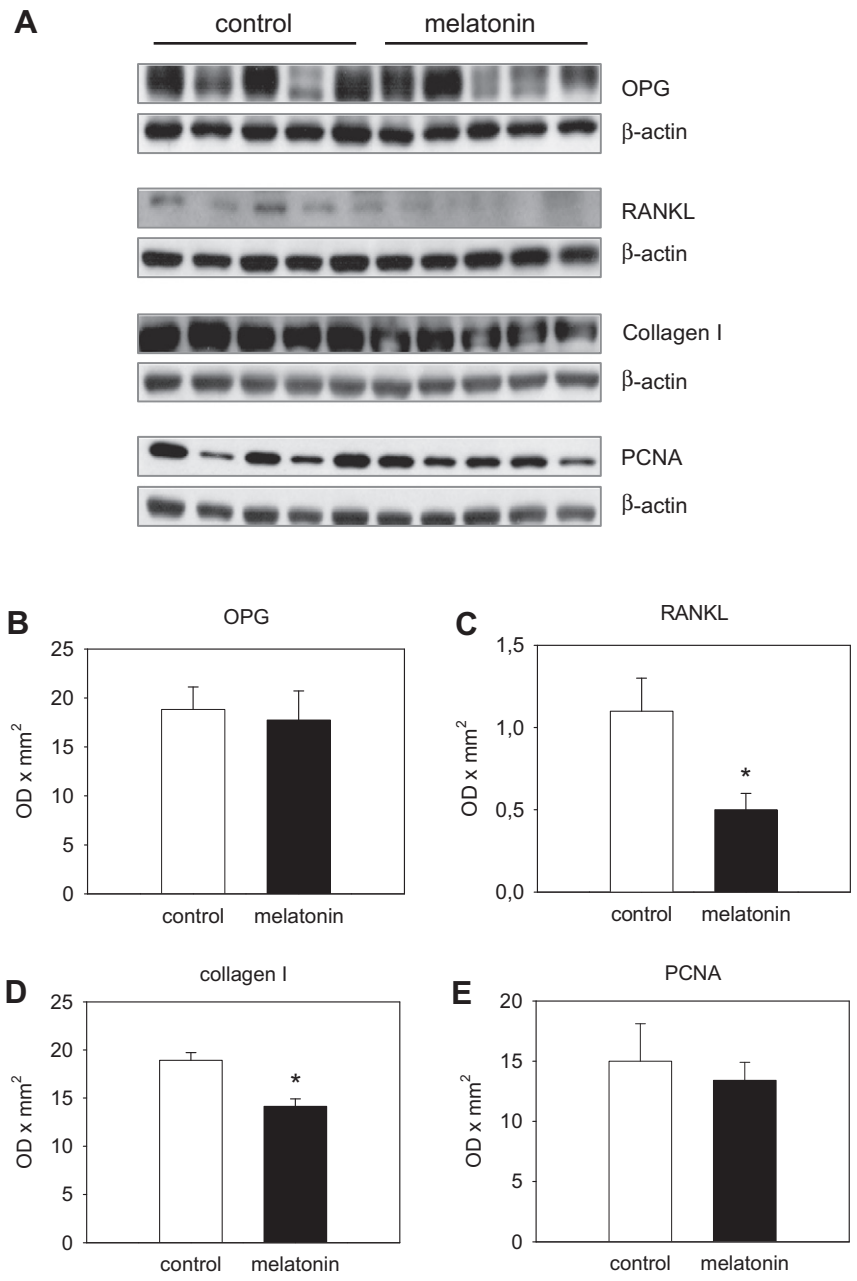

FIG. 5. Western Blot analysis of OPG (A) and (B), RANKL (A) and (C), collagen I (A) and (D), and PCNA (A) and (E) expression in callus of melatonin-treated mice (*black bars*) and controls (*white bars*) after 2 wk of fracture healing. Means ± SEM; *$P < 0.05$ *versus* control.

In general, there is substantial evidence that melatonin may act beneficial in bone [20]. For example, Muñoz *et al.* [21] demonstrated that topical application of melatonin and growth hormone accelerates bone healing around dental implants in dogs. Calvo-Guirado *et al.* [22] and Cutando *et al.* [23] showed also a significantly increased bone formation and bone density after topical application of melatonin of dental implants in Beagle dogs and postulated that melatonin enhances osteointegration. Satomura *et al.* [13] further showed that melatonin stimulates mineralized matrix formation as well as proliferation and alkaline phosphatase activity of human osteoblasts *in vitro* through increased gene expression of type I collagen, osteopontin, bone sialoprotein, and osteocalcin. Of interest, *in vitro* human osteoblasts from younger subjects displayed higher expression of Mel 1aR than those from the elderly, both in males and females. Moreover, intraperitoneal injection of melatonin in mice increased the volume of newly formed cortical bone of femora. Based on these results, the authors suggested that melatonin may be applied to promote bone regeneration during fracture healing [13].

In the present study, we could demonstrate for the first time that osteoblasts within fracture callus are capable of expressing melatonin receptors also *in vivo*. This confirms a possible role of melatonin in bone formation, although melatonin was not effective to accelerate the process of fracture healing.

In contrast to the aforementioned results, Koyama *et al.* [8] found that daily administration of melatonin for 4 wk in mice did not increase bone formation, as indicated by histomorphometric analysis and a lack of a significant increase of serum alkaline phosphatase (ALP). However, this study showed that melatonin increases bone mass through suppression of bone resorption. These results are supported by other studies, demonstrating that melatonin treatment in ovariectomized rats [24] as well as in obese women [25] leads to an acute and marked decrease of bone resorption. In line with the results of these studies, we demonstrate herein that melatonin significantly reduces RANKL expression and, additionally, diminishes the number of TRAP-positive cells. Because remodeling during fracture healing requires osteoclast-mediated bone resorption, melatonin may have negatively affected fracture healing by delaying the process of remodeling, as indicated by a significantly decreased callus stiffness.

Previous studies have indeed demonstrated that inhibition of RANKL affects bone metabolism. Li *et al.* [26] reported that RANKL inhibition prevents orchiectomy-related deficits in trabecular bone mineral density, trabecular architecture, and periosteal bone formation. Of interest, Gerstenfeld *et al.* [27] also showed that RANKL inhibition delays the process of remodeling within the fracture callus, although these authors found an enhanced stiffness of the healing bone. This difference compared with our study may be due to the fact that we herein studied the biomechanics during the early healing period (d 14), while Gerstenfeld *et al.* [27] performed the biomechanical analysis at a very late time point (d 42), when fracture healing in mice is already completed.

In the present study, melatonin did not affect the expression of OPG *in vivo*. Although it has been reported that *in vitro* melatonin is capable of inducing the expression of OPG and suppressing the expression of RANKL [8], results from cross-sectional *in vivo* studies in humans vary from no association between bone mineral density and OPG to a decrease in BMD with increasing OPG levels in both genders [28]. Furthermore, Kon *et al.* [29] showed peaks of OPG expression during bone healing at 24 h and 7 d after fracture. On the other hand, RANKL expression peaked at 3 and 14 d. In line with this, the present study demonstrates expression of OPG and RANKL until 14 d after fracture.

Melatonin may not only affect bone metabolism through control of the RANKL/RANK/OPG system, but also through regulation of biochemical processes. Melatonin is a potent free radical scavenger and, thus, neutralizes free radicals and reactive oxygen species [30]. Because osteoblasts generate high levels of superoxide anions during bone resorption that contribute to the degradative process, the effect of melatonin in preventing osteoclast activity in bone may, in part, be also due to the free radical scavenging properties of the hormone.

The expression of collagen I, a marker of osteoblastogenesis, was significantly reduced after melatonin treatment compared with controls. This result contrasts those of *in vitro* studies. Nakade *et al.* [31] demonstrated that melatonin stimulates the proliferation and type I collagen synthesis in human bone cells *in vitro*. Despite the fact that melatonin increases the proliferation, differentiation, and bone formation *in vitro* [9, 31], the hormone may not be osteogenic *in vivo*. Suzuki *et al.* [32] found that melatonin suppresses the osteoblastic activities by suppressing the ALP activity. This is in line with our result that melatonin significantly reduced collagen I expression.

In conclusion, melatonin may increase bone mass by inhibiting bone resorption rather than increasing bone formation. Thus melatonin may serve as an important regulator of bone mass relating to osteoporosis. However, melatonin is not capable of accelerating fracture healing. In contrast, it delays the time course of bone repair by affecting the process of remodeling.

ACKNOWLEDGMENTS

The authors thank Janine Becker for excellent technical assistance. This study was supported by a grant of the University of Saarland, HOMFOR.

REFERENCES

1. Redman JR. Circadian entrainment and phase shifting in mammals with melatonin. J Biol Rhythms 1997;12:581.
2. Reiter RJ, Tan DX, Manchester LC, et al. Melatonin and reproduction revisited. Biol Reprod 2009;81:445.
3. Cagnacci A, Elliott JA, Yen SS. Melatonin: A major regulator of the circadian rhythmus of core temperature in humans. J Clin Endocrinol Metab 1992;75:447.
4. Drago F, Busa L. Acute low doses of melatonin restore full sexual activity in impotent male rats. Brain Res 2000;878:98.
5. Liebmann PM, Wölfler A, Felsner P, et al. Melatonin and the immune system. Int Arch Allergy Appl Immunol 1997;112:203.
6. Sandyk R. Possible role of pineal melatonin in the mechanisms of aging. Int J Neurosci 1990;52:85.
7. Dijk DJ, Duffy JF, Riel E, et al. Ageing and the circadian and homeostatic regulation of human sleep during forced desynchrony of rest, melatonin, and temperature rhythmus. J Physiol (Lond) 1999;516:611.
8. Koyama H, Nakade O, Takada Y, et al. 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 2002;17:1219.

9. Roth JA, Kim BG, Lin WL, et al. Melatonin promotes osteoblast differentiation and bone formation. J Biol Chem 1999; 274:22041.
10. Sack RL, Lewy AJ, Erb DL, et al. Human melatonin production decreases with age. J Pineal Res 1986;3:379.
11. Uslu S, Uysal A, Oktem G, et al. Constructive effect of exogenous melatonin against osteoporosis after ovariectomy in rats. Anal Quant Cytol Histol 2007;29:317.
12. Lacey DL, Timms E, Tan HL, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165.
13. Satomura K, Tobiume S, Tokuyama R, et al. Melatonin at pharmacological doses enhances human osteoblastic differentiation *in vitro* and promotes mouse cortical bone formation *in vivo*. J Pineal Res 2007;42:231.
14. Holstein JH, Matthys R, Histing T, et al. Development of a stable closed femoral fracture model in mice. J Surg Res 2008;153:71.
15. Goldberg VM, Powell A, Shaffer JW, et al. Bone grafting: Role of histocompatibility in transplantation. J Orthop Res 1985;3:389.
16. Schoen M, Rotter R, Schattner S, et al. Introduction of a new interlocked intramedullary nailing device for stabilization of critically sized femoral defects in the rat: A combined biomechanical and animal experimental study. J Orthop Res 2008;26:184.
17. Histing T, Garcia P, Matthys R, et al. An internal locking plate to study intramembranous bone healing in a mouse femur fracture model. J Orthop Res 2010;28:397.
18. Gerstenfeld LC, Wronski TJ, Hollinger JO, et al. Application of histomorphometric methods to the study of bone repair. J Bone Miner Res 2005;20:1715.
19. Garcia P, Holstein JH, Histing T, et al. A new technique for internal fixation of femoral fractures in mice: Impact of stability on fracture healing. J Biomech 2008;41:1689.
20. Sandyk R, Anastasiadis PG, Anninos PA, et al. Is postmenopausal osteoporosis related to pineal gland functions? Int J Neurosci 1992;62:215.
21. Muñoz F, López-Peña M, Miño N, et al. Topical application of melatonin and growth hormone accelerates bone healing around dental implants in dogs. Clin Implant Dent Relat Res 2009;29. Epub ahead of print.
22. Calvo-Guirado JL, Gómez-Moreno G, Barone A, et al. Melatonin plus porcine bone on discrete calcium deposit implant surface stimulates osteointegration in dental implants. J Pineal Res 2009;47:164. Epub 2009 Jun 29.
23. Cutando A, Gómez-Moreno G, Arana C, et al. Melatonin stimulates osteointegration of dental implants. J Pineal Res 2008;45:174.
24. Ostrowska Z, Kos-Kudla B, Swietochowska E, et al. Assessment of the relationship between dynamic pattern of nighttime levels of melatonin and chosen biochemical markers of bone metabolism in a rat model of postmenopausal osteoporosis. Neuro Endocrinol Lett 2001;22:129.
25. Ostrowska Z, Kos-Kudla B, Marek B, et al. Assessment of the relationship between circadian variations of salivary melatonin levels and type I collagen metabolism in postmenopausal obese women. Neuro Endocrinol Lett 2001;22:121.
26. Li X, Ominsky MS, Stolina M, et al. Increased RANK ligand in bone marrow of orchiectomized rats and prevention of their bone loss by the RANK ligand inhibitor osteoprotegerin. Bone 2009;45:669.
27. Gerstenfeld LC, Sacks DJ, Pelis M, et al. Comparison of effects of the bisphosphonate alendronate *versus* the RANKL inhibitor denosumab on murine fracture healing. J Bone Miner Res 2009; 24:196.
28. Abrahamsen B, Hjelmborg JV, Kostenuik P, et al. Circulating amounts of osteoprotegerin and RANK ligand: Genetic influence and relationship with BMD assessed in female twins. Bone 2005; 36:727.
29. Kon T, Cho TJ, Aizawa T, et al. Expression of osteoprotegerin, receptor activator of NF- κB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. J Bone Miner Res 2001;16:1004.
30. Reiter RJ, Tan DX, Manchester LC, et al. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: A review of the evidence. Cell Biochem Biophys 2001;34:237.
31. Nakade O, Koyama H, Ariji H, et al. Melatonin stimulates proliferation and type I collagen synthesis in human bone cells *in vitro*. J Pineal Res 1999;27:106.
32. Suzuki N, Hattori A. Melatonin suppresses osteoclastic and osteoblastic activities in the scales of goldfish. J Pineal Res 2002; 33:253.