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Effect of melatonin on bone metabolism in ovariectomized rats

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Abstract

To assess the effect of pharmacological dose of melatonin on bone metabolism in ovariectomized rats, urinary deoxypyridinoline (a marker of bone resorption) and calcium excretion, circulating levels of calcium, phosphorus and bone alkaline phosphatase activity (a marker of bone formation), and bone mineral density (BMD), mineral content (BMC) and bone area (BA) of total body, were measured in adult rats for up to 60 days after surgery. Rats received melatonin in the drinking water (25 μg/ml water) or drinking water alone. Urinary deoxypyridinoline increased significantly after ovariectomy by 51% (30 days after surgery) and by 47% (60 days after surgery). The increase in urinary deoxypyridinoline found 30 days after ovariectomy was not observed in melatonin-treated rats. Urinary calcium concentration was similar in the 4 experimental groups studied, as was the circulating calcium concentration at every time interval examined. Fifteen days after surgery, a significant increase in serum phosphorus and bone alkaline phosphatase levels occurred in ovariectomized rats receiving melatonin as compared to their controls. Sixty days after surgery BMD, BMC and BA decreased significantly in ovariectomized rats, an effect not modified by melatonin. Serum estradiol decreased significantly by 30 days after ovariectomy to attain values close to the limit of detection of the assay by 60 days after ovariectomy. The results support the conclusion that a pharmacological amount of melatonin modifies bone remodeling after ovariectomy and that the effect may need adequate concentrations of estradiol.

Keywords: Melatonin; Bone remodeling; Bone mineral content; Ovariectomy; Estradiol

Introduction

Bone metabolism is dependent on cells of the osteoblast and osteoclast lineage. These cells play a major role in the synthesis and degradation of osteoid and in its subsequent min-

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eralization and demineralization, and are under the influence of various systemic and local auto/paracrine factors. For example, estrogen deficiency induces a high turnover of bone remodeling in which the accelerated bone resorption and formation simultaneously occur. During this process bone resorption exceeding bone formation results in bone loss in animals and humans [1–8].

The pineal secretory product melatonin may be another circulating factor modifying bone remodeling. Melatonin increased the proliferation in normal human bone cells and osteoblastic cells and increased procollagen type I c-peptide production by the cells [9]. Melatonin also augmented gene expression of sialoprotein and other bone marker proteins like alkaline phosphatase and osteocalcin in bone cells [10]. Suppression of the major source of circulating melatonin by pinealectomy produced scoliosis in chickens displaying a number of similarities to human adolescent idiopathic scoliosis [11].

The objective of the present study was to determine whether a pharmacological dose of melatonin administered in the drinking water could modify the augmented bone remodeling occurring in estrogen-deprived rats after ovariectomy. To assess this, urinary concentration of deoxypyridinoline (a marker of bone resorption) and of calcium, the circulating levels of calcium, phosphorus and bone alkaline phosphatase activity (a marker of bone formation) were measured in adult rats receiving or not melatonin in the drinking water for up to 60 days after ovariectomy or its sham-operation. At the time of sacrifice, bone mineral density (BMD), bone mineral content (BMC) and bone area (BA) of total body were also assessed.

Methods

Female Wistar rats (5 month-old) raised in our colony were kept under a 12:12h light-dark cycle (lights off at 1800 h) with access to food and water ad libitum. Ovariectomy or the corresponding sham-operation was performed under equitesine (0.3 ml/100 g body weight). Adequate measures were taken to minimize pain or discomfort, in accordance with the principles and procedures outlined in European Communities Council Directives (86/609/EEC).

Melatonin (Sigma Chemical Co., St. Louis, MO) dissolved in ethanol was added to the drinking water at a concentration of 25 μg/ml; the final ethanol concentration was 0.01% for both melatonin-treated and control rats. The water bottles were covered with aluminum foil and fresh solutions prepared twice weekly. As reported elsewhere, adult rats drank about 20 ml/day with 90–95% of this total daily water taken up during the dark period [12]. Thus, the melatonin dosage used provided approximately 500 μg melatonin/day. Nocturnal water consumption was not different between melatonin-treated and control rats.

The rats were divided into 4 groups (10 animals each), as follows: 1) sham-operation + water; 2) sham operation + melatonin; 3) ovariectomy + water; 4) ovariectomy + melatonin. Immediately before surgery and at 15, 30 and 45 days after surgery blood samples were taken from a tail wound under light ether anesthesia. A final sample was taken at 60 days, at the time when rats were killed by excess exposure to diethylether. Twenty-four h urine samples were collected at days –1, 30 and 60 by means of individual metabolic cages.

The concentration of phosphorus was measured in serum and urine by a colorimetric method [13]. Serum and urinary calcium were measured by atomic absorption spectrophotometry [14]. Serum bone alkaline phosphatase activity was estimated by using wheat-germ

agglutinin [15]. Deoxypyridinoline Crosslinks were assessed by using a commercial assay (Pyrilinks-D; Metra Biosystems, Inc. USA). Data on urinary calcium and deoxypyridinoline were expressed as a ratio with urinary creatinine concentration.

Bone mineral content (BMC, bone area (BA) and bone mineral density (BMD) of total body were determined at the time of sacrifice by using a total body scanner and specifically designed software for small animals (DPX L, Small Animal Software, Lunar Radiation Corp., Madison, WI). Results were expressed as g (BMC), cm^2 (BA) or g/cm^2 (BMD). The coefficient of variation (expressed as a percent standard deviation of the mean) was 3.0 % for BMC and 0.9 % for BMD [16]. Estradiol was measured in diethylether-extracted serum samples of an independent group of 3 month-old female rats killed 30 or 60 days after ovariectomy or its sham-operation. A commercial kit was employed following the instructions provided with the kit (Diagnostic Products Corporation, Los Angeles, CA).

Statistical analysis of results was performed by a one-way analysis of variance (ANOVA) followed by a Tukey's or a Dunnett's t test, by a factorial ANOVA or by a Student's t test. Mean values were considered significantly different if $p<0.05$.

Results

Figure 1 shows urinary calcium and deoxypyridinoline excretion, expressed as ratio to urinary creatinine excretion, in ovariectomized and sham-operated rats receiving or not melatonin. Calcium excretion was similar in the 4 experimental groups when examined 30 and 60 days after surgery.

In sham-operated rats not receiving melatonin an age-dependent modification in urinary concentration of deoxypyridinoline was found, with significantly lower values at 7 months of age than at 6 months of age or the basal level ($p< 0.01$, ANOVA, Dunnett's t test). As compared to sham-operated rats, ovariectomized rats showed significantly higher deoxypyridinoline/creatinine ratios when assessed at 30 days after surgery (51% increase) and at 60 days after surgery (47% increase) (p[0.001, ANOVA). The increase in urinary deoxypyridinoline excretion found 30 days after ovariectomy was not observed in melatonin-treated rats whereas that found 60 days after ovariectomy remained unaffected by melatonin (Fig. 1).

Figure 2 depicts the results on serum calcium and phosphorus levels, and on serum bone alkaline phosphatase activity. By 15 days after surgery, circulating phosphorus levels augmented significantly in ovariectomized rats treated with melatonin as compared to similarly treated sham-operated rats ($p< 0.05$, ANOVA). By 15 days after surgery, a significant increase in serum alkaline phosphatase activity was found in ovariectomized rats receiving melatonin as compared to their respective sham-operated controls ($p< 0.01$, ANOVA).

Figure 3 shows BMD, BMC and BA of total body in the 4 experimental groups at the time of sacrifice (60 days after surgery). As a main factor in factorial ANOVA, ovariectomy decreased significantly the three parameters examined, an effect that was not modified by melatonin treatment (Fig. 3).

Estradiol levels after ovariectomy or its sham-operation, as determined in an independent group of rats 30 and 60 days after surgery, are depicted in Fig. 4. Circulating estradiol decreased significantly by 30 days after ovariectomy to attain values close to the limit of detection of the assay by 60 days after ovariectomy.

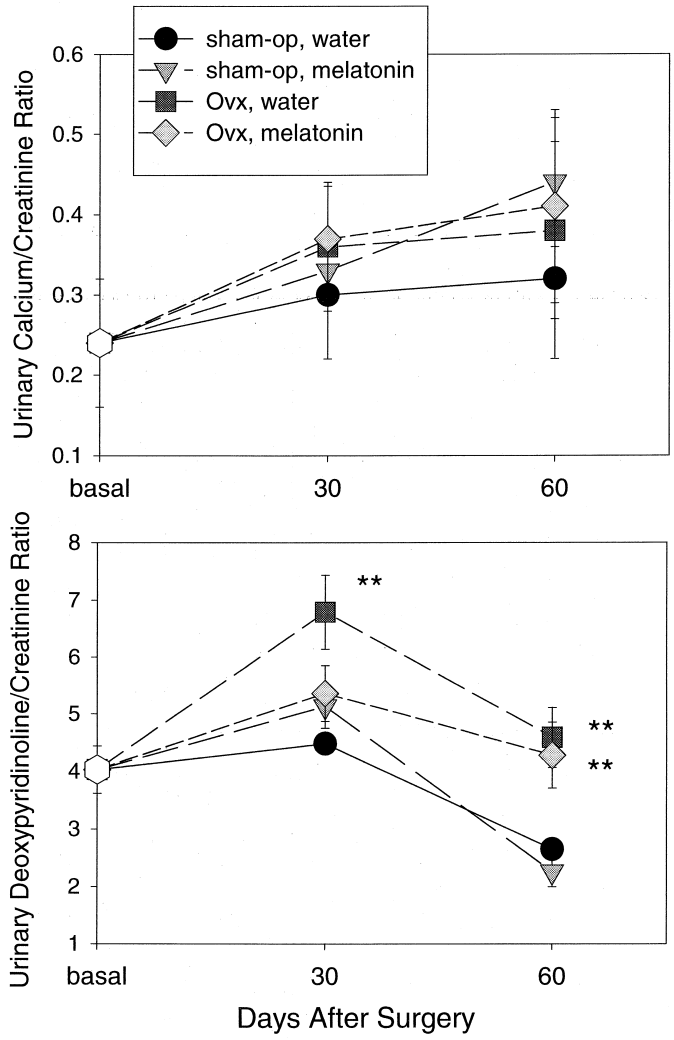

Fig. 1. Urinary calcium and deoxypyridinoline excretion in rats subjected to ovariectomy (Ovx) or its sham-operation and administered or not with melatonin in the drinking water for 60 days, as described in Methods. Shown are the means ± SEM of 8–10 rats/group. Values at 0 time represent the sum of the 4 experimental groups (n= 40). ** $p < 0.01$ as compared to the respective sham-operated group (ANOVA, Tukey's test).

Discussion

The foregoing results support the hypothesis that melatonin can assist, when administered in pharmacological amounts, in preventing bone remodeling after ovariectomy in rats. The increase in urinary concentration of deoxypyridinoline (a marker of bone resorption) observed 30 days after ovariectomy was curtailed by giving melatonin (about 500 μg/day) in drinking water to the rats whereas that found 60 days after ovariectomy remained unaffected. Also a marker of bone formation like serum bone alkaline phosphatase activity, as well as

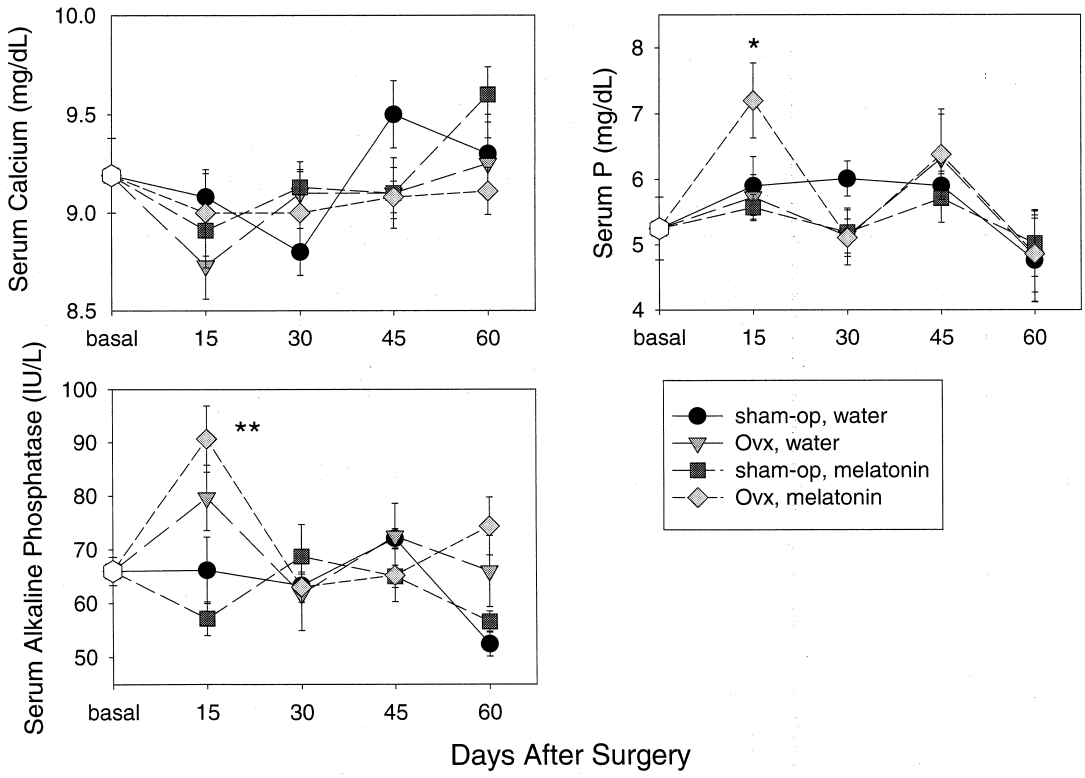

Fig. 2. Serum levels of calcium, phosphorus and bone alkaline phosphatase activity in rats subjected to ovariectomy (Ovx) or its sham-operation and administered or not with melatonin in drinking water for 60 days, as described in Methods. Shown are the means ± SEM of 8–10 rats/group. Values at 0 time represent the sum of the 4 experimental groups (n= 40). * $p < 0.05$, ** $p < 0.01$ as compared to sham-operated rats drinking melatonin (ANOVA, Tukey's test).

serum phosphorus levels, augmented in melatonin-treated rats at an early time (15 days) after ovariectomy, but not later. At the time of sacrifice, ovariectomy decreased significantly BMD, BMC and BA of total body, an effect that was unmodified by melatonin treatment.

Serum estradiol decrease after ovariectomy is biphasic, with an impending decrease a few days after surgery and a slower decline thereafter to attain values not differing from assay limit after 45 days [17]. Indeed, the residual levels of estradiol reported herein 30 days after ovariectomy had been seen in other studies [17,18]. Hence, one possible explanation for the time-related effect of melatonin after ovariectomy could be that the effect of melatonin is dependent on the presence of a sufficient estrogen concentration in the circulation. Relevant to this, receptors for melatonin are modulated by gonadal steroids in animals [19,20] and some of the biologic responses to melatonin are also modulated by gonadal steroids in women [21,22]. For example, a reduced adrenergic response to melatonin was observed in hypoestrogenic postmenopausal women even when the circulating levels of melatonin were kept in the pharmacological range [23]. Further studies are needed to assess whether estradiol

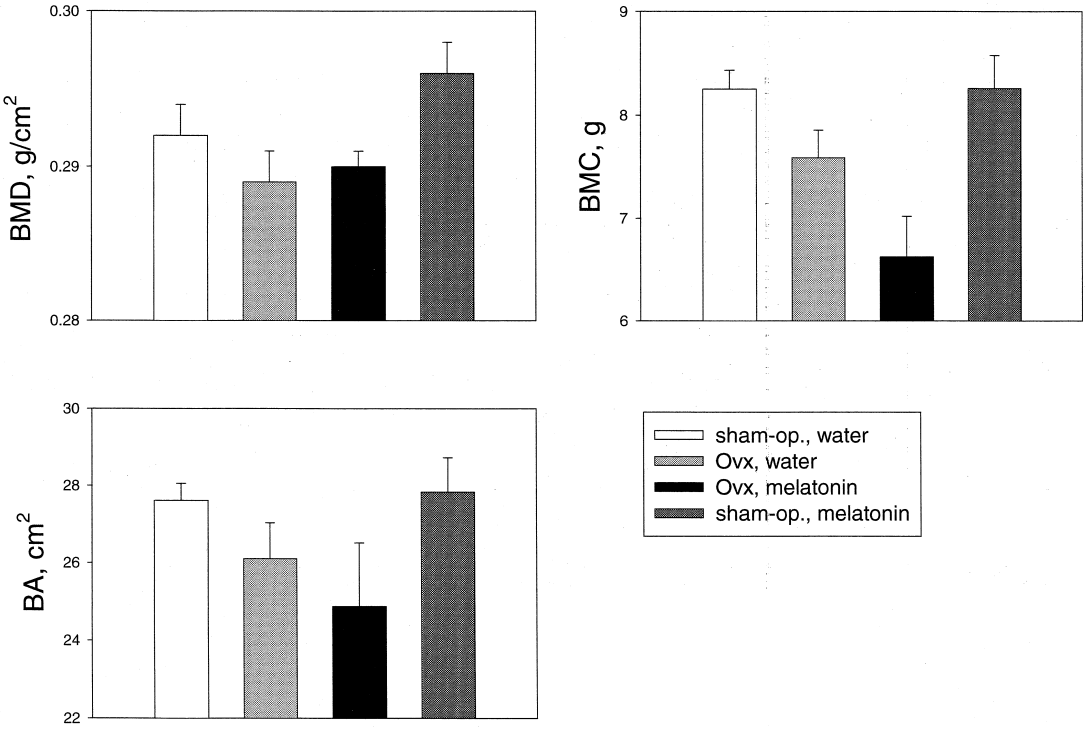

Fig. 3. Total body bone mineral content (BMC), bone mineral density (BMD) and bone area (BA) in rats subjected to ovariectomy (Ovx) or its sham-operation and administered or not with melatonin in the drinking water for 60 days, as described in Methods. Shown are the means ± SEM of 8–10 rats/group. As a main factor in factorial ANOVA, ovariectomy decreased significantly the three parameters examined (BMD: $F = 6.71$, $p < 0.02$; BMC: $F = 14.07$, $p < 0.002$; BA: $F = 5.83$, $p < 0.03$). The effect was unmodified by melatonin treatment, as shown by a non-significant factor interaction in factorial ANOVA.

treatment of rats ovariectomized 60 days earlier can restore the response to melatonin observed by 30 days after ovariectomy.

Two in vitro studies gave credit to the existence of a significant effect of melatonin in bone. Melatonin dose-dependently increased the proliferation in normal human bone cells and human osteoblastic cell lines [9]. Melatonin also increased procollagen type I c-peptide production (a measure of type I collagen synthesis) in these cells. The maximal effect in that study was observed at a 50 μM melatonin concentration [9]. In another study performed to determine whether melatonin could modulate in vitro the expression of rat bone sialoprotein in pre-osteoblast and rat osteoblast-like osteosarcoma cell lines, an increased gene expression of sialoprotein and other bone marker proteins, including alkaline phosphatase, osteopontin, secreted protein and osteocalcin was reported at a melatonin concentration as low as 10 nM [10]. Therefore, melatonin appears to be capable of promoting osteoblast differentiation and mineralization of matrix in vitro.

Osteoclasts use a variety of chemical agents to degrade bone. One important component of this process is the generation of free radicals [24–26]. Osteoclasts generate high levels of

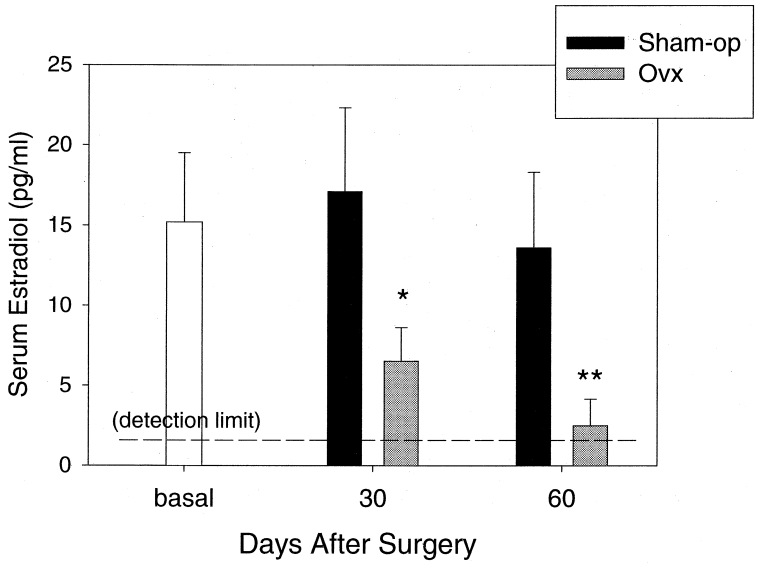

Fig. 4. Serum estradiol levels in rats subjected to ovariectomy (Ovx) or its sham-operation. Shown are the means ± SEM of 6 rats/group. Value at 0 time represents the sum of the 2 experimental groups (n= 12). * $p< 0.05$, ** $p< 0.01$ as compared to sham-operated rats, Student's t test.

superoxide anions during bone resorption that contribute to the degradative process. One mechanism for their removal is via the protective superoxide-scavenging enzyme superoxide dismutase [27].

Melatonin is a significant free radical scavenger and antioxidant at both physiological and pharmacological concentrations [28]. Besides its ability to directly neutralize a number of free radicals and reactive oxygen and nitrogen species, melatonin stimulates several antioxidative enzymes that increase its efficiency as an antioxidant. Therefore, the effect of melatonin in ovariectomy-induced changes in bone may depend in part on the free radical scavenging properties of melatonin.

Conclusion

Post-ovariectomy disruption of bone remodeling (a highly regulated process in the mammalian skeleton) could be prevented in rats by administering a pharmacological amount of melatonin in a way apparently related to the levels of circulating estradiol. Further studies are needed to define the precise roles that melatonin plays in bone development and in formation and degradation of bone and their dependence of circulating estrogen concentration.

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