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Sequential Histomorphometric Changes in Cancellous Bone from Ovariohysterectomized Dogs

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ABSTRACT

To evaluate potential pharmacologic agents for the prevention or treatment of the bone loss associated with ovarian insufficiency, a predictable animal model is needed. To assess the potential utility of the ovariohysterectomized dog as a model of this condition, we characterized the sequential histomorphometric changes in canine cancellous bone in response to the loss of ovarian function. A group of 25 adult beagle dogs were ovariohysterectomized and terminated at 1, 3, 6, and 10 months following surgery. Iliac biopsies were performed following double-fluorochrome labeling at the time of surgery and at termination. Static and dynamic histomorphometry was performed on undecalcified sections. By 3 months postovariohysterectomy, there was activation of cancellous bone remodeling as indicated by significant increases in mineralizing surface and bone formation rate. Increases in osteoid surface, mineralizing surface, and bone formation rate were also apparent at 1 month postovariohysterectomy, and although not statistically significant, these trends suggest the skeletal response to acute loss of ovarian function was rapid. This increase in bone remodeling was transient. By 6 months, mineralizing surface and bone formation rate were depressed below presurgical levels. In addition to a reduction in bone formation, a reduction in osteoblast function characterized by reduced labeling of osteoid and a disproportionate increase in eroded surface also occurred. By 10 months postovariohysterectomy, cancellous bone remodeling was not significantly different from presurgical levels. At no time was a significant reduction in bone volume detected. These data suggest that the changes in cancellous bone remodeling in the ovariohysterectomized dog are a series of transient phenomena. The duration and/or magnitude of these changes do not appear to be sufficient to effect a sizable or significant reduction in bone volume. The brief nature of the transients in bone remodeling and the lack of a sizable bone loss in the ovariohysterectomized dog limit the utility of this model for the routine assessment of agents for either the prevention or treatment of bone disease associated with ovarian insufficiency.

INTRODUCTION

ALTHOUGH MANY FACTORS may contribute to the ultimate bone loss associated with the menopause, the reduction in circulating estrogen appears to play a major role in the pathogenesis of the osteopenia. A predictable and reproducible animal model of bone loss associated with ovarian insufficiency is needed to evaluate pharmacologic agents that may be of potential therapeutic value in the prevention or treatment of this condition in humans. Because common laboratory animals do not experience a natural menopause, preclinical studies examining the effect of estrogen deficiency on the skeleton have used surgical ovariectomy or ovariohysterectomy to create an artificial menopause. The oophorectomized rat has been used extensively.[1,2] Although oophorectomy results in a significant bone loss in this species, the use of this model is restricted by the fact the rat has somewhat limited basic multicellular unit-based remodeling. The dog, however, possesses a remodeling system similar to that of the human, and several

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studies have evaluated the effects of ovariectomy or ovariohysterectomy on canine cancellous bone to assess the dog's potential utility as a model of postmenopausal osteoporosis.[3-5] Based on these studies, the histologic response of the dog to loss of ovarian function appears to be heterogeneous. The apparent variations in these data may in part be owing to a variation in the duration of the post-ovariectomy period. To address the issue of a time-dependent variation in cancellous bone remodeling in response to acute loss of ovarian function in the dog, this study was conducted to characterize the sequential histomorphometric changes in canine cancellous bone at 1, 3, 6, and 10 months following ovariohysterectomy and determine the potential utility of the ovariohysterectomized dog as a model of postmenopausal osteoporosis.

MATERIALS AND METHODS

A group of 25 female beagle dogs, 18 months of age, was obtained from Laboratory Research Enterprises, Inc. (Kalamazoo, MI). Dogs were randomized by weight into four groups and housed in pairs in 5 × 11 foot runs. A complete physical examination, including routine hematology and serum biochemistries, was performed. Dogs were acclimated to the environment for 4 months. Standard Purina dog chow (1.8% calcium, 1.0% phosphorus, 25.5% protein, 9% fat, 3.1% fiber, and 9.8% ash) and water were given ad libitum during the acclimation and study periods.

A routine surgical ovariohysterectomy[6] and iliac bone biopsy was performed on each animal. Dogs were preanesthetized with acepromazine (0.2 mg/kg IM) and atropine (0.06 mg/kg IM), and surgical anesthesia was induced and maintained with pentobarbital (28 mg/kg IV). Postoperatively dogs received pentazocine (0.7 mg/kg IM, Winthrop Lab, NY, NY) every 6 h for the first 24 h and ampicillin (5 mg/lb per os every 12 h, A.H. Robins, Richmond, VA) for 5 days. At the time of surgery, an 8 mm diameter transcortical bone biopsy specimen was taken from the right ilium (approximately 0.5 cm immediately ventral to the anterior iliac spine) by means of a Michele vertebral trephine.

Dogs were weighed at the time of surgery and at monthly intervals following ovariohysterectomy.

Serum estradiol and progesterone (Immunochem Estradiol and Direct Progesterone Kits, Immunochem Corp., Carson, CA) were determined in samples collected at the time of ovariohysterectomy and 1 and 3 months after surgery.

Dogs were terminated in groups by an intravenous overdose of pentobarbital at 1 (n = 7), 3 (n = 6), 6 (n = 6), and 10 (n = 6) months following ovariohysterectomy. At termination, a bone saw was used to collect a wedge biopsy specimen from the left ilium at the level of the anterior iliac spine in the anatomic region corresponding to the first biopsy site.

Double-fluorochrome labeling was performed before bone biopsies. The labeling schedule consisted of 2 days of label, 10 days without label, and 2 days of label followed by 4 days without label (2-10-2-4 protocol). Before the bone biopsies, each group of dogs received either tetracycline hydrochloride (Lederle Labs, Pearl River, NY) or calcein green (Sigma Chemical Co., St. Louis, MO), 20 mg/kg/day IV administered slowly over 10–15 minutes in 60 ml normal saline.

Bone biopsy specimens were processed undecalcified, embedded in methylmethacrylate, and sectioned at 5 and 12 mcm. All embedding, sectioning, and staining procedures were performed as described by Schenk et al.[7] The parameters described below were measured with a semiautomatic image analyzer (Bioquant II, R & M Biometrics, Nashville, TN) interfaced with an Olympus BH2 microscope (Olympus Optical Company LTD, Tokyo, Japan) fitted with an Integrationplatte II ocular reticule (Carl Zeiss Co., Thornwood, NY). The reticule was used to sample the bone surfaces for direct measurement of individual distances, that is, apposition rate and wall thickness.[8] A minimum of 50 mm cancellous bone surface was quantitated for both static and dynamic histomorphometric parameters. Cancellous bone within 350 mcm of the endocortical surface was excluded from measurement.

Static parameters were quantitated at a magnification of ×300 on 5 mcm sections stained with Goldner's modified trichrome. Dynamic parameters were quantitated at a magnification of ×150 and mineral apposition rate at ×300 on unstained 12 mcm sections. Static, dynamic, and calculated parameters[9] were selected for evaluation:

1. Osteoid surface (OS/BS, %): surface extent of osteoid expressed as percentage of total bone surface
2. Mineralizing surface, bone surface referent (MS/BS, %): surface extent of fluorochrome labels (double labels and half-single labels) expressed as the percentage of the total bone surface
3. Mineralizing surface, osteoid surface referent (MS/OS, %): surface extent of fluorochrome labels (double and half-single labels) expressed as the percentage of osteoid surface
4. Mineral apposition rate (MAR, mcm/day): mean distance between the double-fluorochrome labels divided by the labeling interval and multiplied by II/4[8]
5. Adjusted apposition rate (Aj.AR, mcm^3/mcm^2 per day): amount of mineralized bone formed per day per unit of osteoid surface:

$$Aj.AR = [(MS/BS) \times MAR/(OS/BS)]$$

6. Bone formation rate (BFR/BS, mcm^3/mcm^2 per day): amount of mineralized bone formed per day per unit of trabecular surface:

$$BFR/BS = [(MS/BS) \times MAR/100]$$

7. Eroded surface (ES/Md.S, %): surface extent of resorption lacunae expressed as a percentage of mineralized bone surface
8. Wall thickness (W.Th, mcm): mean distance from the cement line to the quiescent bone surface multiplied by Π/4
9. Bone volume (BV/TV, %): volume of mineralized bone and osteoid expressed as a percentage of total bone tissue, determined on 5 mcm von Kossa-stained sections with

an automatic image analyzer (Quantimet 720, Cambridge, Instruments, Deerfield, IL)

Individual values for each histomorphometric variable were determined pre- and postovariohysterectomy for each group of dogs. The difference in actual change between pre- and postovariohysterectomy values within a group was tested for significance ($p < 0.05$) with a paired t-test. The significance ($p < 0.05$) of the differences in actual change between groups was based on Fisher's least significant difference test.

RESULTS

Mean body weight and mean change for each group are given in Table 1. Body weight increased significantly in dogs 3, 6, and 10 months postovariohysterectomy.

Serum estradiol and progesterone values are given in Table 2. Serum estradiol and progesterone levels decreased significantly in dogs 1 and 3 months postovariohysterectomy.

The values of the static and dynamic histomorphometric parameters of cancellous bone remodeling obtained pre- and postovariohysterectomy for all groups are given in Table 3. No significant changes in histomorphometric parameters were noted in dogs evaluated 1 month following ovariohysterectomy, although mean values for osteoid surface, mineralizing surface, total surface referent, and bone formation rate were increased. Mineralizing surface, bone surface referent, and bone formation rate were increased in dogs examined 3 months following ovariohysterectomy. In dogs examined 6 months following ovariohysterectomy, mineralizing surfaces, bone and osteoid surface referent, and adjusted apposition rate were decreased and eroded surface was increased. Bone volume was increased in dogs evaluated 10 months after ovariohysterectomy. No other significant changes were noted in this group.

The mean changes in mineralizing surface, bone and osteoid surface referent, adjusted apposition rate, bone formation rate, and eroded surface are compared between groups in Table 3 and presented graphically in Fig. 1. The mean changes in mineralizing surface, bone surface referent, bone formation rate, and eroded surface at 1 and 3 months postovariohysterectomy were significantly different from the mean changes at 6 and 10 months postovariohysterectomy. The mean changes in mineralizing surface, osteoid surface referent, and adjusted apposition rate at 6 months were significantly different from the mean changes at 1 and 3 months but not from mean changes at 10 months postovariohysterectomy.

DISCUSSION

In the present study, the cancellous bone from dogs responded acutely to ovariohysterectomy with an initial increase in bone remodeling. The activation of cancellous bone remodeling was reflected in the significant increases in mineralizing surface, bone surface referent, and bone formation rate and a parallel but not significant increase in osteoid surface by 3 months postovariohystewrectomy. Osteoid surface, mineralizing surface, bone surface referent, and bone formation rates were also increased, although

TABLE 1. BODY WEIGHT (kg, MEAN ± SD) BEFORE AND AT 1, 3, 6, AND 10 MONTHS FOLLOWING OVARIOHYSTERECTOMY (OHE)[a]

	1 Month	*3 Months*	*6 Months*	*10 Months*
Mean weight pre-OHE	11.3 ± 1.1	12.1 ± 0.9	12.2 ± 1.4	11.0 ± 1.3
Mean weight post-OHE	11.4 ± 1.3	12.6 ± 1.1	14.3 ± 2.3	14.7 ± 1.6
Mean weight change	+0.2 ± 0.3	+0.6 ± 0.4[a]	+2.1 ± 1.5[a]	+2.9 ± 1.7[a]

[a] $P < 0.05$, paired t-test.

TABLE 2. SERUM ESTRADIOL AND PROGESTERONE (MEAN ± SD) BEFORE AND AT 1 AND 3 MONTHS POSTOVARIOHYSTERECTOMY (OHE)

	Pre-OHE	*1 Month post-OHE*	*3 Months post-OHE*
Estradiol, pg/ml	31.9 ± 10.1 (n = 25)	22.6 ± 6.6[a] (n = 25)	22.0 ± 3.7[a] (n = 6)
Progesterone, pg/ml	6.0 ± 8.2 (n = 19)	0.4 ± 0.3[b] (n = 8)	0.1 ± 0.1[b] (n = 3)

[a] $p < 0.001$, two-sample t-test.
[b] $p < 0.01$, two-sample t-test.

TABLE 3. STATIC AND DYNAMIC HISTOMORPHOMETRIC PARAMETERS BEFORE AND AT 1, 3, 6, AND 10 MONTHS FOLLOWING OVARIOHYSTERECTOMY (OHE)[a]

Post-OHE period	OS/BS (%)	MS/BS (%)	MS/OS (%)	MAR (mcm/day)	Aj.AR (mcm³/mcm per day)	BFR/BS (mcm³/mcm² per day)	ES/Md.S (%)	W.Th (mcm)	BV/TV (%)
1 Month									
Pre-OHE	26.5 ± 4.9	19.4 ± 8.4	72.0 ± 21.2	0.93 ± 0.09	0.69 ± 0.25	0.19 ± 0.09	18.8 ± 4.6	39.3 ± 4.1	21.6 ± 3.0
Post-OHE	34.9 ± 11.6	28.3 ± 11.2	83.5 ± 27.8	0.95 ± 0.10	0.78 ± 0.24	0.27 ± 0.09	18.2 ± 2.8	39.3 ± 4.0	23.0 ± 4.0
Mean change	8.5 ± 13.4	8.9 ± 10.9[b,c]	11.4 ± 13.7[b]	0.02 ± 0.16	0.09 ± 0.13[b]	0.08 ± 0.09[b]	−0.6 ± 4.7[b,c]	0.0 ± 5.9	1.4 ± 2.6
3 Months									
Pre-OHE	20.1 ± 8.2	18.8 ± 4.4	99.7 ± 21.1	1.04 ± 0.14	1.06 ± 0.28	0.20 ± 0.06	17.2 ± 2.9	40.5 ± 1.9	20.4 ± 2.3
Post-OHE	29.4 ± 6.0	30.6 ± 6.0	104.7 ± 13.8	1.08 ± 0.04	1.13 ± 1.18	0.33 ± 0.07	17.6 ± 6.7	42.9 ± 2.2	22.2 ± 3.3
Mean change	8.4 ± 13.8	11.8 ± 8.9[b,c,d]	4.2 ± 25.8[b]	0.04 ± 0.13	0.05 ± 0.24[b]	0.13 ± 0.10[b,c,d]	−1.8 ± 6.8[c]	2.0 ± 3.0	2.6 ± 2.7
6 Months									
Pre-OHE	29.8 ± 5.8	28.2 ± 5.2	95.8 ± 14.3	0.95 ± 0.17	0.89 ± 0.16	0.26 ± 0.04	17.1 ± 5.4	40.7 ± 2.5	23.7 ± 3.1
Post-OHE	27.6 ± 8.9	15.6 ± 5.9	58.6 ± 18.0	0.88 ± 0.16	0.53 ± 0.20	0.13 ± 0.04	25.5 ± 8.8	44.1 ± 3.2	24.5 ± 4.7
Mean change	−2.2 ± 7.7	−12.7 ± 8.4[d,e,f]	−37.2 ± 25.6[d,e,f]	−0.06 ± 0.27	−0.37 ± 0.27[d,e,f]	−0.13 ± 0.05[d,e,f]	8.3 ± 7.9[d,e,f]	3.5 ± 3.4	0.8 ± 3.9
10 Months									
Pre-OHE	25.3 ± 7.4	28.1 ± 5.4	115.5 ± 20.9	1.01 ± 0.10	1.18 ± 0.31	0.28 ± 0.05	12.9 ± 3.2	39.6 ± 4.6	18.7 ± 3.0
Post-OHE	23.8 ± 5.4	24.8 ± 8.2	102.9 ± 16.4	0.92 ± 0.09	0.98 ± 0.22	0.24 ± 0.09	14.3 ± 3.3	39.5 ± 4.9	22.0 ± 3.4
Mean change	−1.5 ± 10.1	−3.3 ± 7.9[e,f]	−12.6 ± 30.2	0.09 ± 0.16	−0.22 ± 0.44	−0.05 ± 0.09[e,f]	1.4 ± 5.5[e,f]	0.2 ± 2.3	3.3 ± 2.3[d]

[a]Values are presented as mean ± SD.
[b]Different from mean change at 6 months, $p < 0.05$.
[c]Different from mean change at 10 months, $p < 0.05$.
[d]$p < 0.05$.
[e]Different from mean change at 1 month, $p < 0.05$.
[f]Different from mean change at 3 months, $p < 0.05$.

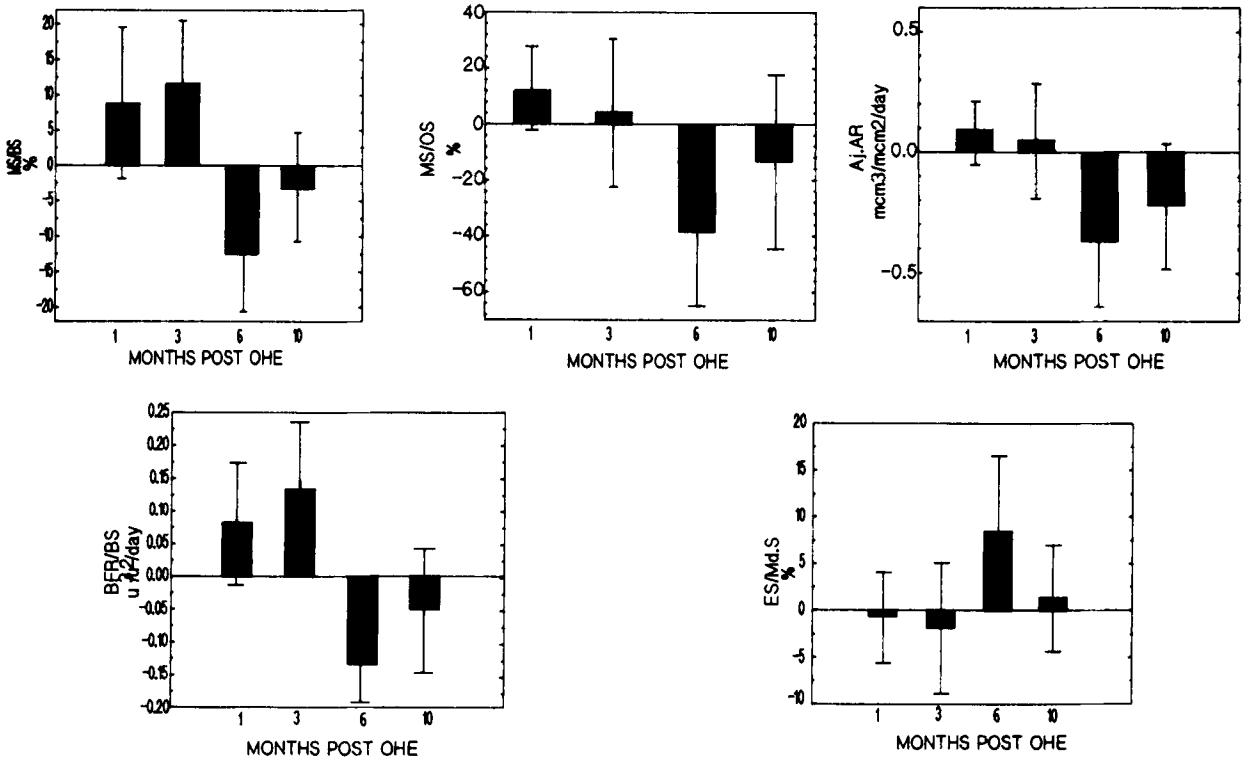

FIG. 1. Mean change ± SD in MS/BS, MS/OS, BFR/BS, Aj.AR, and ES/Md.S at 1, 3, 6, and 10 months postovariohysterectomy (OHE).

not significantly, 1 month postovariohysterectomy. These trends suggest that the skeletal response to the acute loss of ovarian function was rapid and occurred within the first month. This increase in activation and increase in bone turnover was transient, however. By 6 months postovariohysterectomy, there was no evidence of a sustained increase in mineralizing surface, bone surface referent, or bone formation rate; in fact, values for these parameters fell below preovariohysterectomy levels by 6 months. A concomitant and disproportionate increase in eroded surface was also evident at this time. These changes were associated with significant reductions in mineralizing surface, osteoid surface referent, and adjusted apposition rate. By 10 months postovariohysterectomy, cancellous bone remodeling was not significantly different from that observed preovariohysterectomy. Together, these data suggest that following ovariohysterectomy the initial increase in bone turnover is not sustained within the first 6 months. By 6 months, an imbalance in surface-referent bone formation and resorption evolved that was associated with reduced osteoblast function at the cellular level. By 10 months, the remodeling parameters returned to baseline values. This suggests that the changes in cancellous bone remodeling observed at 1, 3, and 6 months postovariohysterectomy are transient phenomena.[10]

The effect of ovariohysterectomy was apparent in the dogs by the decrease in mean values of serum estradiol and progesterone 1 and 3 months postovariohysterectomy. Also, mean body weight increased significantly in dogs 3, 6, and 10 months postovariohysterectomy. Body weight increases following ovariectomy have also been reported in rats[1,2] and dogs.[11]

In our study, bone volume did not decrease at any time point following ovariohysterectomy. In the 10 month group, there was a significant increase in bone volume when compared with preovariohysterectomy values, but this small increase is of questionable biologic significance. The apparent increase was probably owing to the relatively low preovariohysterectomy mean value for bone volume in this group. If bone loss occurred in response to the loss of ovarian function, it was not of the magnitude to be detected by histologic means. It should be noted that more sensitive physical measurement techniques for cancellous bone may have detected some bone loss in this model.

Many of the changes in cancellous bone remodeling observed in this study have been described in previous investigations evaluating the response of the canine skeleton to acute loss of ovarian function. The increase in cancellous bone turnover observed at 1 and 3 months in the present study corroborates the findings of Dannucci et al.,[4] who reported an increase in cancellous bone turnover 6 months after ovariectomy in 6-year-old beagle dogs.

Reduced bone formation rates and osteoblast function in cancellous bone have also been previously described in oophorectomized bitches. Malluche et al.[3] described osteopenia associated with osteoblastic insufficiency in 2-year-old beagle dogs evaluated 4 months postovariectomy. The osteoblastic insufficiency was characterized by

reduced apposition rate, mineralizing surface, osteoid surface referent, bone formation rate (BFR/BV and BFR/OB), wall thickness, and bone volume. In the present study, a reduction in mineralizing surface, osteoid surface referent, and bone formation rate (BFR/BS) was evident at 6 months postovariohysterectomy, but a reduction in wall thickness and bone volume was not detected. Martin et al.[11] also reported a 15% reduction in cancellous bone volume in the spine in 3- to 7-year-old beagle dogs at 11 months postovariectomy.

Snow et al.[5] compared bone remodeling in the first and third lumbar vertebrae from 4-year-old beagles, intact or 12 months following ovariectomy. These authors reported no significant differences in histomorphometric parameters of bone remodeling or bone volume between ovariectomized and intact bitches. Similarly, in the present study no significant changes in remodeling were noted in dogs 10 months postovariohysterectomy.

The apparent heterogenous response of the canine skeleton to loss of ovarian function may be due to several factors. The data from the present study suggest that the apparent heterogeneity in the response of canine cancellous bone to loss of ovarian function is at least in part related to the fact that these changes are transient phenomena that have a temporal sequence. Therefore, the time point selected for evaluation postovariectomy influences the results.

In addition to the duration of the postovariectomy period, the age and stage of the estrous cycle of the dog at the time of ovariectomy may greatly influence the temporal response of the skeleton to the loss of ovarian function. Concomitant hysterectomy may also have some influence on the skeletal response to ovariectomy. The present study differs from previous studies in that the dogs were younger and were ovariohysterectomized rather than ovariectomized.

Body weight could also influence the response of the skeleton to the loss of ovarian function, particularly changes in bone mass. An association between body weight, bone loss, and fracture incidence has been reported in postmenopausal women.[12] The lack of a measureable osteopenia in the ovariohysterectomized dogs in the present study may be in part be due to the increase in body weight that occurred in the dogs at 3, 6, and 10 months postovariohysterectomy. This weight gain may have protected the skeleton against bone loss. However, in older beagles, Martin et al.[11] reported a 15% bone loss in the spine in dogs that experienced approximately a 22% increase in body weight 11 months following ovariectomy. The increase in body weight in the present study was approximately 24%, 10 months following ovariohysterectomy. Wronski et al.[13] have demonstrated that the increase in body weight in ovariectomized rats only partially protects the long bones against osteopenia and that a substantial osteopenia still develops in obese ovariectomized rats. However, it cannot be totally excluded that the changes in body weight may have influenced in some way the skeletal response of the dogs in the present study.

The acute histologic response of human cancellous bone to the loss of ovarian function has not been extensively investigated, but calcium balance studies in women during natural menopause support an acute increase in bone turnover in response to loss of ovarian function.[14] Changes in biochemical markers of bone remodeling also support an increase in bone turnover in response to loss of ovarian function. Biochemical indices are increased in ovariectomized women; however, a disproportionate increase in serum markers of bone resorption occurs relative to serum markers of bone formation. The peak dissociation between the markers of resorption and formation is found to coincide with the maximum rate of bone loss following ovariectomy, thus underscoring the importance of the imbalance of formation and resorption in the genesis of the osteopenia.[15] This imbalance in remodeling is also apparent in histomorphometric data obtained from postmenopausal women, which demonstrate excessive resorption relative to formation.[16-18]

The data from the present study suggest the canine skeleton responds to ovariohysterectomy with a transient increase in bone turnover followed by a temporary imbalance in surface-referent bone formation and resorption and reduced osteoblast function. However, in this study, the duration and/or magnitude of these changes did not appear to be sufficient to effect an appreciable reduction in cancellous bone volume. In contrast, the increase in bone turnover and tissue-based imbalance in bone resorption and formation that occurs in the ovariectomized rat is of sufficient magnitude and duration to effect a dramatic reduction in bone volume.[1,2,19] The variability between species in sensitivity of cancellous bone to estrogen deficiency may relate to differences in the proportion of cancellous bone that is estrogen dependent.[20] This proportion may relate to the frequency of the estrous cycles. The rat is polyestrus, with a cycle length of 4–6 days. In contrast the dog is diestrus, cycling usually in the spring and fall.[21] An ovariectomized animal model using a higher animal with frequent estrous cycles may prove to be a more useful model of osteopenia associated with estrogen deficiency. This hypothesis is supported by the findings that a significant reduction in vertebral cancellous bone volume in response to ovariectomy occurs in the macaque,[22] a species that cycles monthly.[21]

In conclusion, we believe that the utility of the ovariohysterectomized dog as a model of postmenopausal osteoporosis is limited in the routine assessment of pharmacologic agents for the prevention and/or treatment of bone disease associated with the loss of ovarian function because of the transient and brief nature of the changes in remodeling and the apparent lack of a sizable bone loss.

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