Ovariectomy-Induced Changes in Aged Beagles: Histomorphometry and Mineral Content of the Rib

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Running title: Histomorphometry of Ribs from Ovariectomized Dogs

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ABSTRACT

The effects of ovariectomy on the aged beagle skeleton were studied by histomorphometric analysis of the cortical bone in sequential rib biopsies. Biopsies were taken from each ovariectomized (OV) or sham-operated (SO) dog at the time of surgery and at 1, 4, and 8.5 months after surgery. Tetracycline, calcein, and xylenol orange, respectively, were administered by a fluorochrome labeling procedure (2d-10d-2d) just prior to each postoperative biopsy to provide markers of bone formation. Analysis of sequential biopsies provided a means to follow the response to ovariectomy over time and compare each animal against its own baseline. Examination of sequential biopsies indicated that cortical porosity increased by the fourth month after ovariectomy and remained high at 8.5 months. Ovariectomy did not influence histomorphometric indices at one month after surgery, but substantial differences were observed at later times. Ovariectomy stimulated a transient increase in bone formation and was increased six-fold over that of SO dogs at four months. Ribs were also analyzed for mineral content at necropsy. The rib was heterogenous along its length for calcium content and concentration. In the midrib where biopsies for histomorphometric analysis were taken, ovariectomy induced a decrease in mass and mineral content; total calcium was decreased by approximately 31%. These data demonstrate that the rib cortical bone is a responsive site for the effects of ovariectomy in female dogs.

Key words: cortical bone, dog, estrogen-depletion, histomorphometry, ovariectomy, osteoporosis

INTRODUCTION

Bone loss associated with estrogen depletion has been documented in a number of experimental and naturally occurring situations in many species. Ovariectomy is the most common experimental model of estrogen depletion and in many species the changes that occur replicate many aspects of the peri- and post-menopausal human skeleton. Ovariectomy is associated with transient increases in the rate of cancellous bone turnover that result in losses of bone mass in rats, dogs and primates (for reviews, 15,20). Similar changes have been described following oophorectomy in humans (10,12,24).

While the effects of gonadal deficiency on cancellous bone in many species is well documented, especially in the rat, the effects on cortical bone are less clear. Short-lived species, such as rodents, exhibit changes in cortical bone following ovariectomy attributable to changes on the periosteal and endocortical surfaces (13,14,27). However, the rat is not a suitable model to address changes that may occur in the intracortical bone envelope as it lacks Haversian systems. In the dog, some of the changes that have been reported to occur in cortical bone following ovariectomy include a transient loss of bone mineral at 2 months (17) and bone loss at 7 months (11) as measured by photon absorptiometry and increased resorption spaces one year after surgery (17,23). Ovariectomy of nonhuman primates also resulted in significant changes in intracortical bone after two years (16). Cortical bone loss in humans in the first year after oophorectomy was approximately 2.8% (24), with the rate of loss decreasing with time after surgery (12,24).

This study examines the effects of estrogen depletion at 1,4, and 8.5 months after ovariectomy in cortical bone of the ribs of mature beagles. The rib specimens were obtained from sequential biopsies, providing time-response information from each animal. The histomorphometric and biochemical analyses presented in this manuscript are part of a larger study that investigated the effects of low-level cadmium exposure and estrogen depletion on the skeletons of aged female beagles (21,22).

MATERIALS AND METHODS

Animals

Seven female beagles obtained from the Argonne National Laboratory breeding colony and Laboratory Research Enterprises (Kalamazoo, MI) were housed individually in metabolism cages. All dogs were 7–9 years old at the start of the experiment and were meal fed for one hour daily (0800-0900 hours) with approximately 300 g of Purina Scientific Animal Feeds Special Canine Diet (0.5% calcium, 0.5% phosphorous, 4.4 IU vitamin D/g). Water was provided *ad libitum*. The animals were housed in AALAC-inspected and approved facilities, and the experiment was approved by the Argonne Institutional Animal Care and Use Committee.

To determine the release of calcium from the skeleton after experimental treatment, the animals were given three biweekly, subcutaneous injuctions of ^{45}Ca two months prior to ovariectomy as described (22). This time course permitted the ^{45}Ca to become equilibrated in the skeleton and released from other stores such that the bone was the only major depot of the isotope. Four dogs were bilaterally ovariectomized (OV)

and three were sham-operated (SO). Dogs were matched in their respective groups by age, number of successful breeding cycles, and number of pups per litter. Surgery was performed under general anesthesia (4 - 8 mg Serutal/kg; 15 ml methoxyflurane/l O_2), and sham surgery followed the same protocol as ovariectomy, excluding ovarian extrusion. Dogs were injected with a series of three fluorochromes (2d-10d-2d) as markers of *de novo* bone formation. Tetracycline (25 mg/kg in saline), calcein (15 mg/kg in 2% $NaHCO_3$), and xylenol orange (50 mg/kg in 2% $NaHCO_3$) were administered intramuscularly at 1, 4 and 8.5 months after surgery, respectively.

Tissue collection and preparation

Blood was collected weekly during the experiment and analyzed for calcium, phosphorus, and osteotropic hormone levels (21).

Five rib biopsies were performed on each dog: at the time of surgery, after each labeling period, and at necropsy (approximately 9 months after surgery). Care was taken to remove analogous samples from the midsection of each rib, sampling ribs #10 - 14 from alternate sides to avoid alterations in bone remodeling rate that can occur in adjacent ribs (1). The variability of histomorphometric parameters (1) and biochemical parameters (3) on sequential rib biopsies is minimal. The midsection of each rib was excised, fixed in 70% ethanol, dehydrated in 90% and 100% ethanol, defatted in xylene, and embedded undecalcified in methylmethacrylate (2). Cross sections from the embedded midrib were cut with a bone saw (Isomet, Buechler Inc., Lake Bluff, IL), mounted on plastic slides, ground to approximately 20 μm in thickness, and polished.

These sections were used for histomorphometric analyses. Some sections were also stained by the von Kossa method for bone mineral.

At necropsy, the fifth rib from each dog was excised and frozen at -20°C for subsequent biochemical analysis.

Serum mineral and hormone assays

Serum concentrations of calcium were determined by atomic absorption spectrophotometry, and phosphorus was determined using a diagnostic kit (Sigma Diagnostics, St. Louis, MO) as previously described (22). Serum concentrations of 17β-estradiol and progesterone were determined by radioimmunoassay using commercially available kits (ICN Corp., Costa Mesa, CA; Diagnostic Products Corp., Los Angeles, CA) validated for use in the dog as previously described (22). Intact parathyroid hormone and calcitonin were measured using commercially available radioimmunoassays (Corning-Nichols Institute, San Juan Capistrano, CA) validated for use in the dog as previously described (22).

Bone mineral analyses

The entire fifth rib of each dog (obtained at necropsy) was processed for mineral content and ^{45}Ca content as described (25). The ribs were cleaned of extraneous tissue, precisely quartered, fixed in 70% ethanol for 4 d, defatted in chloroform-methanol (2:1, v:v) for 2 d, and dried at 110°C for 2 d. The rib quarters were cooled in a nitrogen-flow desiccator for 1–6 h before fat-free dry weights were taken. The pieces were ashed in

a muffle furnace (525°C) for 4 d and weighed as described above. (Variability in measurements of control rib pieces was less than ±1 mg, irrespective of the presence of dry nitrogen or the length of the cooling period.) Bone ash was dissolved in 10 ml of 6N HCl and allowed to settle for 24 h. Duplicate aliquots were analyzed for ^{45}Ca content by scintillation counting and for total Ca content by atomic absorption spectrophotometry as described previously (22). For each bone piece, dry and ash weights, the ratios of ^{45}Ca and calcium content to dry and ash weights and the specific activity of each bone (^{45}Ca/total Ca) were determined.

Histomorphometry

Static and dynamic histomorphometry was performed on cross sections of the 1-, 4-, and 8.5-month midrib biopsies. Cortical porosity was measured from digitally captured images of von Kossa-stained sections using a Bioquant image analysis system (R&M Biometrics, Inc. Nashville, TN) and was the fraction of cortical bone area accounted for by empty space of any kind; this could include resorption osteons, incompletely formed osteons, and marrow-filled spaces completely surrounded by cortical bone.

Histomorphometric measurements were made on at least three sections from each animal using cortical bone methods as described (9). The primary measured indices included total osseous tissue area (B.Ar), osteoid area (O.Ar), osteoid thickness (O.Wi), number of single- and double-labeled osteons, number of reversal osteons, number of resorption cavities, perimeter of single (sL.Pm)- and double-labels (dL.Pm), interlabel width, and mean wall thickness (MWT). The MWT was measured only on mature

osteons that contained a single or double label to ensure that these had formed during the experimental period. The periosteal and endocortical surfaces were excluded from analyses. From these primary indices the following secondary indices were calculated: number of single, double, resorption and reversal osteons per square millimeter of osseous tissue area, mineralizing area, mineral appositional rate (MAR), area-referent bone formation rate expressed as bone turnover (%/yr), average MWT, relative osteoid volume, mean osteoid thickness (O.Th), osteoid maturation time (Omt), and formation period (FP). The bone formation rates (BFR) were calculated from new bone area that was calculated using both the dL.Pm and the mineralizing surface perimeter (M.Pm). The M.Pm was calculated as dL.Pm plus one-half the sL.Pm. The Omt was calculated as O.Th/MAR. The FP was calculated as MWT/MAR and is expressed as days.

Statistics

Data requiring multiple group comparisons were analyzed by analysis of variance (ANOVA) followed by Fischer's least significant difference test (LSD). The one-tailed Student's t-test was applied to determine the significance of SO vs. OV differences. Levels of significance are indicated for $p < 0.10$ and $p \leq 0.05$, but only the latter values were considered statistically significant. Some histomorphometric data are presented through time to illustrate time-dependent changes.

RESULTS

Serum and mineral hormone levels

The serum levels of calcium, phosphorus, estrogen, progesterone, PTH, $1,25(OH)_2$ vitamin D_3, and calcitonin from the dogs used in this study have been previously published (22); they are presented here in summary form to provide comparisons to related studies. Six weeks after surgery, all OV animals were anestrous (OV vs. SO, mean $\pm$ SE, n = 3–4; estradiol, 8.9 $\pm$ 1.6 vs. 8.2 $\pm$ 2.5 pg/ml; progesterone, 0.12 $\pm$ 0.4 vs. 0.33 $\pm$ 0.2 ng/ml). None of the OV dogs (but all three SO dogs) showed signs of estrus or had elevated progesterone levels during the 9 month experiment. Ovariectomy did not affect serum total calcium or phosphorus levels (OV vs. SO, integrated mean $\pm$ SE for five timepoints; Ca, 10.53 $\pm$ 0.10 vs. 10.48 $\pm$ 0.05 mg/dl; P, 4.7 $\pm$ 0.1 vs. 4.5 $\pm$ 0.1 mg/dl). No changes in intact PTH or $1,25(OH)_2$ vitamin D_3 were observed in either condition over the course of the experiment (OV vs. SO, integrated mean $\pm$ SE for seven timepoints; PTH, 30.6 $\pm$ 2.4 vs. 37.9 $\pm$ 9.0 pg/ml; $1,25(OH)_2$ vitamin D_3, 53.5 $\pm$ 1.7 vs. 47.0 $\pm$ 4.7 pg/ml). Calcitonin levels were increased in the ovariectomized animals (OV vs. SO, integrated mean $\pm$ SE for seven timepoints; 15.9 $\pm$ 1.2 vs. 6.0 $\pm$ 0.6 pg/ml, $p <$ 0.001).

Effects of ovariectomy on rib calcium content

At necropsy, the entire fifth rib of each dog was quartered and analyzed by region and compared to the rib as a whole. As in bovine ribs (3), the rib of the SO dogs was heterogenous along its length (Fig. 1). The sternal end (E2) was lower in Ca content and concentration (Fig. 1A and B) than other regions of the rib. This same area had a higher turnover activity, inferred by a higher level of ^{45}Ca labeling of the bone (Fig. 1C). In

ovariectomized dogs, the sternal end of the rib also had a lower Ca content and higher ^{45}Ca content than other rib sections (data not shown). Comparison of OV and SO dogs indicated that ovariectomy caused a decrease in mass and mineral content of the midrib pieces (Fig. 2A and B). Midrib data are presented in Fig. 2 because this rib section was taken at biopsy for the histomorphometric analyses reported later. Total Ca was decreased approximately 31%. The Ca/dry ratio (Fig. 2C) and the specific activity of the midrib (Fig. 2D) did not change significantly in response to ovariectomy. The midrib data reflected that of the rib analyzed as a whole; ovariectomy decreased the bone mass and mineral of the entire rib without a significant change in Ca/dry or specific activity (data not shown).

Effects of ovariectomy on cortical porosity

In the aging SO animal, overall cortical porosity was 1–2% and did not change significantly over 8.5 months (Fig. 3). Cortical porosity was significantly increased by the 4th month following ovariectomy, and remained high at 8.5 months post surgery. The large standard errors for the OV animals at 4 and 8.5 months (Fig. 3A) were due to the variation between individual dogs in the time course of response to ovariectomy (Fig. 3B). Cortical porosity was greatest at 4 months in three of the four OV dogs, whereas one other dog showed a delayed response due to ovariectomy (8.5 months). The cortical porosity of the rib biopsies is further illustrated in Fig. 4 for a single SO and a single OV dog over time. Bone loss was evident microscopically as increased porosity in the rib sections of ovariectomized dogs. Although the loss was varied in location, increased

medullary space, a thinning of the cortical bone (especially at the lateral aspects), and the presence of resorption cavities at the periosteal surface were observed (Fig. 4).

Effects of ovariectomy on intracortical bone formation, mineralization, and turnover

Histomorphometric analysis was performed on biopsies taken from the midsection of the rib. Ovariectomy did not affect any of the histomorphometric indices at 1 month after surgery, but substantial differences were observed at later times when compared to the sham-operated dogs.

At 4 months after ovariectomy, examination of fluorescent markers of bone formation indicated a striking increase in cortical bone turnover (Fig. 5); the M.Pm BFR of rib cortical bone in OV dogs was increased approximately sixfold over that of SO dogs (Fig. 6A) with little variability between animals (Fig. 6B). There were also greater numbers of single- and double-labeled and reversal osteons in the OV dog ribs (Table 1). The MAR, dL.Pm BFR, osteoid seam thickness, and osteoid volume were all significantly greater in the OV dog ribs relative to the SO ribs (Fig. 7, Table 1).

At 8.5 months, most of the bone formation indices (single- and double-labeled osteons, reversal osteons, BFR-dL.Pm, BFR-M.Pm, O.Th, Omt, osteoid volume) had decreased in both the OV and SO groups compared with their values in the same dogs at 4 months (Table 1). When the OV animals were compared against the SO group, the number of single-labeled osteons and the MAR were still significantly elevated in the OV dogs ($p < 0.025$). The number of double-labeled osteons, number of reversal osteons, BFR, and osteoid volume also remained elevated in the OV group ($p < 0.10$). Statistical

significance was not achieved between groups because of a single outlier (for BFR and osteoid, see Dog ■ in Figs. 6B and 7B). The magnitude of the bone turnover response to ovariectomy of the outlier dog was similar to the other three dogs but lagged behind temporally (Dog ■, Fig. 3B). The changes in O.Th and proportion of osteoid in cortical bone paralleled the changes in bone formation rates (Figs. 6 and 7, Table 1). The FP was also reduced at 8.5 months in the OV group. When the data were analyzed for differences within each group over time, histomorphometrical indices of bone formation did not significantly change in the SO group. However, in the OV group, the number of single- and double-labeled osteons, number of reversal osteons, BFR, MWT, O.Th, and osteoid volume showed significant and transient increases over time (Table 1).

DISCUSSION

This report details the histomorphometric analysis of cortical bone from sequential rib biopsies taken at 1, 4, and 8.5 months after ovariectomy in aged female beagles. The transient nature of the response to ovariectomy is strongly supported by the bone formation data (Figs. 5-7; Table 1). Bone formation variables increased significantly by 4 months after ovariectomy and returned toward baseline levels by 8.5 months. Although the number of resorption osteons was also increased at 4 months, the presence of increased reversal and formation osteons at this time suggests that the activation of bone remodeling units with its accompanying resorption phase occurred earlier to create the osteons that were being filled, a situation similar to that of the earlier cancellous bone response (7). The transient increase observed for cortical bone turnover in this study had

a later onset after ovariectomy than that described for cancellous bone (4,7). The formation period stimulated by OV may also have been slightly longer than observed with cancellous bone, but the increased duration may be explained by the increased length of formation periods of cortical vs. cancellous bone (19). The demonstration that the ribs sustained a net decrease in calcium content at necropsy (Fig. 2) suggests that the increase in bone resorption and formation caused by ovariectomy became unbalanced, resulting in an increase in net resorption and a more porous cortex (Fig. 4). This decrease in rib calcium content occurred with no change in Ca/dry ratio (Fig. 2B vs. Fig. 2C), indicating that there was a loss of bone (osteopenia) rather than a decrease in bone mineral. Cortical porosity was still increased over the levels in controls at the end of the study (Figs. 3 & 4), which may be indicative of a new steady state in basal bone turnover. Alternatively, the formation period for the rib bone in OV dogs may not have been completed, since the study extended through a single remodeling cycle (Fig. 6, Ref. 19). Results of the ^{45}Ca analyses demonstrated that ovariectomy-induced bone mineral losses (Fig. 2B) tapped both new ^{45}Ca-labeled and old stable Ca bone pools to nearly the same extent. This is indicated by similar specific activities of the SO and OV dog ribs (Fig 2D), because decreased ^{45}Ca/stable Ca ratios would indicate a preferential loss of newly labeled ^{45}Ca surfaces in response to ovariectomy.

The extent of bone turnover is dependent on its skeletal location. Rib cortical bone in the young adult beagle turns over approximately 18% per year, whereas the cortical midshafts of long bones turn over at less than 1% per year (19). The higher levels of bone turnover in a small bone such as the rib increase the chances that

histomorphometric labels will be seen and that small changes can be detected. Indeed, histomorphometric analysis of human ribs has demonstrated the responsiveness of this site to the normal aging process (6). Our rib cortical turnover values for SO dogs were approximately 6% (Fig. 4), a level most probably indicative of an aged skeleton. The demonstration of a six- to eight-fold increase in bone turnover due to ovariectomy suggests that the rib of the aged female beagle is still very responsive to hormonal manipulation. It also became evident over the course of this experiment that the rib was more responsive to ovariectomy than other bones. The results from the biochemical analysis of the rib (Fig. 2) are in contrast to those for the midshaft and ends of the humeri and tibiae and for the L2–4 vertebrae, where the effects of ovariectomy on bone mineral content were not statistically significant (21). The rib is also sensitive to dietary deficiencies. In heifers, biochemical bone properties and breaking load analyses of ribs were sensitive to a low phosphorus diet (26).

The rib was chosen as the site of histomorphometrical analysis in part because a series of biopsies could be performed on analogous sites on alternating ribs. The observed results in the SO dogs indicated that a substantial regional accelerating phenomenon (8) due to the sequential biopsies was not observed (Figs. 3-7; Table 1). Sequential biopsies have the advantage that each animal can be compared to its own baseline and can be followed through time, minimizing the problem of large variations between individual dogs at a single timepoint that often obscure small changes in bone variables. Clearly, in our study, all the dogs responded to ovariectomy, measured by the extent of the cortical porosity (Figs. 3 and 4) and by histomorphometry (Table 1, Figs. 5-

7) but they did not do so to the same extent or at identical times. For example, when individual values for cortical porosity overtime are plotted (Fig. 3B) it is not surprising that the outlier dog is not the same at 4 and 8.5 months: Dog ■ did not become porous until after 4 months, and Dog ● demonstrated increased porosity at 4 months but decreased back to control levels at 8.5 months (Fig. 3B). The delayed response of Dog ■ to ovariectomy reflected in the porosity data was also reflected in the BFR (Fig. 6) and the amount of osteoid (Fig. 7).

To maximize the skeletal response to ovariectomy while providing adequate calcium for maintenance of bone in mature, aged dogs, the dogs in our study were fed a diet containing 0.5% calcium and phosphorus. Standard canine chow (Purina Mills Inc., Richmond, IN) contains 1.6% calcium and 1% phosphorus. The dogs were meal fed approximately 300 g/d, resulting in an intake of 150 mg Ca/kg/d, still above the recommended requirements established for healthy canine metabolism by the National Research Council (18). The SO dogs did not lose bone mineral density over the entire course of the experiment (21) nor were serum levels of calcium, phosphorus, or osteotropic hormones affected (22), suggesting that this level of calcium intake was sufficient for calcium homeostasis without detriment to the bone. In contrast, OV dogs showed a dramatic cortical bone response in the midrib when analyzed by histomorphometry (Figs. 3-8, Table 1), a response that might have been masked if calcium and phosphorus had been higher.

Although the functional effects of cortical bone loss due to ovariectomy occur later and to a lesser magnitude than cancellous bone loss at some skeletal sites (4,5,7,17,23),

our data demonstrate that early changes in cortical bone turnover occur. This study provides histomorphometrical data to demonstrate the transient nature of the response from 1 - 8.5 months after ovariectomy. The transient nature of the response may explain the lack of change (17) or the minimal changes (23) observed at approximately one year after surgery. Our data demonstrate that the rib cortical bone is a responsive site for the effects of ovariectomy in female dogs and occurs even in the aged dog where cortical turnover rates are extremely low.

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Table 1. Cortical Bone Histomorphometric Indices Measured in the Rib at 1, 4 and 8.5 Months After Ovariectomy or Sham Operation.

		Months After Surgery		
		1	4	8.5
Single labeled osteons (no./mm^2±SE)	SO	1.05 ± 0.17	1.45 ± 0.42	0.20 ± 0.03
	OV	1.28 ± 0.45	^{b}3.27 ± 0.41^{y}	1.29 ± 0.32^{y}
Double labeled osteons (no./mm^2±SE)	SO	0.42 ± 0.12	0.78 ± 0.23	0.20 ± 0.03
	OV	0.53 ± 0.22	^{b}3.59 ± 0.26^{z}	1.36 ± 0.59^{x}
Resorption osteons (no./mm^2±SE)	SO	0.46 ± 0.09	0.83 ± 0.30	0.41 ± 0.05
	OV	0.62 ± 0.09	1.17 ± 0.29	0.82 ± 0.27
Reversal osteons (no./mm^2±SE)	SO	0.04 ± 0.04	0.20 ± 0.08	0.00 ± 0.00
	OV	0.05 ± 0.02	^{b}0.73 ± 0.08^{z}	0.27 ± 0.14^{x}
Mineral appositional rate (MAR) (µm/d±SE)	SO	1.13 ± 0.14	0.93 ± 0.08	0.90 ± 0.03
	OV	0.95 ± 0.14	1.21 ± 0.05^{y}	1.18 ± 0.09^{y}
Bone formation rate (dL.Pm) (%/y±SE)	SO	3.30 ± 1.75	4.64 ± 1.44	0.87 ± 0.10
	OV	3.16 ± 1.43	^{b}34.73 ± 4.51^{z}	8.98 ± 4.52^{x}
Bone formation rate (M.Pm) (%/y±SE)	SO	6.35 ± 2.44	8.07 ± 1.35	1.27 ± 0.05
	OV	6.61 ± 2.65	^{b}48.71 ± 4.51^{z}	12.40 ± 5.54^{x}
Mean wall thickness (MWT) (µm±SE)	SO	59.61 ± 4.99	63.67 ± 2.93	61.95 ± 0.98
	OV	58.59 ± 2.17	^{a}65.97 ± 1.80	61.71 ± 1.33
Formation period (FP) (d±SE)	SO	54.8 ± 9.82	69.8 ± 8.02	69.13 ± 1.13
	OV	67.8 ± 14.2	54.6 ± 2.46	53.38 ± 3.79^{x}
Osteoid Thickness (O.Th) (µm±SE)	SO	8.40 ± 0.99	6.38 ± 0.50	4.40 ± 2.20
	OV	7.81 ± 0.47	^{b}10.50 ± 0.61^{z}	6.65 ± 0.86
Osteon maturation time (Omt) (d±SE)	SO	7.45 ± 0.18	7.08 ± 1.20	5.06 ± 2.53
	OV	8.68 ± 1.07	8.66 ± 0.47	^{a}5.61 ± 0.35
Osteoid volume (%±SE)	SO	0.21 ± 0.08	0.16 ± 0.03	0.01 ± 0.01
	OV	0.34 ± 0.10	^{b}1.57 ± 0.11^{z}	0.42 ± 0.18^{x}

Values are mean ± SE; SO, n=3; OV, n=4.
$^{a}p < 0.05$. $^{b}p < 0.01$ by multiple comparison in a given row over time with ANOVA + LSD.
$^{x}p < 0.1$, $^{y}p < 0.025$, $^{z}p < 0.005$ versus SO at the indicated time after surgery by student's t-test.

FIGURE LEGENDS

Figure 1. Biochemical Indices of Rib Sections from Control Dogs.

At necropsy, the fifth rib from each untreated dog was excised, quartered, and biochemically analyzed as described in the Materials and Methods. Rib quarters extended from the vertebral end (E1) to the curved midsections (M1 and M2) to the sternal end (E2). For each rib quarter, the amount of calcium (A), the ratio of calcium content to fat-free dry weight (B) and the specific activity (C) are illustrated. Values are mean $\pm$ SE, $n = 3$ (different from the other rib sections, ANOVA + LSD, $^{**}p < 0.05$; $^{*}p < 0.10$).

Figure 2. Effects of Ovariectomy on Biochemical Indices in the M1 Quarter of the Rib.

At necropsy, the fifth rib from SO and OV dogs was excised, quartered and biochemically analyzed as described in the Materials and Methods. The fat-free dry mass (A), amount of calcium (B), the ratio of calcium content to fat-free dry weight (C), and the specific activity (D) of the M1 rib quarter midsection on the vertebral side are illustrated. The changes illustrated here are essentially the same as for the other midrib midsection (M2)

and reflect those obtained for the rib analyzed as a whole. Values are mean ± SE, n = 3-4 (*$p < 0.10$, Student's t-test).

Figure 3. Overall Cortical Porosity.

Von Kossa-stained sections were measured for the area of holes in the cortical bone at successive timepoints by image analysis. (A) Values of mean ± SE for the number of dogs shown in parentheses (* significantly increased from 1 month value, ANOVA + LSD, $p < 0.05$). (B) Porosity of individual dog ribs over time. (open symbols, SO; filled symbols, OV). Symbols for each dog are consistent with Figs. 6 and 7.

Figure 4. Overall Cortical Porosity.

Images of von Kossa-stained sections of sequential rib biopsies for a single SO (A,C,E) and OV (B,D,F) dog at 1, 4 and 8.5 months post surgery, respectively. The OV dog illustrated here (Dog ▾) has a porosity value of 5.9% whereas the SO dog (Dog ◊) has a porosity value of 2.0% at 4 months (Fig. 3B). Notice the overall increased cortical porosity in the OV rib at 4 and 8.5 months and the periosteal resorption sites at 4 months in the OV rib.

Figure 5. Fluorescent Markers of Cortical Bone Formation.

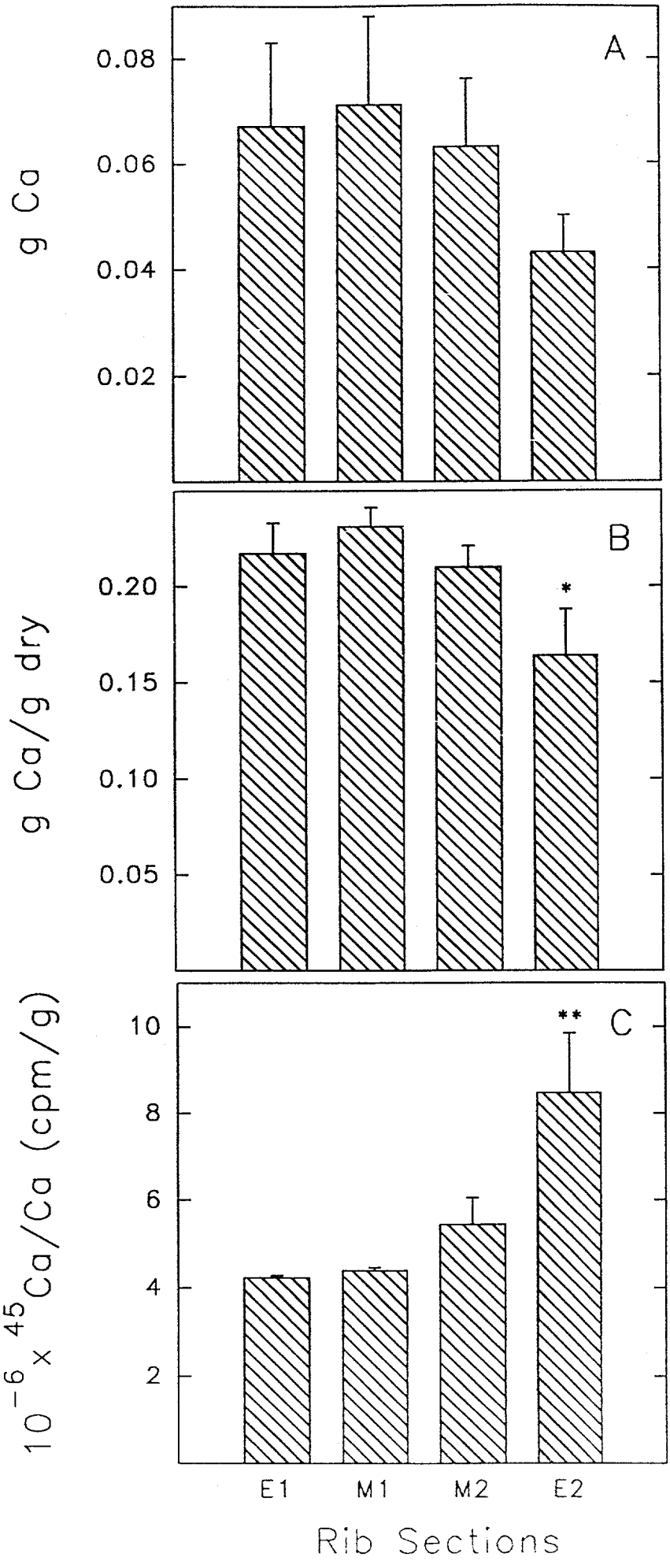
Dogs were injected (2d-10d-2d) at 1 month with tetracycline and at 4 months with calcein as described in the Materials and Methods. Low-magnification fluorescent micrographs show a portion of midrib biopsied just after the second label was administered from an SO (A) or OV (B) dog, illustrating the OV-induced increase in cortical BFR. (66x magnification)

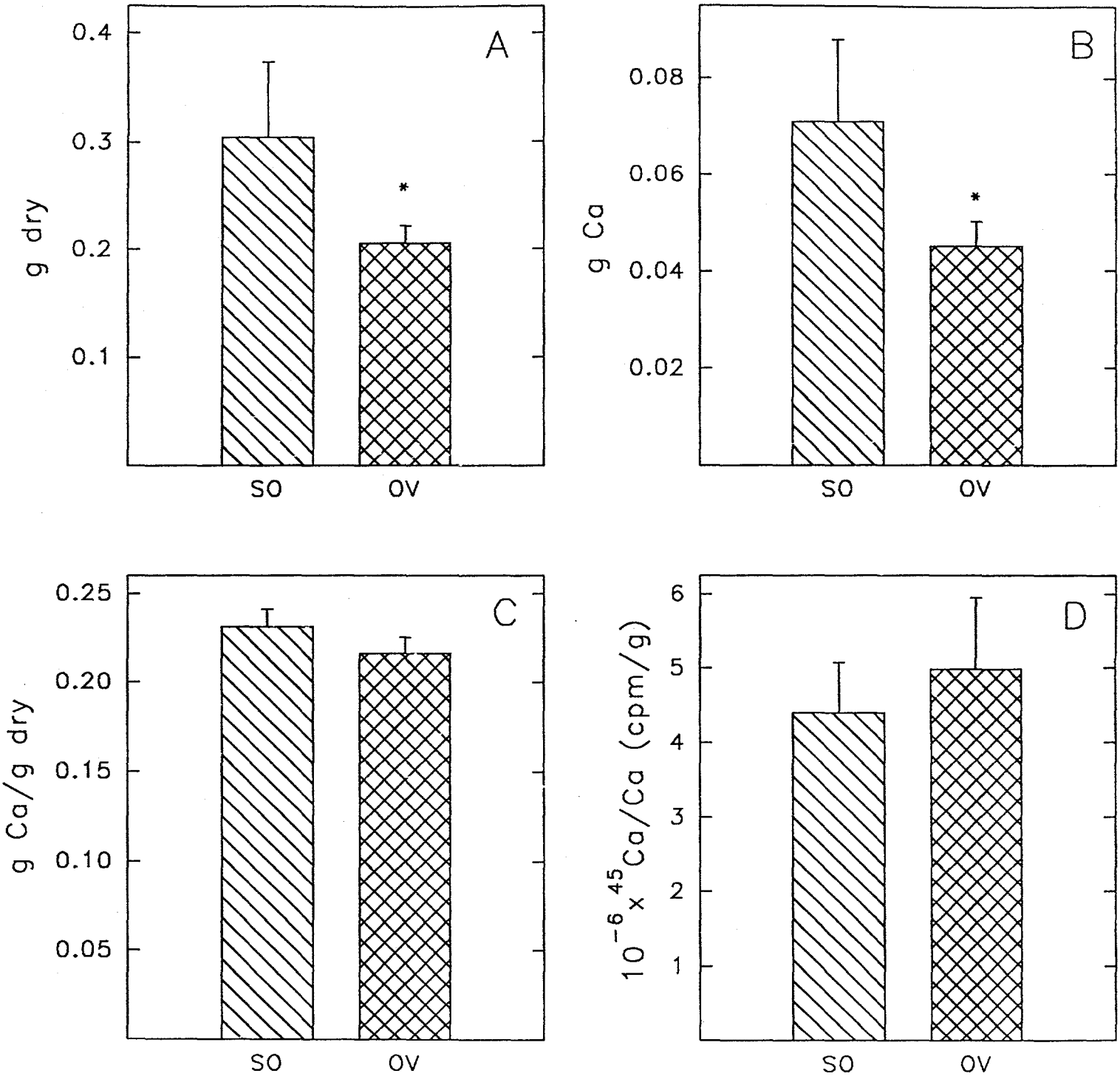
Figure 6. Cortical Bone Formation Rate.

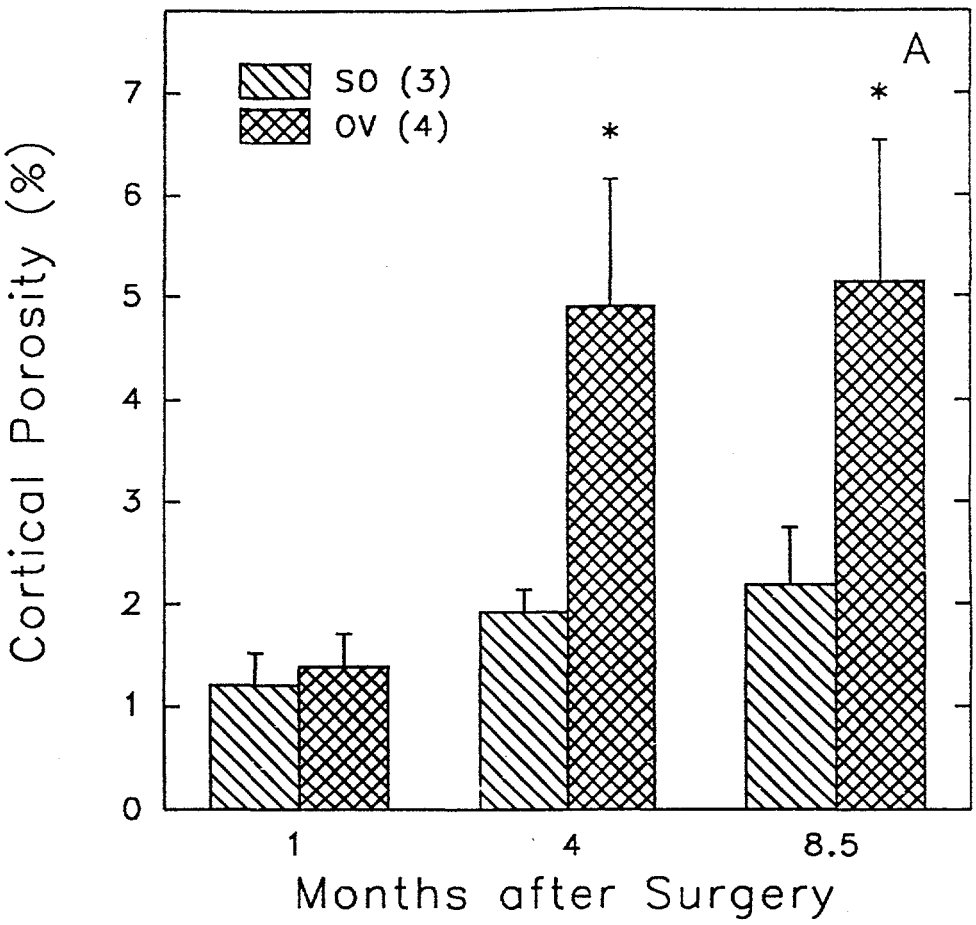
The M.Pm BFR was calculated as described in the Materials and Methods. (A) Values are mean $\pm$ SE for the number of dogs shown in parentheses (*significantly increased over SO, Student's t-test, $p < 0.001$; ** significantly increased from 1 month value, ANOVA + LSD, $p < 0.001$); (B) BFR of individual dog ribs over time (open symbols, SO; filled symbols, OV). Symbols for each dog are consistent with Figs. 3 and 7.

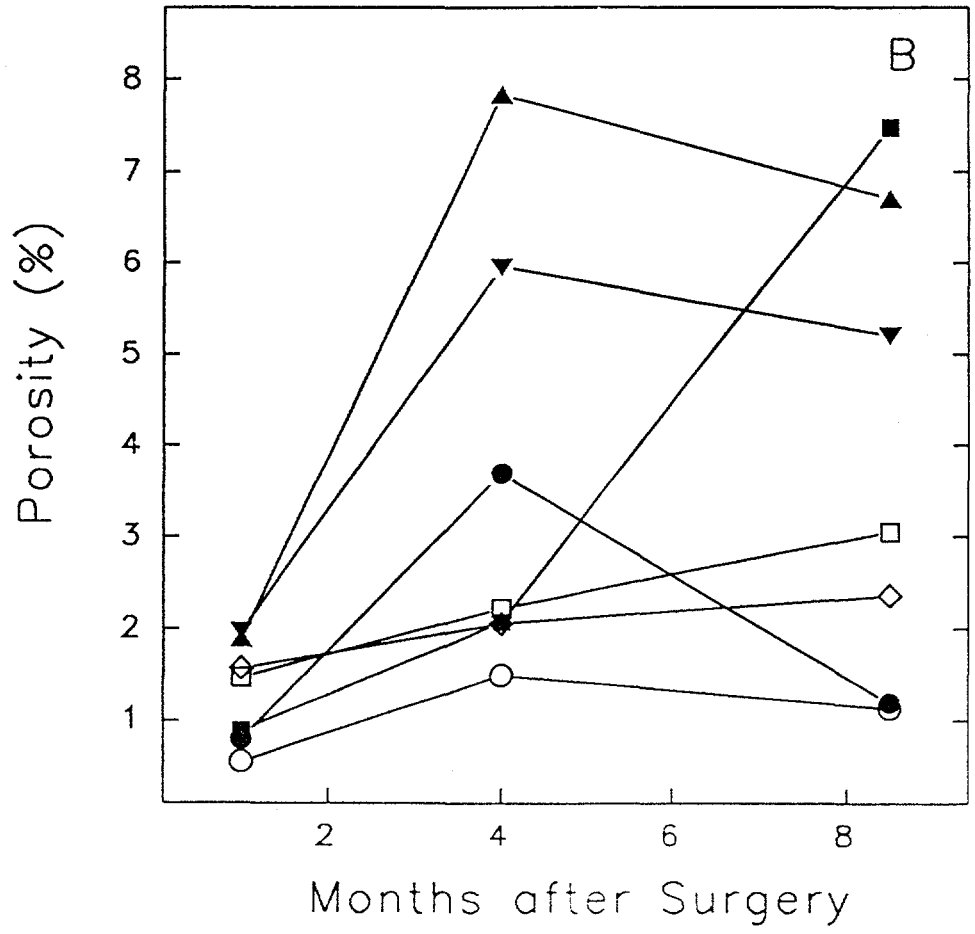
Figure 7. Percentage of Cortical Osteoid Volume.

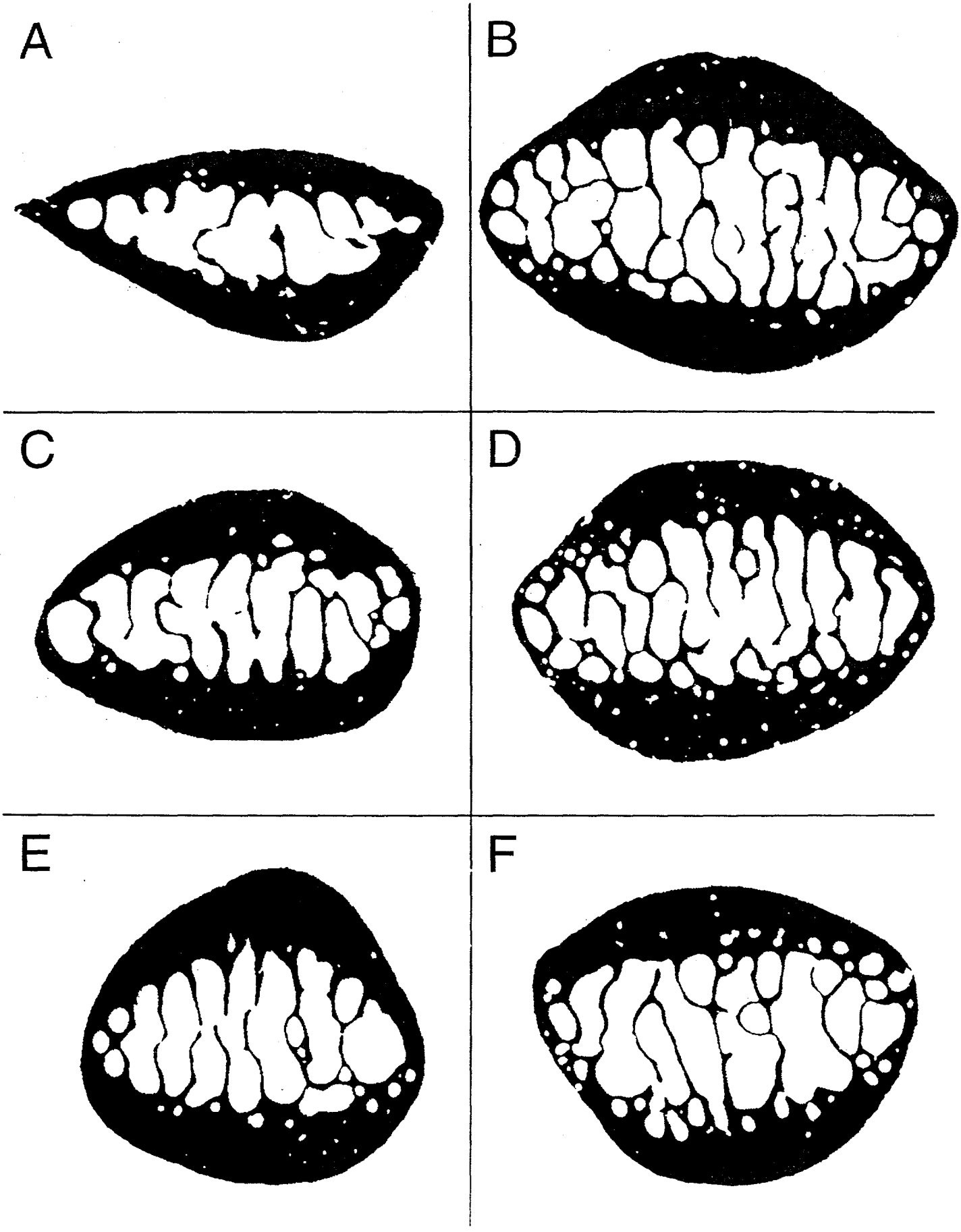
Osteoid (cortical osteoid area/total cortical area) was measured from von Kossa-stained sections. (A) Values are mean $\pm$ SE for the number of dogs shown in parentheses (*significantly increased over SO, Student's t-test, $p < 0.005$; **significantly increased over baseline values, ANOVA + LSD, $p < 0.01$); (B) osteoid values of individual dog ribs over time (open symbols, SO; filled symbols, OV). Symbols for each dog are consistent with Figs. 3 and 6.

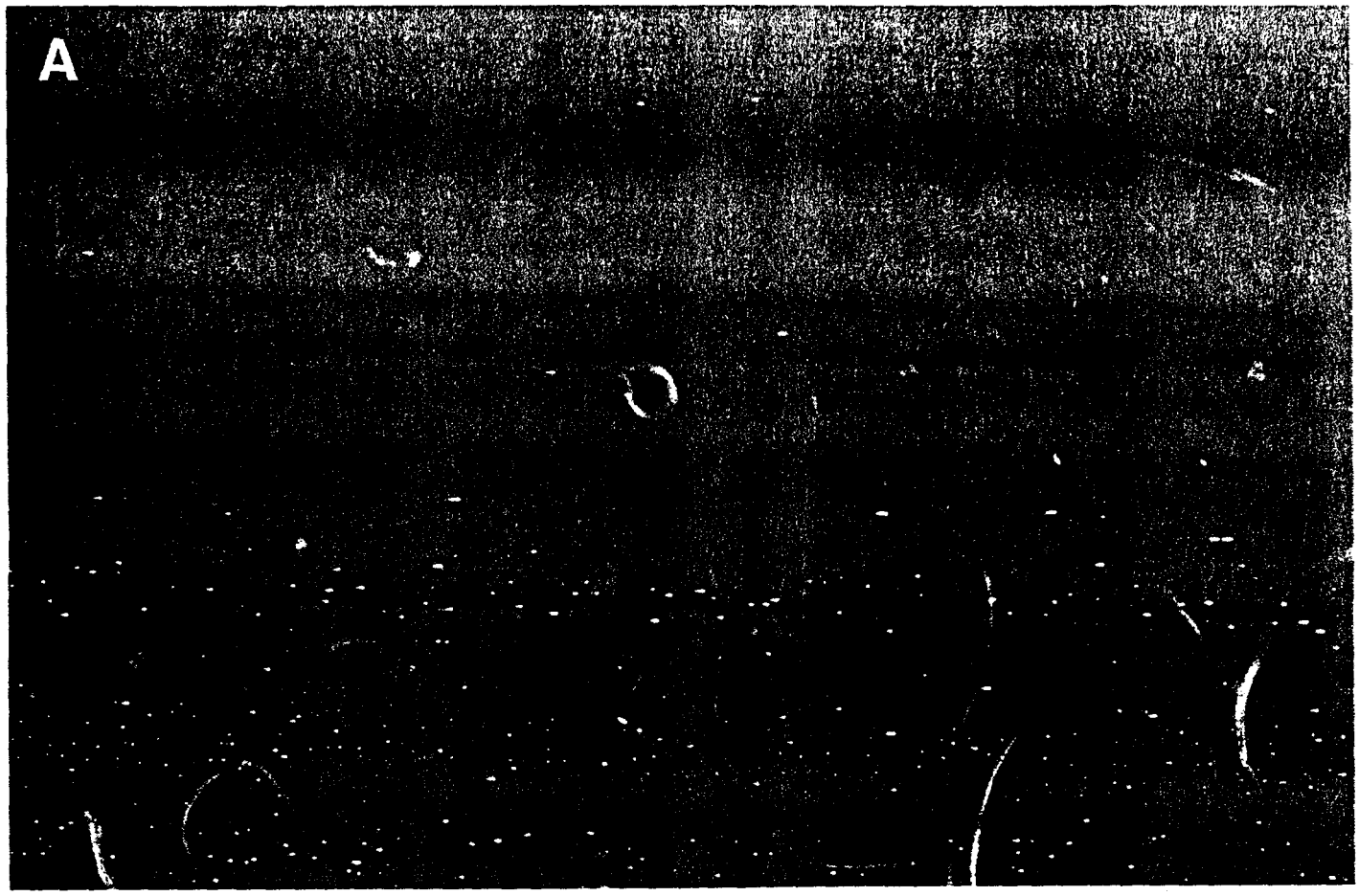

A
0.08
0.06
0.04
0.02
g Ca
B
0.20
0.15
0.10
0.05
g Ca/g dry
*
C
10
8
6
4
2
$10^{-6} \times {}^{45}Ca/Ca$ (cpm/g)
**
E1
M1
M2
E2
Rib Sections

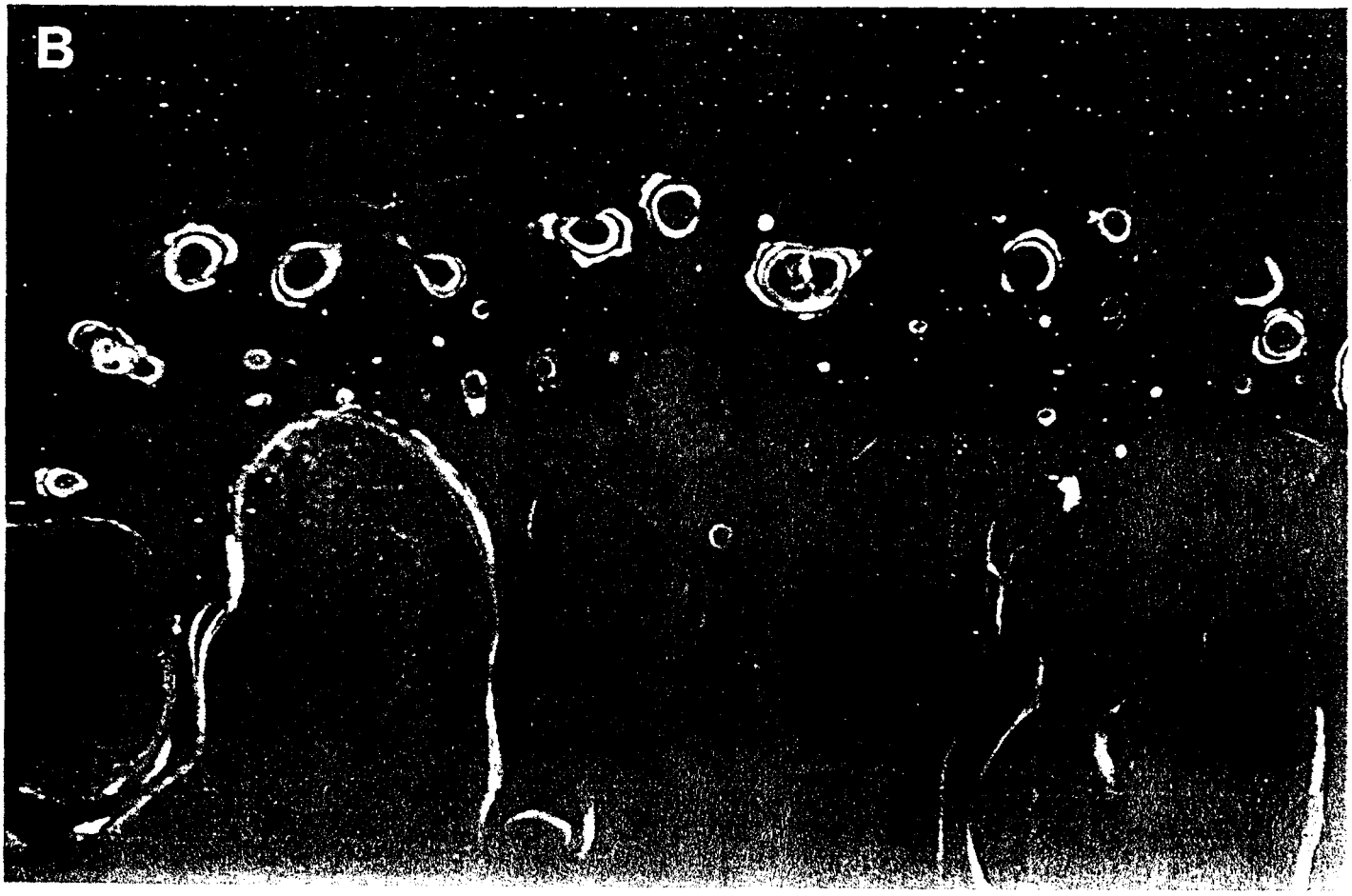

A
B
C
D
g dry
g Ca
g Ca/g dry
$10^{-6} \times {}^{45}Ca/Ca$ (cpm/g)
0.4
0.3
0.2
0.1
0.08
0.06
0.04
0.02
0.25
0.20
0.15
0.10
0.05
6
5
4
3
2
1
*
*
SO
OV
SO
OV
SO
OV
SO
OV

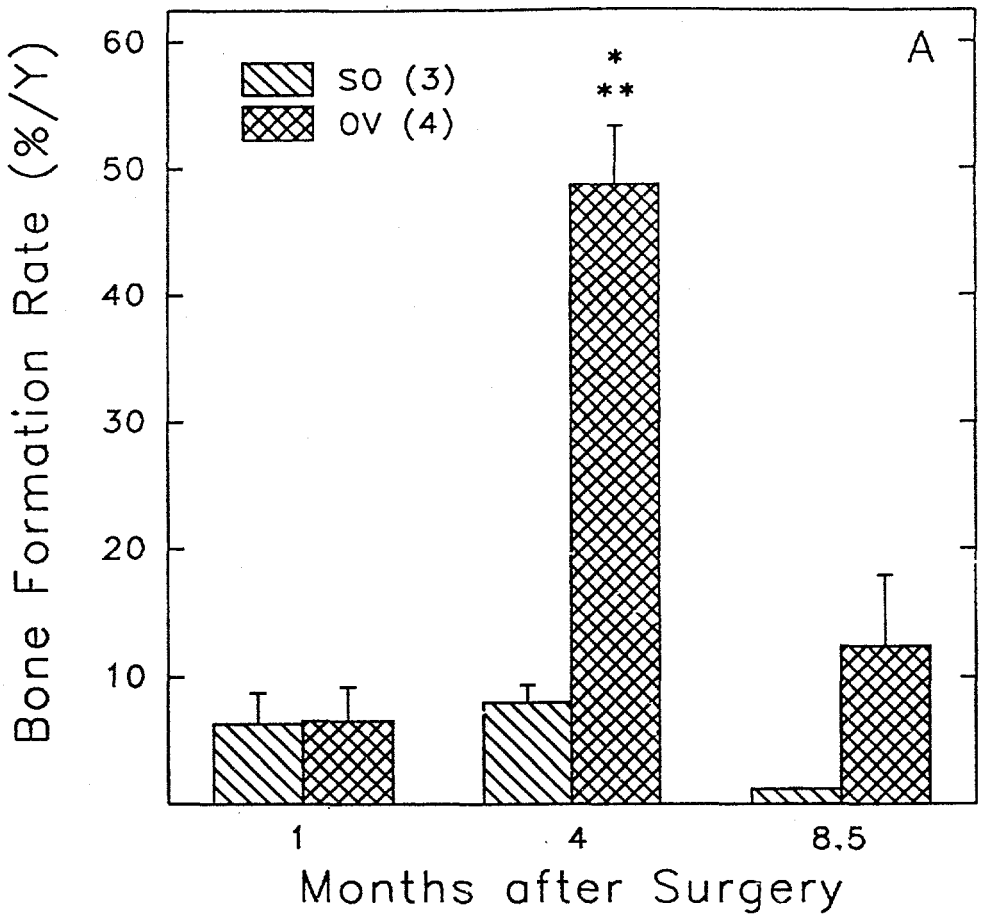

A
SO (3)
OV (4)
*
*
Cortical Porosity (%)
0
1
2
3
4
5
6
7
1
4
8.5
Months after Surgery

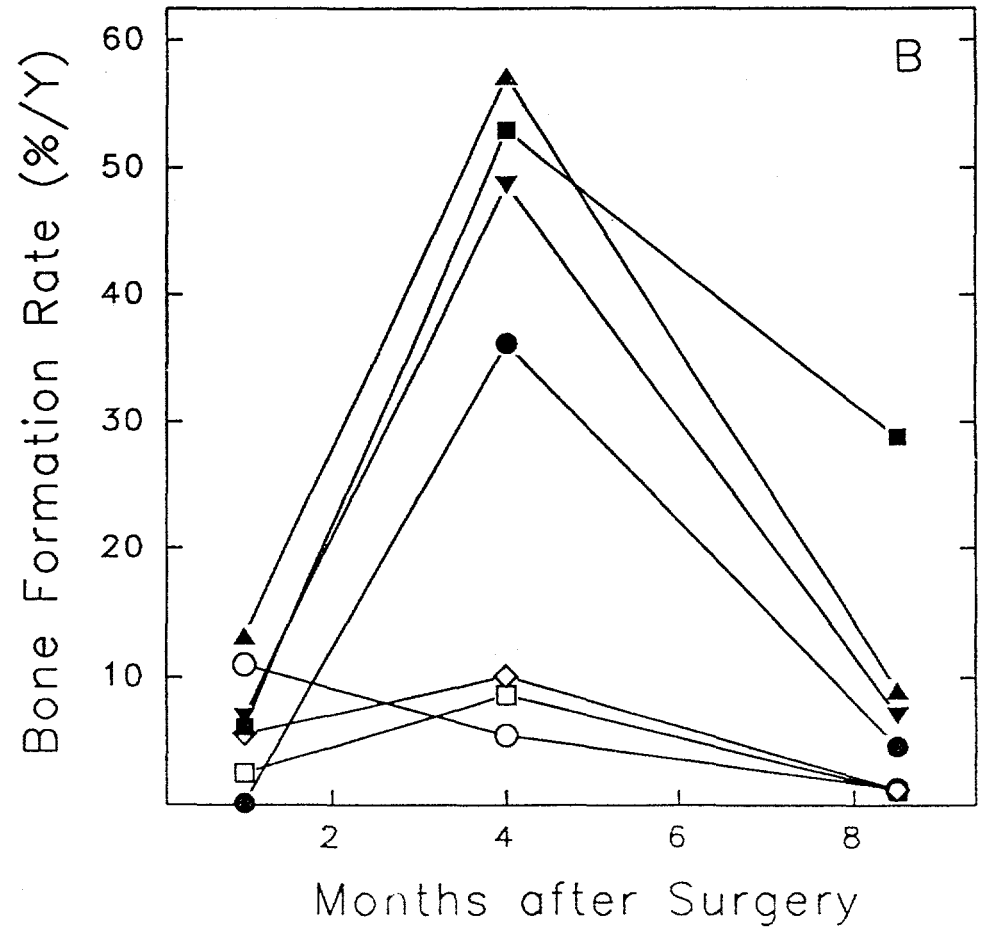

B
Porosity (%)
1
2
3
4
5
6
7
8
2
4
6
8
Months after Surgery

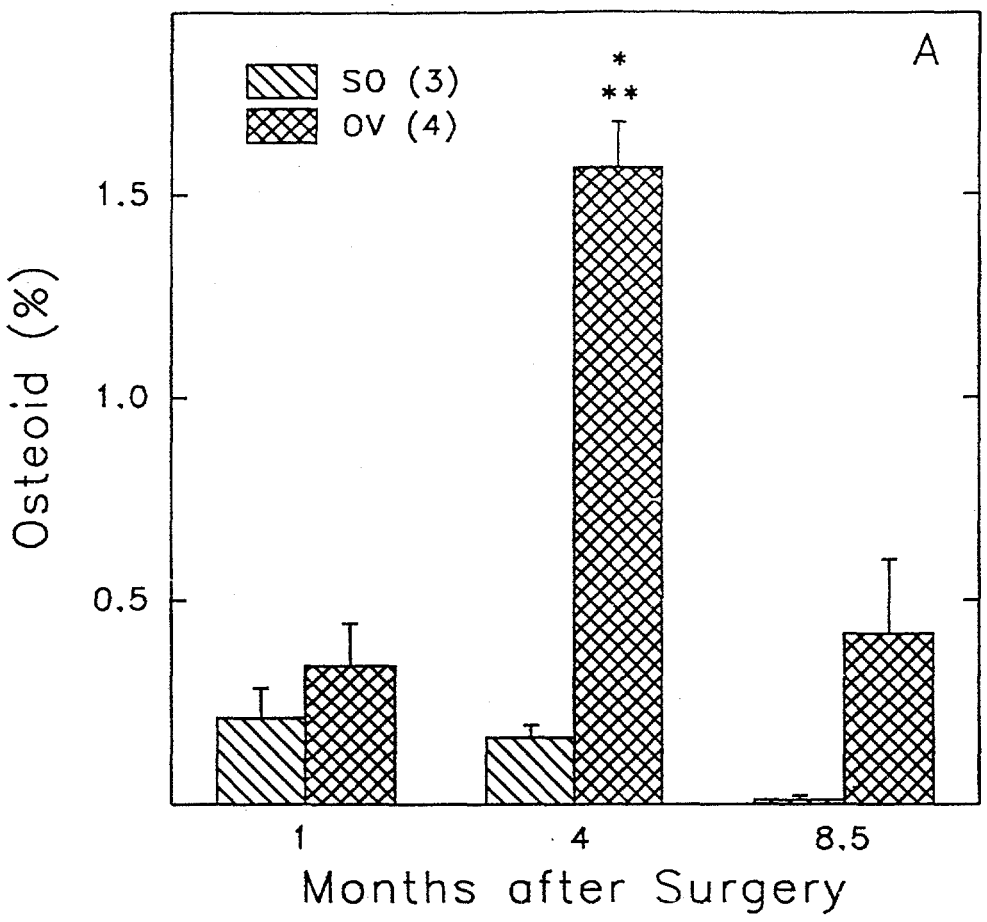

A
B
C
D
E
F

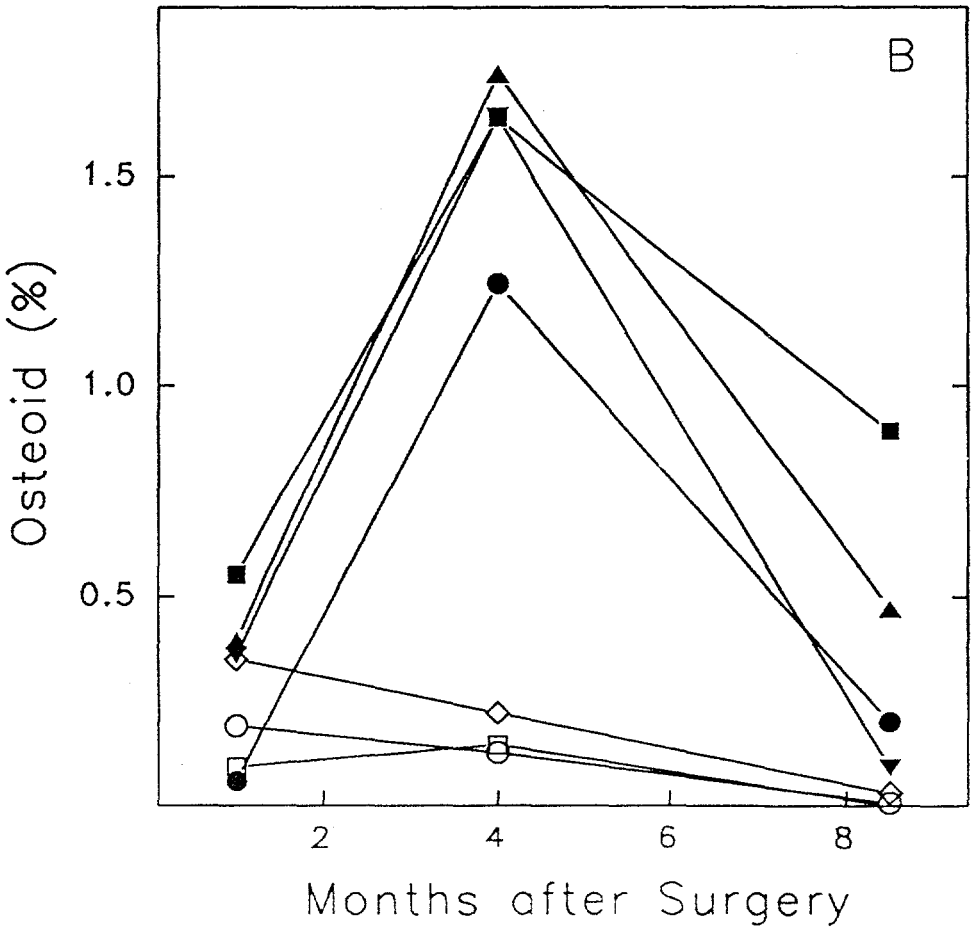

A


B


A
SO (3)
OV (4)
*
**
Bone Formation Rate (%/Y)
60
50
40
30
20
10
1
4
8.5
Months after Surgery


B
Bone Formation Rate (%/Y)
60
50
40
30
20
10
2
4
6
8
Months after Surgery


A
SO (3)
OV (4)
*
**
Osteoid (%)
1.5
1.0
0.5
1
4
8.5
Months after Surgery


B
Osteoid (%)
1.5
1.0
0.5
2
4
6
8
Months after Surgery