INTRODUCTION:
The secretion of melatonin, the major pineal hormone, plays a critical role in the control of the circadian rhythm, the reproductive function, the body temperature, immunomodulation and aging. Besides, it has been indicated that melatonin also influences bone metabolism through regulation of osteoblast and osteoclast activity. 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-κ B ligand (RANKL) / receptor activator of nuclear factor-κ B (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 inhibits RANKL binding. 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.

METHODS:
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 weeks old CD-1 mice were used. Thirty mice were treated daily with 50 mg/kg body weight (BW) melatonin i.p. 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 [1]. Thirty vehicle-treated mice served as controls. Bone healing was studied in a murine closed femur fracture model using radiological, biomechanical, histomorphometric and protein biochemical analysis at 2 and 5 weeks after fracture. 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 two experimental groups was performed by Student’s t-test.

RESULTS:
Radiological analyses 2 and 5 weeks after fracture could not demonstrate significant differences between melatonin-treated animals (n=10) and controls (n=10) (p>0.05). Biomechanical analysis at 2 weeks after fracture healing showed a significantly lower bending stiffness in melatonin-treated animals (n=10) compared to controls (n=10). After 5 weeks, the melatonin-treated animals still showed a lower bending stiffness compared to controls, however, the difference did not prove statistically significant. Histologically, all samples demonstrated a typical pattern of secondary fracture healing with callus formation, including intramembranous and endochondral ossification. At 2 weeks after fracture healing, the size of the total callus of the melatonin-treated animals (n=10) was almost the same as that of controls (n=10). After 5 weeks, however, the callus size was significantly larger in melatonin-treated animals compared to controls. This indicates a delay in bone remodeling after melatonin treatment. Analysis of callus composition revealed that at 2 weeks bone formation was slightly reduced and cartilage formation was increased in melatonin-treated animals compared to controls. Analysis of tartrate-resistant acid phosphatase (TRAP) activity demonstrated a significantly reduced number of TRAP-positive cells after melatonin treatment compared to controls. TRAP activity was detected predominately in multinuclear osteoclasts within the central region of the callus. Of interest, TRAP-positive cells could neither be found in the periosteon 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. At 2 weeks, Western blot analysis (n=5, each group) demonstrated that expression of OPG, which is an inhibitor of osteoclastogenesis, was not affected by melatonin treatment. 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 to controls.

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 when compared to 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. In general, there is substantial evidence that melatonin may act beneficial in bone. Satomura et al. 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. 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 [2]. In contrast to the aforementioned results, Koyama et al. [1] found that daily administration of melatonin for 4 weeks 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. In line with the results of this study, 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.

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.
