Relative Effects of Prolactin Excess and Estrogen Deficiency on Bone in Rats

Robert A. Adler, Renuka Evani, Arash Mansouri, and Richard J. Krieg, Jr

Humans with prolactinoma are at risk for osteoporosis. The relative contributions of hyperprolactinemia-induced hypogonadism and the prolactin (PRL) excess per se have been unclear from clinical studies. To determine the effects of PRL excess, two models of chronic hyperprolactinemia were used. In one, mild hyperprolactinemia was produced in rats bearing extra anterior pituitary glands under the kidney capsule. Severe hyperprolactinemia was produced by subcutaneously transplanting the PRL-secreting MMQ tumor into other rats. To control for estrogen deficiency, the rats were ovariectomized. In some experiments, estrogen replacement was provided. Urinary calcium excretion was increased in hyperprolactinemic rats compared with controls, regardless of severity of PRL excess and estrogen status. This suggested that PRL excess itself had some effect on calcium balance. More importantly, however, the spinal bone mineral density (BMD; measured by dual-energy x-ray densitometry) of mildly hyperprolactinemic ovariectomized rats was the same as control ovariectomized rats. Similarly, tibial dry weight and ash weight were affected by the estrogen status, but not by the severe PRL excess of the tumor-implanted rats. Thus, despite the evidence for an increase in urinary calcium excretion in hyperprolactinemic rats, estrogen deficiency is much more important in determining bone mineral. Therefore, the present data indicate that the osteoporosis of hyperprolactinemia is likely due to PRL-induced hypogonadism, rather than a direct effect of PRL on calcium homeostatis.

HUMANS WITH PROLACTINOMA are at risk for osteoporosis. In 1980, Klibanski et al[1] reported that young hyperprolactinemic women had bone mineral density similar to that of postmenopausal women. This report was followed by several others[2-4] in which the relative contributions of estrogen deficiency caused by the prolactin (PRL) excess and a more direct effect of PRL were debated. Biller et al[2] found that women with hyperprolactinemia had considerable bone loss that was related to estrogen status. In contrast, Schlechte et al[3] found no correlation between the magnitude of bone loss and estrogen level. The finding that women with hyperprolactinemia lost bone implied that treatment of the PRL excess was necessary to prevent further bone loss. However, as discussed by Wardlaw and Bilezikian,[5] the clinical studies did not determine the mechanism of the bone loss. For several years, our laboratory has been using rat models of human prolactinoma to explore the contributions of chronic PRL excess and hypogonadism to the loss of bone mineral.[6-9] Hyperprolactinemic rats excrete excess calcium in the urine[7-10] and have some evidence of bone loss.[9,10] The purpose of the present study was to induce chronic PRL excess in an accepted rat model of postmenopausal osteoporosis, the 6-month-old ovariectomized rat.[11,12] Using two separate models of chronic PRL excess, we found that estrogen deficiency is the more important contributor to bone loss.

METHODS AND MATERIALS

Model of Mild Hyperprolactinemia

The classic model of chronic hyperprolactinemia has been the rat implanted with extra anterior pituitary glands under the kidney capsule.[13] The advantage of this model is that the rats eat and grow normally[14] and, as previously summarized,[13] have generally normal endocrine function. The model was modified for these experiments because an accepted model of postmenopausal osteoporosis is the ovariectomized 6-month-old rat.[11,12] Ovariectomized 6-month-old Fischer 344 rats (Hilltop Laboratories, Scottville, PA) were used as host rats. Donors were younger Fischer 344 rats that had been previously implanted with a diethylstilbestrol pellet (Innovative Research of America, Sarasota, FL) for 10 days before harvesting the pituitary. This pretreatment of the donor rat resulted in considerable growth of the pituitary gland, which was subsequently implanted under the kidney capsule of the host rats. Control ovariectomized rats were implanted with muscle tissue from the estrogen-treated donors. Studies from Esquifino et al[15] have shown that there is no carryover of estrogen to the host rats after receiving pituitary grafts. In an additional group of ovariectomized 6-month-old Fischer 344 rats, 2.5 pituitary glands or muscle tissue from estrogen-primed donors were implanted under the kidney capsule. Using the inbred strain of Fischer 344 rats prevents rejection of the pituitary graft[13]; the kidney capsule was assessed for the presence of hypophyseal tissue when the rats were killed.

Model of Severe Chronic Hyperprolactinemia

In contrast to the anterior pituitary grafted rat, which has mild hyperprolactinemia, rats bearing transplantable pituitary tumors often have serum PRL levels four or five orders of magnitude higher than controls. The Buffalo rat bearing the PRL-only tumor, MMQ, has been characterized as having levels of serum PRL of 1,000 to 20,000 ng/mL.[16] To study the effects of estrogen lack and replacement, 3-month-old ovariectomized Buffalo rats (Harlan Sprague Dawley, Indianapolis, IN) were divided into four groups: tumor plus estradiol pellet (approximate physiologic levels from 0.1 mg pellet; Innovative Research of America), tumor plus placebo pellet, control plus estradiol pellet, and control plus placebo pellet. Tumors grew vigorously in both estradiol- and placebo-treated rats. Tumor-bearing rats grow normally for the first 2 or 3 weeks after implantation,[15] after which the gross weight of the tumor-bearing rats exceeds that of controls. However, the weight of the rat minus the tumor weight is generally less than the body weight of control rats.

From the Endocrinology Section, Medical Service, McGuire Veterans Affairs Medical Center; and Departments of Internal Medicine and Anatomy, Medical College of Virginia/Virginia Commonwealth University, Richmond, VA.

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Address reprint requests to Robert A. Adler, MD, Chief, Endocrinology and Metabolism (111-P), McGuire Veterans Affairs Medical Center, 1201 Broad Rock Blvd, Richmond, VA 23249.

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Studies of Urinary Calcium Excretion

Approximately 1 month after pituitary graft or muscle implantation, the Fischer 344 rats were placed in individual metabolic cages for two 3-day periods for collection of urine, while the rats ate normal laboratory chow (Purina Laboratory Chow 5001, 1.2% calcium; Purina Mills, Richmond, IN) and tap water ad libitum. As described in previous studies,[6,8,9] urine was collected, measured, acidified with 1*N* HCl, and an aliquot was frozen at -20°C for later assay of calcium using the standard clinical laboratory Ektachem method. Calcium excretion is expressed as milligrams per 100 grams body weight per 24 hours.

Studies of Bone Mineral Density

Two to 6 months after pituitary or muscle implantation, Fischer 344 rats were anesthetized with pentobarbital or brevital (50 mg/kg intraperitoneally) and ketamine (10 mg/kg intramuscularly). Two or three consecutive measurements of bone mineral density (BMD) of lumbar vertebrae 1-4 were performed using a Hologic (Waltham, MA) 1000-W dual-energy x-ray densitometer and small animal software supplied by the manufacturer.[11,12] The coefficient of variation of repeated BMDs in the same rats was 2.3%.

Studies of Bone Mineral Content

Ovariectomized Buffalo rats, with and without severe hyperprolactinemia, were euthanized 1 month after tumor or sham implantation. The right tibia was removed, dried, weighed, and ashed.

Measurement of Serum PRL Levels

When the rats were killed, blood was obtained under anesthesia with pentobarbital and ketamine from the aorta. It was frozen at -20°C for later immunoassay for PRL, as previously described.[13]

Statistics

Data are expressed as means ± SEM. Differences between groups were determined using Student's *t* test for unpaired data using Instat software (Graph Pad Software, San Diego, CA). A *P* value of less than .05 was considered statistically significant.

RESULTS

Effects of Mild Hyperprolactinemia on Urinary Calcium Excretion

Fischer 344 rats were placed in individual metabolic cages for determination of urinary calcium excretion. As shown in Table 1, urinary calcium excretion was increased in rats with mild hyperprolactinemia. This was a function of the pituitary graft, because all rats had been ovariectomized and none had received hormone replacement. Although the rats were not pair-fed, previous studies have shown that the increased urine calcium excretion in similar rats is not due to alterations in eating behavior in pituitary-grafted rats.[6] In tumor-bearing rats with PRL levels of 1,000 to 2,000 ng/mL, the excess urinary calcium excretion is also not due to altered feeding behavior.[9] Pituitary-grafted and control rats had similar body weights at the time of urine collection and BMD measurements (data not shown).

Table 1. Urinary Calcium and Serum PRL in Fischer 344 Rats

Ovariectomized Rat Group	Urinary Calcium Excretion (mg/100 g BW/24 h)	Serum PRL (ng/mL)
Control (n = 8)	4.8 ± 0.5	23.4 ± 1.3
Pituitary graft (n = 8)	8.2 ± 1.0*	45.0 ± 7.0*

Abbreviation: BW, body weight.
*$P < .01$.

Table 2. Lumbar BMD in Fischer 344 Rats

Ovariectomized Rat Group	BMD L1-4 at 3 Months (g/cm²)	BMD L1-4 at 5 Months (g/cm²)
Group I (pituitary-grafted rats received 1 AP under the kidney capsule)		
Control (n = 8)	0.168 ± 0.002	
Pituitary graft (n = 8)	0.164 ± 0.003	
Group II (pituitary-grafted rats received 2.5 AP under the kidney capsule)		
Control (n = 6)	0.171 ± 0.006	0.166 ± 0.005
Pituitary graft (n = 6)	0.165 ± 0.002	0.155 ± 0.002

Abbreviation: AP, anterior pituitary.

Effects of Mild Hyperprolactinemia on BMD

As shown in Table 2, at approximately 2 months after pituitary or muscle implantation, BMD measurements by dual-energy x-ray densitometry of the lumbar spine (L1-4) were slightly lower in two groups of ovariectomized pituitary-grafted rats compared with respective ovariectomized control rats, but the differences were not statistically significant. The second group of pituitary-grafted rats was studied again, 5 months after pituitary or muscle grafting. At this time, the BMD in L1-4 (Table 2) was slightly lower in the grafted rats compared with controls, which was at the edge of statistical significance ($P = .0572$).

Effects of Severe Hyperprolactinemia on Bone

Buffalo rats implanted with the subcutaneous MMQ tumor cannot undergo BMD measurements by dual-energy densitometry because the tumor itself prevents the anesthetized rat from being placed supine on the densitometer table. Thus, measurements of tibial bone were substituted for densitometry. When the rats were killed approximately 1 month after tumor or sham implantation, the right tibia was removed. Table 3 shows the tibial dry weight and ash weight in a group of ovariectomized Buffalo rats. Rats with the MMQ tumor had lower tibial and ash weights. Estrogen-replaced rats had higher tibial weights than those that received a placebo pellet. Nonetheless, compared with the effect of estrogen deficiency, the tumor had little effect on ash weight.

DISCUSSION

In ovariectomized Fischer 344 rats implanted with pituitary or muscle grafts at 6 months of age, mild hyperprolactinemia was associated with elevated urinary calcium excretion. This

Table 3. Tibial Weights in Buffalo Rats

Rat Group	Tibia Dry Weight (mg)	Tibia Ash Weight(mg)
Control ovariectomy + placebo (A) (n = 5)	214.6 ± 3.5	0.1388 ± 0.0023
Control ovariectomy + estradiol (B) (n = 5)	221.2 ± 4.2	0.1487 ± 0.0029
Tumor ovariectomy + placebo (C) (n = 5)	203.2 ± 3.8	0.1341 ± 0.0025
Tumor ovariectomy + estradiol (D) (n = 6)	222.8 ± 4.7	0.1477 ± 0.0037

	Dry Weight *P* Values	Ash Weight *P* Values
A *v* B	.2663	.0271
A *v* C	.0590	.2092
A *v* D	.2106	.0856
B *v* C	.0132	.0054
B *v* D	.8100	.8315
C *v* D	.0114	.0185

confirms a previous study in younger Fischer rats[8] similarly studied. Thus, this small alteration in calcium balance was not due to PRL-induced hypogonadism, because all rats had been ovariectomized. Both male and female Buffalo rats implanted with the pituitary tumor MMQ have severe hyperprolactinemia and increased urinary calcium excretion.[9,16] In these rats, the urinary calcium excretion is closely correlated to the serum PRL levels.[9] In earlier studies,[6-9] the filtered load of calcium was found to be unaltered by PRL excess, suggesting an intrarenal mechanism for the hypercalciuria. Thus, it can be concluded that at least part of the calcium abnormalities of hyperprolactinemia can be ascribed to the PRL excess itself, rather than to estrogen deficiency.

Nonetheless, measures of bone mineral in ovariectomized rats were not affected markedly by the presence or absence of hyperprolactinemia. In two groups of rats studied 2 months after pituitary or muscle implantation, there was no difference in lumbar BMD between normoprolactinemic and hyperprolactinemic rats. Only after 5 months of hyperprolactinemia was a small difference in BMD possibly demonstrated. In contrast, ovariectomy leads to a much larger decrease in BMD after only 4 weeks.[11] Fiore [17] reported that male rats made hyperprolactinemic by implantation of two extra anterior pituitary glands under the kidney capsule had lower femoral bone density (as measured by single photon absorptiometry) than sham-operated rats. Although serum testosterone levels are generally normal in pituitary-grafted male rats,[13] other data suggest more subtle changes of androgens in such animals. Thus, the relative contribution of sex hormone deficiency to PRL excess cannot be ascertained in the study of Fiore[17] The hypercalciuria of the pituitary-grafted rats might lead to secondary hyperparathyroidism, which in turn could affect trabecular and cortical bone differently. However, we have demonstrated that cyclic adenosine monophosphate (AMP) excretion in not affected by the presence of pituitary grafts[6] in Fischer rats or by the MMQ tumor[9] in Buffalo rats. Thus, abnormal parathyroid hormone secretion is unlikely in these two models.

Similar to the findings in the mildly hyperprolactinemic rats, in a model of severe hyperprolactinemia, we find that estrogen deficiency was clearly more important than PRL excess in determining tibial dry weight and ash weight. In this latter model, the study lasted only 1 month, and although the weights of the tumor-bearing rats (minus the weight of the tumor itself) and that of the controls are comparable, more subtle nutritional differences could play a role. However, in pituitary grafted animals, BMD was not significantly lower even after 5 months of hyperprolactinemia. In addition, nutritional factors are unlikely to have played a role in bone because pituitary-grafted rats eat and grow normally.[14] Furthermore, dietary calcium intake does not play a mechanistic role in the hypercalciuria of either pituitary-grafted[6] or tumor-implanted[9] rats.

In addition to the studies of osteoporosis in prolactinoma patients, another group of hyperprolactinemic humans have been tested for bone loss. Halbreich et al[18] measured BMD in the spine and hip of psychiatric patients, many of whom were on neuroleptic medications that raise the serum PRL level. They found several correlations between hormone levels and BMD, although there was evidence to relate both PRL and sex hormone levels to various measures of bone density.

In a recent study of humans with prolactinoma,[19] Stiegler found that levels of parathyroid hormone–related peptide (PTHrP) correlated with BMD in hyperprolactinemic patients. In addition, Sowers[20] recently hypothesized that PTHrP is responsible for bone loss found in women with normal lactation. Studies are planned to assess this new calciotropic hormone in various rat models of PRL excess.

In conclusion, hyperprolactinemia, regardless of estrogen status, is responsible for a minor part of the calcium abnormalities in rat models of human prolactinoma. Estrogen deficiency, while not responsible for all of the calcium loss, is clearly the more important determining factor of bone mineral in these models. Thus, the present data indicate that PRL-induced estrogen (or testosterone) deficiency is the more important determinant of bone loss in hyperprolactinemia.

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