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Osteoporotic-like Effects of Cadmium on Bone Mineral Density and Content in Aged Ovariectomized Beagles*

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ABSTRACT

The purpose of this study was to evaluate the effects of ovariectomy in conjunction with cadmium (Cd) exposure on bone. Aged female beagles with ^{45}Ca-labeled skeletons were divided into four groups: sham-operated controls (SO-); ovariectomized (OV-); shams exposed to Cd (SO+); ovariectomized exposed to Cd (OV+). Total Cd exposure period was 1 month by capsules (1, 5, 15, 50 ppm) and 6 months by drinking H_2O (15 ppm). Successive vertebral scans (by dual photon absorptiometry) monitored changes in bone mineral density (BMD) in each dog with time. Results showed that ovariectomy or Cd exposure alone caused significant decreases in BMD (-4.1 ± 1.9%; -5.3 ± 1.2%, respectively, mean ± SE, n=3-4); ovariectomy with Cd exposure caused the greatest decrease (-7.2 ± 1.2%). Ovariectomy alone did not decrease BMD in the distal end or mid-shaft of the tibia. However, BMD of the distal tibia decreased significantly due to Cd exposure alone (-11.9 ± 1.6%). Combination treatment resulted in significant decreases in BMD of both tibial regions (distal tibia: -15.4 ± 4.3%; midshaft: -10.8 ± 2.6%). At necropsy, tibiae, humeri, lumbar vertebrae and ribs were obtained for biochemical analysis. No group-to-group differences in bone weights (wet, dry, ash), in ash/dry ratios, or in long bone and vertebral Ca/dry or Ca/ash ratios were observed. Significantly higher total ^{45}Ca content and ^{45}Ca/dry and ^{45}Ca/ash ratios were observed in long bones and vertebrae of OV- and OV+ groups. In contrast, intact ribs showed significantly decreased Ca/dry and Ca/ash ratios compared to the SO- group. Quartered ribs demonstrated regional responses to specific treatment; decreases in total Ca content were greatest in the mid-rib region (-36 to -46%). Results suggest that in the aged female beagle, bone mineral loss associated with

estrogen depletion is not only related to bone type (trabecular versus cortical) but also to bone Ca pools (older stable versus newer labeled). Our results also suggest that a regional heterogeneity of bone plays a role in responsiveness to ovariectomy and Cd exposure. These aspects suggest that Cd is an exogenous factor affecting bone mineral loss independently of estrogen depletion. However, estrogen depletion "primes" bone for responsiveness to Cd-induced bone mineral loss.

INTRODUCTION

Osteoporosis is a debilitating bone disease marked by significantly decreased bone mass and strength, leading to increased risk of fractures from minimal trauma. While there is agreement that the disease is multifactorial in nature (1,2), the interaction of factors such as estrogen depletion with age, cigarette smoking, alcohol, caffeine consumption or cadmium exposure is unclear (2-7). In fact, there is some disagreement as to interpretation of bone density changes at different skeletal sites that are related to estrogen depletion but independent of age (6). Generally, trabecular bone is especially sensitive to hormonal deficiencies and is the more appropriate bone type for assessing postmenopausal osteoporosis (8,9). On the other hand, changes in bone mineral density of cortical bone are related to the aging process (10-12).

Using noninvasive bone densitometry, histomorphometry, or bone biochemical analysis, investigators have reported decreased trabecular bone mass in young adult ovariectomized rats, although there is no consensus as to whether these changes are due solely to increased bone resorption (13-16). However, primates and dogs have skeletal structural and bone-remodeling units more comparable to human skeletons than rodents. Pharmacologically induced amenorrhea in young adult monkeys (17) and ovariectomy in young adult beagles (18-21) leads to decreased bone mineral content and density, increased numbers of resorption spaces, increased osteoclast activity and decreased osteoblast function.

Itai-Itai, an osteomalacic disease occurring in postmenopausal Japanese women, has been linked to cadmium exposure as a result of environmental pollution by area

industry (4). Cigarette smoke is another known source of Cd exposure (22,23), and in fact, female smokers have low bone mass and increased incidence of fractures, as well as early tooth loss (24-26). Studies have shown Cd-induced skeletal lesions, increased resorptive surfaces, decreased bone calcium content, and increased fecal ^{45}Ca excretion in animals with radiolabeled bones (7, 27-32). Cd intoxication also causes decreased bone formation rates in young adult beagles (33-34). Cd may act directly on bone and induce bone mineral loss by stimulating differentiation of mononucleated bone marrow cells into active multinucleated osteoclast-like cells which in turn resorb bone at an accelerated rate (35,36).

Knowing that 95% of Itai-Itai cases were postmenopausal women, we decided to investigate a possible interaction between ovarian hormone depletion and Cd effects on bone. Our current study in dogs attempts to verify earlier results with ovariectomized mice (7) in a species whose skeleton is more closely related to humans. We recently reported that the combined effect of ovariectomy and Cd exposure in aged beagles increased skeletal release and fecal excretion of ^{45}Ca independently of calciotropic hormone interactions or renal dysfunction (37). This report presents osteoporotic changes in bone mineral density, bone mineral distribution, and specific bone calcium pool responses (labeled vs. unlabeled Ca) due to ovariectomy, Cd exposure alone, or Cd in combination with estrogen depletion in aged beagles. Regional effects on trabecular versus cortical bone are presented in order to determine selective responses following ovariectomy, Cd exposure, or both.

MATERIALS AND METHODS

Protocol

Fourteen female beagles (7-9 years old) were housed individually in metabolism cages and meal fed as previously described (37). Skeletons were labeled with ^{45}Ca via three biweekly subcutaneous injections of $^{45}CaCl_2$ (9.42 mCi/mg Ca; Amersham, Arlington Heights, IL). The total dose of the radioisotope was 3.7×10^6 Bq/kg body weight. For this experiment animals were matched by age, weight and breeding history in four groups: sham-operated controls (SO-; n=3), ovariectomized (OV-; n=4), shams exposed to cadmium (SO+; n=3), and ovariectomized exposed to cadmium (OV+; n=4). Eight weeks after the final ^{45}Ca injection, ovariectomies were done on 8 dogs (groups OV- and OV+), and sham surgery was done on the remaining 6 (groups SO- and SO+). Table 1 shows the timetable for the cadmium exposure protocol initiated 6 weeks after surgery for groups SO+ and OV+. The total Cd exposure period was 7 months: 1 month by oral ingestion of Cd-containing capsules (1, 5, 15, 50 ppm) followed by 6 months via drinking water containing Cd (15 ppm). Groups SO- and OV- were given a cornstarch placebo instead of Cd capsules and plain water instead of water containing 15 ppm Cd. Details of capsule and water preparation have been described previously (37).

Bone Mineral Density

Periodic bone densitometry scans were carried out on each dog for the duration of the experiment. Lumbar vertebrae and tibia scans were obtained prior to ovariectomy or sham surgery, 3 weeks prior to Cd capsule exposure, 3 weeks into Cd·H_2O I exposure

cycle, and 3 and 14 weeks into $Cd{\cdot}H_2O$ III exposure cycle. No more than 3 months transpired between consecutive scans. During scans dogs were anesthetized (4-8 mg Serutal/kg; 15 mL methoxyflurane/L O_2) in order to position each animal properly and prevent any sudden movements that would affect the measurements of bone density.

Bone densitometry was carried out on the spine and tibia using dual photon absorptiometry (DPA) with ^{153}Gd (LUNAR DP-4; Madison, WI). Since the source was new and all scans were done within a 12 month period, the only adjustment made for source decay was changing the collimator from 2 to 3 mm for the last scans. Systems quality assurance scans were done at least twice 24 h prior to the start of each scan period and also daily during scan periods. A whole body scan on a non-experimental embalmed dog was done prior to baseline scanning of experimental animals and at the end of the experiment (9 months later). Reproducibility between these scans was ±2% relative standard deviation (RSD). Quality control for data analysis was also carried out. For each experimental dog, a specific lumbar scan was computer-analyzed (LUNAR DP-4 SOFTWARE, Madison, WI) at 1 week and again at 18 months after the scan was initially obtained. The reproducibility in results was ±4% (RSD). Quality control was also done by evaluating bone mineral density changes over time in the sham dogs not exposed to Cd (SO-). Each dog was used as its own control. For the lumbar vertebrae, reproducibility for 5 sequential bone mineral density measurements over 8 months' time was ±3.8% (RSD); for the whole tibia, reproducibility was ±2.6% (RSD). Specific regional analysis of the tibia (mid-shaft versus distal end) gave ±4.2 and ±4.0% (RSD)

reproducibility, respectively. Results are reported as bone mineral density (BMD; g calcium-hydroxyapatite per cm^2) and percent change in BMD over time.

Bone Biochemistry

At the end of $Cd{\cdot}H_2O$ III exposure cycle, 12 of the 14 experimental dogs were euthanized. During necropsy, the following bones were taken for biochemical analysis: tibiae, humeri, lumbar vertebrae (L_{2-4}, L_5) and ribs. Bones were treated in the following manner in order to analyze total calcium and ^{45}Ca content in each bone. All bones were frozen at -70 °C for a period of four to seven months. At the time of analysis, each bone was cleaned of any remaining extraneous tissue. Tibiae and humeri were divided for analysis into end regions (trabecular bone) and shaft (cortical bone). The lumbar vertebrae were divided into L_2-L_4 (coinciding with DPA scan region) and L_5. One rib per dog was analyzed intact and the next adjacent rib was quartered; no costal cartilage was taken with the ribs. Wet weight of each whole bone was recorded prior to cutting and recorded a second time by weighing individual pieces on a Mettler (PL200) Analytical Balance (±0.01 mg) (Hightstown, NJ). The bones were defatted in 70% ethanol for four to five days and then in chloroform-methanol (2:1; V:V) for 24-48 h (38). Analyses of aliquots of ethanol and chloroform-methanol indicated that no ^{45}Ca leaching occurred.

All defatted bones were placed into tared glass beakers or scintillation vials (rib quarters) and samples treated as follows: (1) dried in drying oven (110 °C) for two days; (2) cooled in a desiccator for at least 1 h; (3) dry weights recorded no sooner than 1 h

and no later than 6 h in desiccator. All bones were ashed in a muffle furnace (525 °C). The lumbar vertebrae and quartered rib pieces were ashed for four days; all other bones were ashed for one week. Ashed bones were again cooled in a desiccator for 1-6 h prior to recording ash weights. For quality control, five empty glass vials and three random rib samples in vials were put through the drying and ashing protocol in order to test the effects of environmental humidity and drying times on accuracy of weights. Dry nitrogen gas flowed through the desiccator system to ensure a humidity-free environment, and weights were recorded at 1 and 6 h after placement of samples in the desiccator. Variability was less than ± 1 mg irrespective of the dry nitrogen gas or difference in cooling period.

Bone ash from tibiae, humeri, vertebrae, and intact ribs was dissolved in 25 mL of 6N HCl and allowed to further hydrolyze and settle for five days. Bone ash from quartered ribs was dissolved in 10 ml of 6N HCl and allowed to settle for 24 h. Samples were covered airtight to prevent evaporation. Duplicate 0.5 or 1.0 mL aliquots of each ash solution were analyzed for ^{45}Ca as previously described (37). Total bone calcium content was analyzed by atomic absorption spectrophotometry (37) using appropriate dilutions in 0.1% lanthanum chloride/0.1N HCl. For each bone piece, wet, dry and ash weights, as well as total ^{45}Ca content per bone, total Ca per bone, and the ratio of mineral content to dry (Ca/dry) and ash (Ca/ash) weights were determined.

Statistical Comparisons

Statistical comparisons were made using the Students' t-test ($p<0.05$ or $p<0.10$) or analysis-of-variance (1-way ANOVA; $p<0.05$) followed by Fischer's least significant difference test (FLSD). ANOVA + FLSD comparisons were made between SO-, OV-, SO+, and OV+ dogs for BMD scans. Paired Students' t-test comparisons and 1-way ANOVA followed by FLSD were made between the OV- and OV+ treatment groups and the SO- group for biochemistry data. Baseline vs. end point BMD scans for a specific group were statistically evaluated using the Students' t-test.

RESULTS

Bone Mineral Density

Results of sequential DPA measurements of bone mineral density are presented in two ways. First, group mean BMD values obtained just before surgery (baseline) are compared to group mean BMD values obtained when the experiment was terminated (end point) 8 months later. Because groups are small, advantage was taken of the opportunity to evaluate changes with time in each individual dog. These results are presented as percentage change in BMD, from baseline to end point, for each dog.

L_2-L_4 DPA Scans

No group-to-group differences in baseline BMD were observed (Fig. 1a, B values), indicating that the dogs did not differ at zero time. Only the SO+ group BMD was significantly decreased at the end point from its own baseline (Fig. 1a). However, using each animal as its own control (Fig. 1b), ovariectomy (OV-) caused a small but statistically significant decrease in lumbar BMD with 3 of 4 dogs responding (-3 to -9%). Cadmium exposure, both alone (SO+) and in combination with ovariectomy (OV+), resulted in a more consistent decrease in L_2-L_4 BMD for each dog (-3 to -10%). Over time, 2 of the 4 OV+ dogs began showing decreases in BMD during Cd·H_2O I (Fig. 2a). During Cd·H_2O III, all 4 OV+ dogs were losing bone mineral density. However, no consistent pattern of change in BMD with time was observed in SO- dogs (Fig. 2b).

Tibia DPA Scans

As with lumbar BMD, no group-to-group differences were observed in baseline whole tibia scans (Fig. 3a). However, in contrast to the lumbar scans, at end point the OV+ group and not the SO+ group BMD was significantly lower than the baseline value (Fig. 3a). Comparing each dog to its own baseline BMD, Cd exposure following ovariectomy resulted in significant and consistent decreases (-6 to -11%) in tibia BMD (3 of 4 OV+ dogs; Fig. 3b). Mean changes for the other groups were not statistically significant. BMD analyses of the distal (trabecular) end of the tibia showed no group-to-group differences at baseline (Fig. 4a). In this specific region of the tibia, both SO+ and OV+ groups had significantly lower BMDs at the end of the experiment compared to respective baseline scans. In fact, all 3 SO+ dogs (-10 to -14%) and all 4 OV+ dogs (-4 to -22%) showed decreases in BMD (distal trabecular end) compared to little or no changes observed for each of the SO- and OV- dogs (Fig. 4b). In contrast, the cortical midshaft showed BMD results similar to what was observed for the whole tibia (Figs. 5a, 5b). The OV+ dogs showed BMD decreases (-6 to -18%), which were reflected by the mean group baseline to end point change (Fig. 5a). No consistent effect on BMD due to ovariectomy or Cd exposure alone was observed in the tibia midshaft when each animal was evaluated as its own control (Fig. 5b).

Over time, the distal end of the tibia of 2 OV+ dogs began showing decreased BMD by $Cd{\cdot}H_2O$ I with one remaining dog also demonstrating decreased BMD during $Cd{\cdot}H_2O$ III scans (Fig. 6a). Little or no change over time was observed in the SO- dogs (Fig. 6b). The cortical midshaft of the tibia had BMD decreases in all the OV+ dogs by

$Cd{\cdot}H_2O$ I, and this loss persisted through $Cd{\cdot}H_2O$ III (Fig. 6c). No such trend of BMD loss was observed in the tibial cortical midshaft of the SO- dogs (Fig. 6d).

Bone Biochemistry

No differences as a result of treatments were observed when making group-to-group comparisons of mean bone weights at sacrifice (wet, dry, ash). No changes in ash/dry ratios were observed in any bones taken at necropsy due to ovariectomy (OV- group) or due to the combined treatment of ovariectomy in conjunction with Cd exposure (OV+ group). Tibiae, humeri, and lumbar vertebrae did not show any changes in Ca/dry or Ca/ash ratios. Significant differences, in OV- and OV+ groups compared to the SO- group, were observed in Ca pools (newer labeled vs. older unlabeled) of the long bones and lumbar vertebrae, as well as in total Ca content, Ca/dry and Ca/ash ratios of the ribs. Heterogeneity in bone type affecting responsiveness was apparent through biochemical analysis of long bone shafts versus ends as well as quartered versus intact ribs.

L_2-L_4 Biochemical Analysis

Total ^{45}Ca content for L_2-L_4 vertebrae was significantly higher in the OV+ than the SO- group (Fig. 7a), but the mean total Ca content for the two groups was similar (Fig. 7b). Specific activity ($^{45}Ca/Ca$) was significantly higher in the OV+ group (Fig. 7c). ^{45}Ca/dry and ^{45}Ca/ash ratios were also significantly higher in the OV+ group than the SO- group (18 and 17%, respectively). No significant effect due to ovariectomy alone

was observed in ^{45}Ca/dry or ^{45}Ca/ash ratios. Results for L_5 were similar to those for L_2-L_4 vertebrae (data not shown).

Tibia and Humerus Biochemical Analysis

Total ^{45}Ca content was significantly higher in the whole tibia and trabecular ends from the OV- and OV+ groups when compared to the SO- group (Fig. 8a). In the cortical shaft, mean ^{45}Ca content of the OV- group, although higher, was not significantly different than that of the SO- group (Fig. 8a). No group-to-group differences, due to ovariectomy alone or in combination with Cd-exposure, were observed for total Ca content of whole tibiae, trabecular ends or cortical shaft (Fig. 8b). Specific activity (^{45}Ca/Ca) was significantly higher in the whole tibiae and trabecular ends of the OV- and OV+ dogs compared to the SO- dogs (Fig. 8c). For the tibia shaft, ovariectomy resulted in significantly higher mean specific activity compared to SO- controls, but the value for OV+ dogs was not significantly different from that of the SO- dogs (Fig. 8c). Specific Ca pool responses to treatments for analogous regions of the humerus were similar to the tibia (data not shown).

OV- dogs had 32% higher ^{45}Ca/dry and ^{45}Ca/ash ratios for the trabecular ends of the tibia compared with the SO- dogs. The OV+ group had 27 to 37% higher ^{45}Ca/dry and ^{45}Ca/ash than the SO- group for the same tibial end region. These significant differences were reflected in similar magnitudes by the whole tibia data. However, no significant group-to-group differences were observed in the tibial cortical shafts, although the ^{45}Ca/dry and ^{45}Ca/ash ratios were always higher in the OV- and

OV+ groups compared to the SO- group (data not shown). For humeri of the OV- and OV+ dogs, the ^{45}Ca/dry and ^{45}Ca/ash ratios were consistently and significantly elevated in both trabecular ends and cortical shaft (15 to 40% higher) compared to the SO- group.

Rib Biochemical Analysis

For the intact ribs, mean total Ca content (g/bone) showed no group-to-group differences between the OV- or OV+ compared to the SO- group (SO-: 0.198 ± 0.037 g; OV-: 0.189 ± 0.006 g; OV+: 0.192 ± 0.025 g). No significant group-to-group differences in total ^{45}Ca content (cpm x 10^{-5}) or specific activity (^{45}Ca/Ca; cpm/g x 10^{-3}) were observed (SO-: 9.94 ± 2.32, 5.97 ± 0.57; OV-: 8.11 ± 0.13, 4.37 ± 0.79; OV+: 9.62 ± 1.86, 4.99 ± 0.85), respectively. However, results from the quartered ribs showed selective regional differences. Both mid-rib quarters had significantly lower mean total Ca contents due to ovariectomy alone and in conjunction with Cd exposure (Figs. 9a, 9b). The average decrease was 36-46% in OV- and OV+ groups compared to the SO- control group (Figs. 9a, 9b). In contrast, for the rib ends, Ca content differences between groups were not statistically significant (data not shown).

Ca/dry and Ca/ash ratios also showed significant differences due to treatments. In the intact ribs, OV- and OV+ resulted in similar and significant decreases in Ca/dry (11%) and Ca/ash (12%) ratios (Table 2). Regional analysis demonstrated that, for the OV+ dogs, decreases in both Ca/dry and Ca/ash ratios observed by using intact ribs were clearly specific to the mid-rib region (Table 2). For the OV- dogs, Ca/dry decreases were also associated with the mid-rib region. However, Ca/ash decreases were

accounted for by changes occurring specifically in end_1 (vertebral) rather than end_2 (sternal) or mid-rib.

DISCUSSION

We have previously shown that Cd exposure caused accelerated but transient skeletal release and fecal excretion of ^{45}Ca in aged ovariectomized beagles (37). In fact, the action of Cd was independent of osteotropic hormone interaction (37) and occurred prior to renal demise (due to Cd intoxication or aging) that could affect bone calcium metabolism (4, 28, 37, 39). In this study, we have now shown in the aged female beagle that Cd induces bone mineral density decreases with significant loss of both labeled and unlabeled Ca, particularly when in combination with estrogen depletion. Cd exposure alone resulted in significant decreases in bone mineral density of trabecular bone (lumbar vertebrae; distal end of tibia), in a manner similar to that observed as a result of ovariectomy (lumbar vertebrae) (Figs. 1, 2). However, it is unclear why the distal tibia was more sensitive to effects of Cd, but less sensitive to effects of OV, than trabecular bone of lumbar vertebrae. The effects of Cd exposure following ovariectomy on bone mineral loss also occurred in cortical bone (tibia shaft) (Fig. 3) and began early during exposure periods ($Cd{\cdot}H_2O$ I). Female ovariectomized mice fed diets containing 50 ppm Cd for 12 months had significantly greater bone Ca loss from femurs than lumbar vertebrae (40). Our data further supports the hypothesis that long bones are more sensitive to the effects of Cd than lumbar vertebrae. Treatment-related increases in

$^{45}Ca/Ca$ ratios of the bones at sacrifice suggest that along with this regional effect, Cd not only induced loss of newly mineralized bone (represented by ^{45}Ca) but, in fact, exacerbated loss of the older calcium pool (represented by unlabeled Ca) following ovariectomy (Figs. 7, 8). All dogs were given the same total dose of isotope per kg body weight at the same time and in the same manner, and no differences in baseline BMD were observed at the start of the experiment.

Concurring with what has been reported by others using younger animal models (12-20), we found that ovariectomy alone in our aged dogs caused a significant decrease in the lumbar vertebral BMD (Fig. 1). The cortical bone of ribs vs. long bones (tibia and humerus) differed in their response to ovariectomy. The mid-rib regions from ovariectomized dogs had 36% less total Ca content compared to the sham-operated controls (Fig. 9). This was in contrast to a previous report suggesting that the detectable decrease in bone density (osteoporotic) in humans is not associated with modifications of cortical bone Ca concentrations (41). On the other hand, cortical bone in the tibia and humerus shafts from our OV dogs did not show any significant decrease in either BMD (Fig. 5) or total Ca content (Fig. 8b). The difference in results for cortical bone may be linked to specific bone sensitivities to estrogen depletion. Ribs from young male beagles show no difference in bone remodeling activity between sim ar sites on alternate ribs (42-44) and regional bone remodeling differences disappear when a beagle is 3 years old (45). However, our study suggests that estrogen depletion in aged female beagles plays a major role in regional bone-turnover activity.

An apparent contradiction in our data set arises when BMD results are considered along with the biochemical measurements; i.e., BMD results for lumbar vertebrae and tibia showed Cd- and OV-induced decreases in bone mineral density (Figs. 1-6), while these same bones taken at sacrifice showed no group-to-group differences in Ca content or concentration due to OV or Cd or the combination (Figs. 7b, 8b). However, treatment-induced decreases in BMD were small for the lumbar vertebrae and tibia (7-15%) -- too small to be determined reliably by group-to-group comparisons of bones at sacrifice with only 3-4 dogs per group. These decreases could only be measured with the sensitivity afforded by serial BMDs, allowing each dog to serve as its own baseline control. In fact, as with values for Ca content at sacrifice, BMDs at end point for the lumbar vertebrae and tibia showed no significant differences based on group-to-group comparisons (Figs. 1a and 2a, comparison of group mean E values, ANOVA + FLSD, $p>0.05$). In addition, for the mid-rib regions in which decreases were large enough to reach statistical significance by group-to-group comparisons, results of biochemical analyses showed significant OV- and Cd-induced decreases in Ca content (Fig. 9, 36% decrease for OV- vs. SO-; 46% decrease for OV+ vs. SO-). Finally, if the lack of group-to-group differences in Ca content for the tibia and lumbar vertebrae at sacrifice indicated no differences in bone mineral loss between treatment groups, then the group-to-group differences in $^{45}Ca/Ca$ ratio should not have occurred (Figs. 7c, 8c). These results and their interpretation should be considered carefully by others who might investigate OV- or Cd-induced changes in bone in a larger species where the number of animals per group is often small.

Use of a skeletally mature aged female beagle, in declining stages of reproductive function, provided a model for better understanding the pathophysiology of postmenopausal bone mineral loss in association with aging. The model is also applicable for evaluation of interactions between specific exogenous factors and estrogen depletion in cases of accelerated bone mineral loss (such as in Itai-Itai disease, occupational and non-occupational exposure to sources of Cd). The use of each dog as its own control (BMDs) provided a more accurate and sensitive method of evaluation of bone changes in our study in which groups were small and the degree of responsiveness varied amongst subjects. Our study showed that bones of the same histological type (cortical) may have been affected by estrogen depletion differently. More importantly, we have shown that Cd exposure alone causes bone mineral loss in trabecular bone. Biochemical and absorptiometric scanning analysis showed that both trabecular and cortical bone loss are exacerbated when Cd exposure follows OV. Ninomiya et al. (46) suggested that there is a profound difference in biochemical composition of cortical and trabecular bone. The view that regions of bone are heterogeneous in composition and metabolic activity is crucial when evaluating the differential effects of OV and subsequent Cd exposure. Regional responses to Cd-induced BMD and mineral content changes may be linked with the proposed differences in bone regulatory mechanisms (46). In fact, differential effects of Cd on bone mineral loss in cultured fetal calvariae versus fetal long bones have been shown (7, 47-49). These two in vitro systems evaluate completely different models of bone development, growth and maintenance.

Our study supports the hypothesis that Cd exposure leads to increased loss of bone mineral and mass, especially in postmenopausal women. We have proposed (37) that the in vivo effect of Cd-induced bone mineral loss may be linked to Cd replacing Ca in hydroxyapatite crystals (50) or induction of osteoresorptive cytokines (51-55). Further evaluation of our model is necessary in order to assess if Cd-induced BMD and mineral content changes might also have been linked to inhibition of bone formation (33,34). Additional mechanistic studies may provide information for understanding and preventing Cd-induced bone loss in women exposed to the metal via cigarette smoke or industrial pollution. Furthermore, this study supports the concept that the multifactorial nature of osteoporosis or Itai-Itai disease may be influenced by the heterogeneous nature of bone tissue. It would appear from our study that although there is some overlap, the mechanism(s) associated with postmenopausal bone mineral loss may differ from those associated with Cd-induced bone mineral loss.

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FIGURE LEGENDS

Figure 1. Comparison of mean baseline (B) versus end point (E) lumbar vertebral bone mineral density (1a) (Students' t-test; $p<0.05$); and evaluation of percent change in bone mineral density for each individual dog (1b) (ANOVA + FLSD; $p<0.05$). B to E time was 8 months. In 1a, bars are mean ± SE for number of dogs shown in parentheses. In 1b, each bar represents a single dog.

Figure 2. Changes in lumbar vertebral BMD during Cd exposure in OV+ dogs (2a) and overtime in control SO- dogs (2b). Shaded areas indicate time-span of Cd exposure periods. Each type of symbol represents BMD for given animal.

Figure 3. Comparison of mean baseline (B) versus end point (E) whole tibia bone mineral density (3a) (Students' t-test; $p<0.05$) and evaluation of percent change in bone mineral density for each individual dog (3b) (ANOVA + FLSD; $p<0.05$). B to E time was 8 months. In 3a, bars are mean ± SE for number of dogs shown in parentheses. In 3b, each bar represents a single dog.

Figure 4. Comparison of mean baseline (B) versus end point (E) bone mineral density of distal tibia (4a) (Students' t-test; $p<0.05$) and evaluation of percent change in bone mineral density for each individual dog (46) (ANOVA + FLSD; $p<0.05$). B to E time was 8 months. In 4a, bars are mean ± SE for number of dogs shown in parentheses. In 4b, each bar represents a single dog.

Figure 5. Comparison of mean baseline (B) versus end point (E) tibial midshaft bone mineral density (5a) (Students' t-test; $p<0.05$) and evaluation of percent change in bone mineral density for each individual dog (5b) (ANOVA + FLSD; $p<0.05$). B to E time was 8 months. In 5a, bars are mean ± SE for number of dogs shown in parentheses. In 5b, each bar represents a single dog.

Figure 6. Changes in BMD of distal end of tibia during Cd exposure in OV+ dogs (6a) and overtime in control SO- dogs (6b). Changes in BMD of tibial midshaft during Cd exposure in OV+ dogs (6c) and overtime for analogous region in control SO- dogs (6d). Shaded areas represent time-span of Cd exposure periods. Each type of symbol represents BMD for given animal.

Figure 7. End point bone mineral content in lumbar vertebrae (L_2-L_4) of control (SO-), ovariectomized (OV-), and ovariectomized dogs exposed to Cd (OV+). Total ^{45}Ca (7a), total stable Ca (7b), and specific activity (7c) are presented for each group (number of dogs per group in parentheses). *Significantly higher than SO- group (Students' t-test; $p<0.10$).

Figure 8. End point bone mineral content of whole tibia, tibial ends and cortical midshaft from control (SO-), ovariectomized (OV-), and ovariectomized dogs exposed to Cd (OV+). Total ^{45}Ca (8a), total stable Ca (8b), and specific activity (8c) are presented for each group (number of dogs per group in parentheses). *Significantly higher than SO- group (Students' t-test; $p<0.10$).

Figure 9. Total Ca content of ribs (mid-regions 1 (9a) and 2 (9b) taken at necropsy from controls (SO-), ovariectomized (OV-), and ovariectomized dogs exposed to Cd (OV+). *Significantly lower than analogous region from SO- dogs (ANOVA + FLSD; $p<0.05$).

Table 2. Bone mineral distribution in ribs (intact and quartered), expressed as mineral content to weight ratios (mg Ca/mg dry or ash weight), from beagles ovariectomized and exposed to cadmium.

	SO- (3)	OV- (4)	OV+ (4)
Intact Rib			
Ca/dry	0.194 ± 0.003	0.175 ± 0.006*	0.170 ± 0.009*
Ca/ash	0.208 ± 0.011	0.182 ± 0.007*	0.190 ± 0.007*
Quartered Rib			
End_1			
Ca/dry	0.217 ± 0.016	0.186 ± 0.016	0.205 ± 0.015
Ca/ash	0.349 ± 0.023	0.307 ± 0.028‡	0.340 ± 0.023
Mid_1			
Ca/dry	0.231 ± 0.016	0.216 ± 0.009‡	0.192 ± 0.012‡
Ca/ash	0.340 ± 0.008	0.351 ± 0.011	0.327 ± 0.021‡
Mid_2			
Ca/dry	0.210 ± 0.011	0.197 ± 0.007‡	0.183 ± 0.009‡
Ca/ash	0.352 ± 0.020	0.342 ± 0.007	0.316 ± 0.010‡
End_2			
Ca/dry	0.164 ± 0.024	0.136 ± 0.024	0.173 ± 0.020
Ca/ash	0.350 ± 0.010	0.322 ± 0.018	0.346 ± 0.012

Values are expressed as mean ± SE for intact or quartered portions of bone taken from number of dogs shown in parenthesis at top of each column. Starred values are significantly different than mean value for SO- group ($p<0.10$; Students' t-test). ‡Values are significantly different when compared with same rib region from SO- group ($p<0.05$; ANOVA; FLSD). End_1 = vertebral end; End_2 = sternal end.

Figure 2a/2b

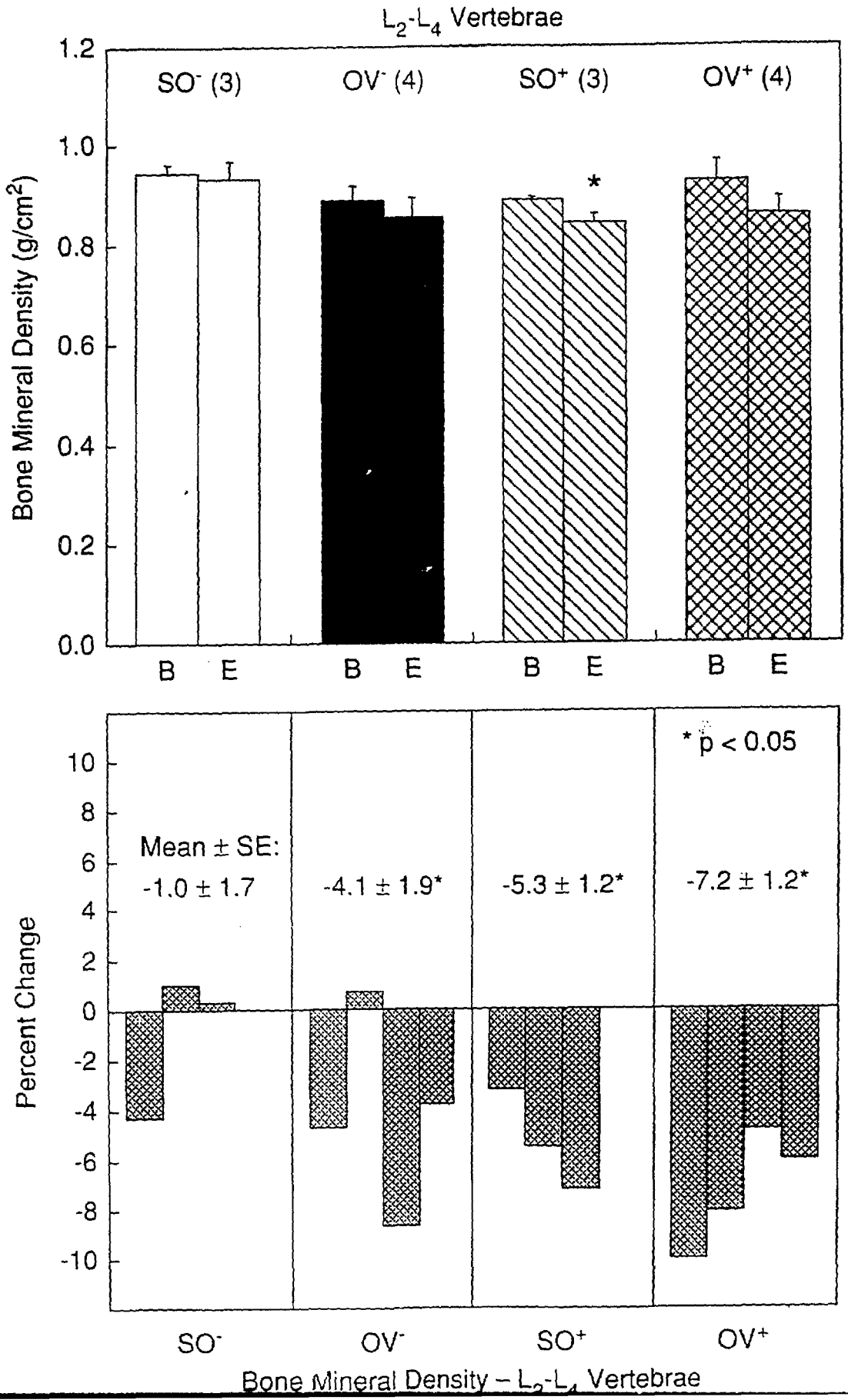

L2-L4 Vertebrae
SO- (3)
OV- (4)
SO+ (3)
OV+ (4)
Bone Mineral Density (g/cm2)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
B E B E B E B E
* p < 0.05
Mean ± SE:
-1.0 ± 1.7
-4.1 ± 1.9*
-5.3 ± 1.2*
-7.2 ± 1.2*
Percent Change
10
8
6
4
2
0
-2
-4
-6
-8
-10
SO-
OV-
SO+
OV+
Bone Mineral Density – L2-L4 Vertebrae

Figure 2a/2b

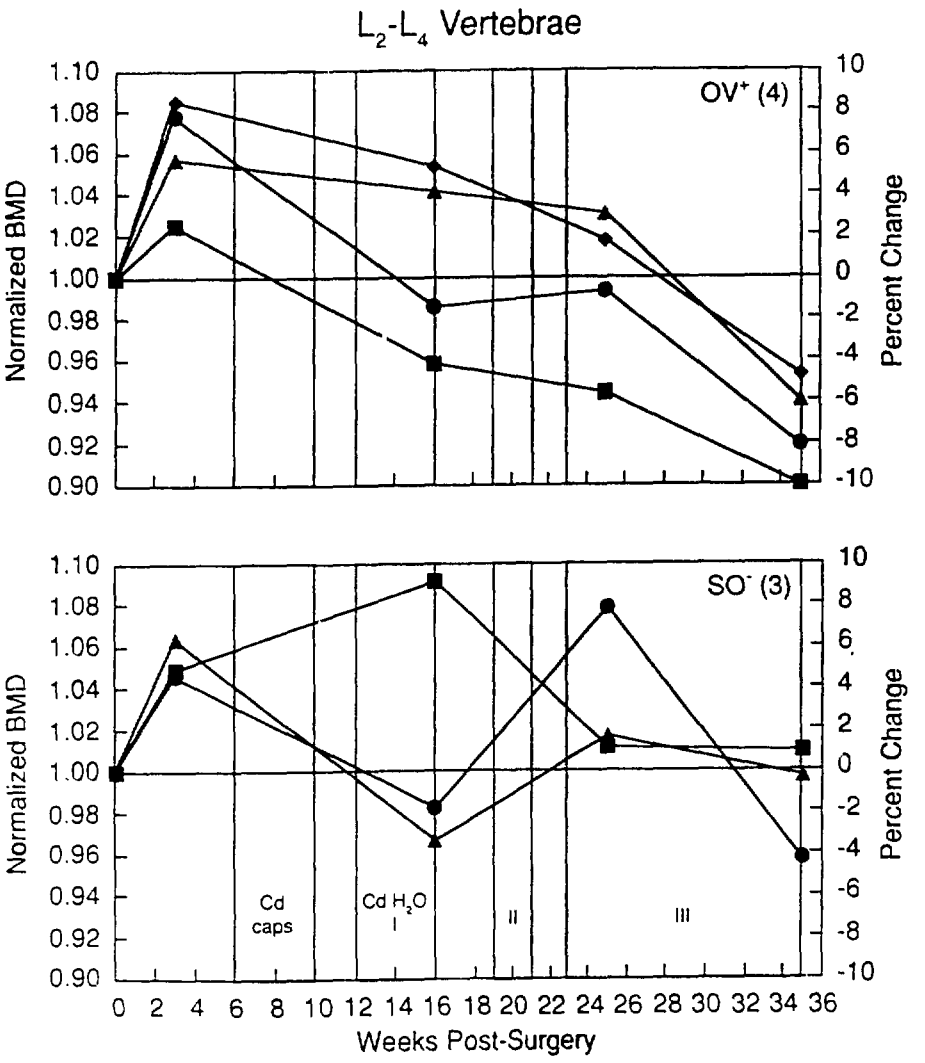

L2-L4 Vertebrae
OV+ (4)
SO- (3)
Normalized BMD
Percent Change
Cd caps
Cd H2O
I
II
III
Weeks Post-Surgery

Figure 2a/2b

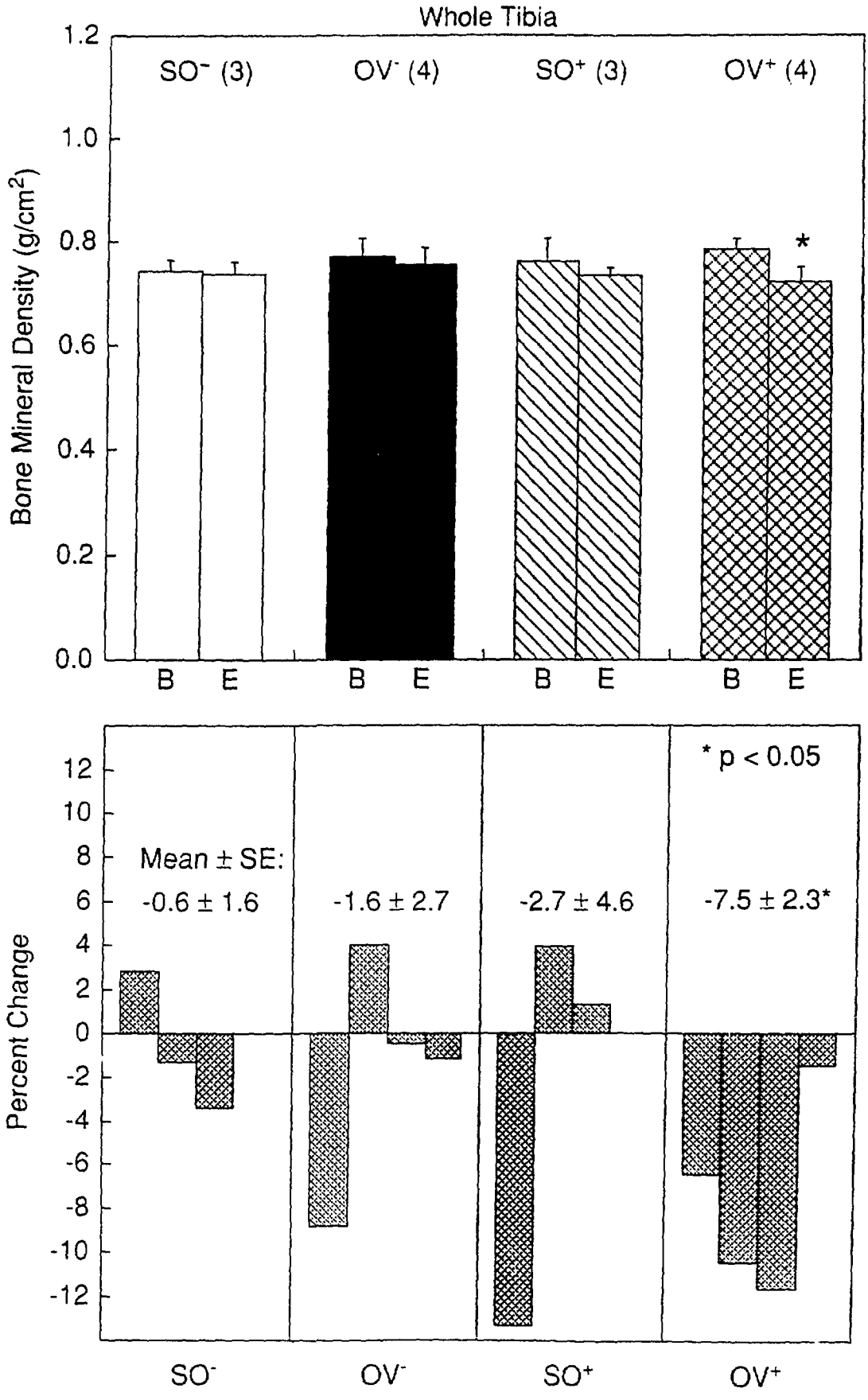

Whole Tibia
SO⁻ (3)
OV⁻ (4)
SO⁺ (3)
OV⁺ (4)
Bone Mineral Density (g/cm²)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B E B E B E B E
*
* p < 0.05
Mean ± SE:
-0.6 ± 1.6
-1.6 ± 2.7
-2.7 ± 4.6
-7.5 ± 2.3*
Percent Change
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
SO⁻
OV⁻
SO⁺
OV⁺
Bone Mineral Density – Whole Tibia

Figure 9a/9b

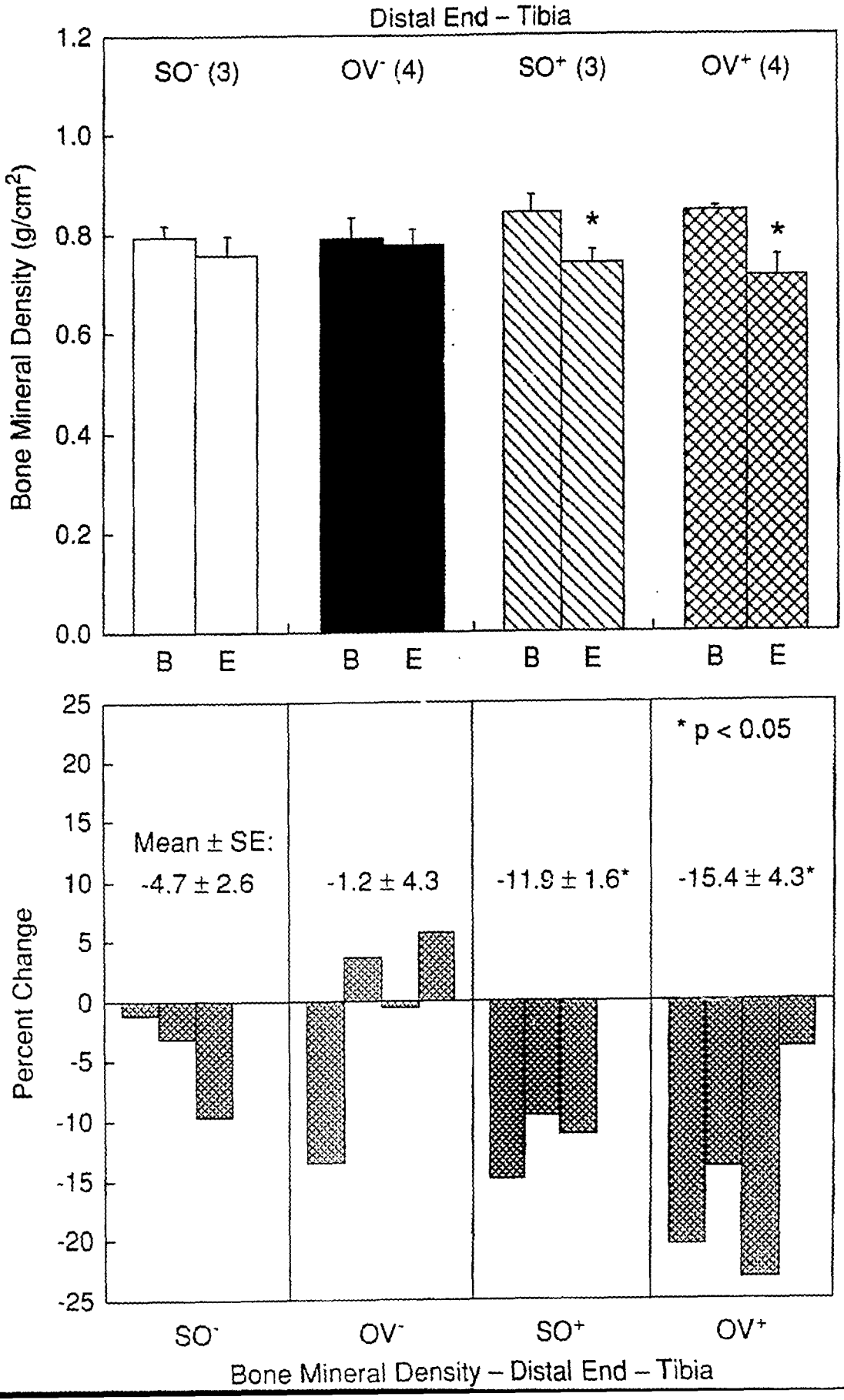

Distal End – Tibia
SO⁻ (3)
OV⁻ (4)
SO⁺ (3)
OV⁺ (4)
Bone Mineral Density (g/cm²)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B E B E B E B E
*
*
25
20
15
10
5
0
-5
-10
-15
-20
-25
Percent Change
Mean ± SE:
-4.7 ± 2.6
-1.2 ± 4.3
-11.9 ± 1.6*
-15.4 ± 4.3*
* p < 0.05
SO⁻
OV⁻
SO⁺
OV⁺
Bone Mineral Density – Distal End – Tibia

Figure 5a/5b

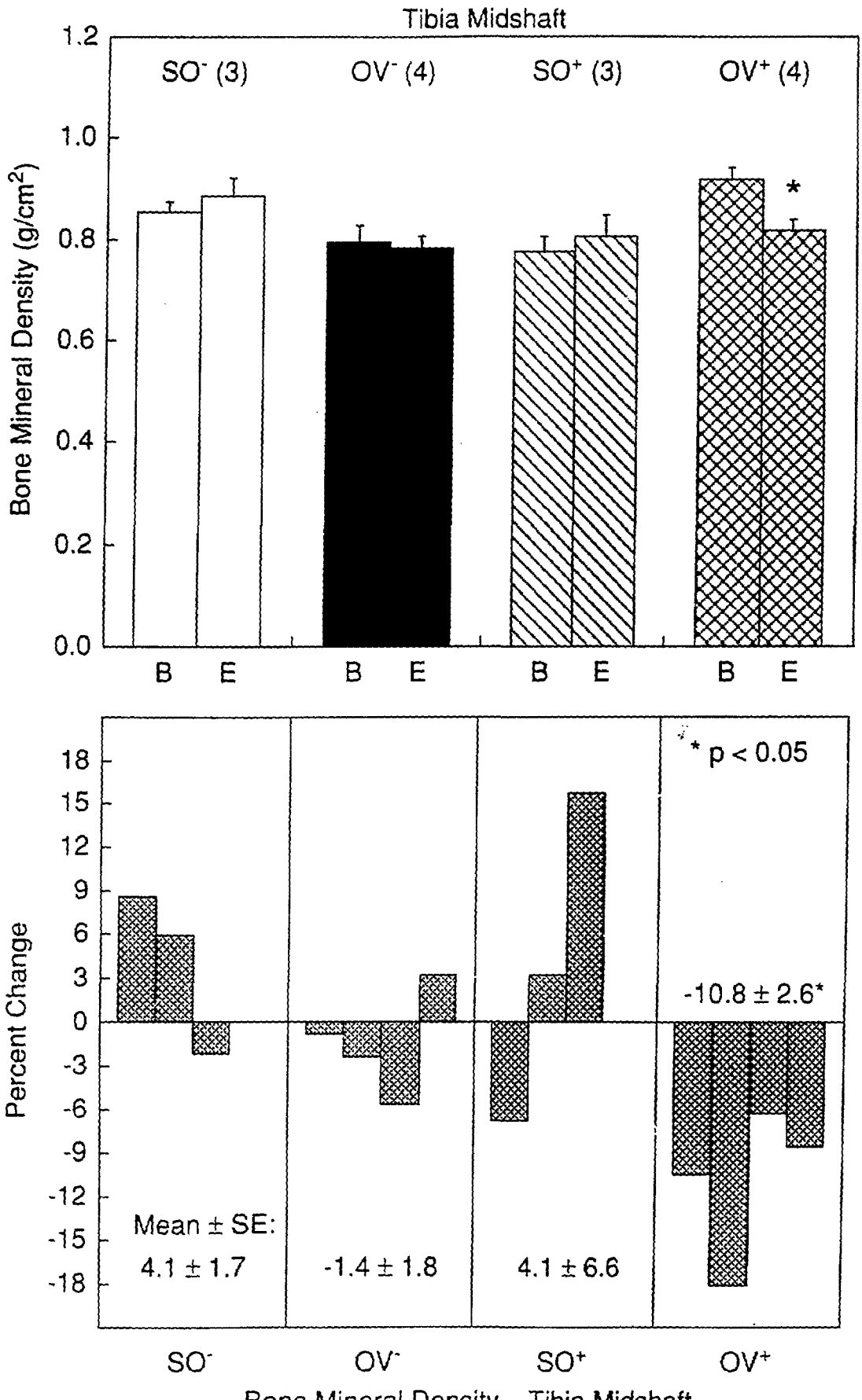

Tibia Midshaft
SO⁻ (3)
OV⁻ (4)
SO⁺ (3)
OV⁺ (4)
Bone Mineral Density (g/cm²)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
B E B E B E B E
* p < 0.05
Percent Change
18
15
12
9
6
3
0
-3
-6
-9
-12
-15
-18
-10.8 ± 2.6*
Mean ± SE:
4.1 ± 1.7
-1.4 ± 1.8
4.1 ± 6.6
SO⁻
OV⁻
SO⁺
OV⁺
Bone Mineral Density – Tibia Midshaft

Figure 6a/6b

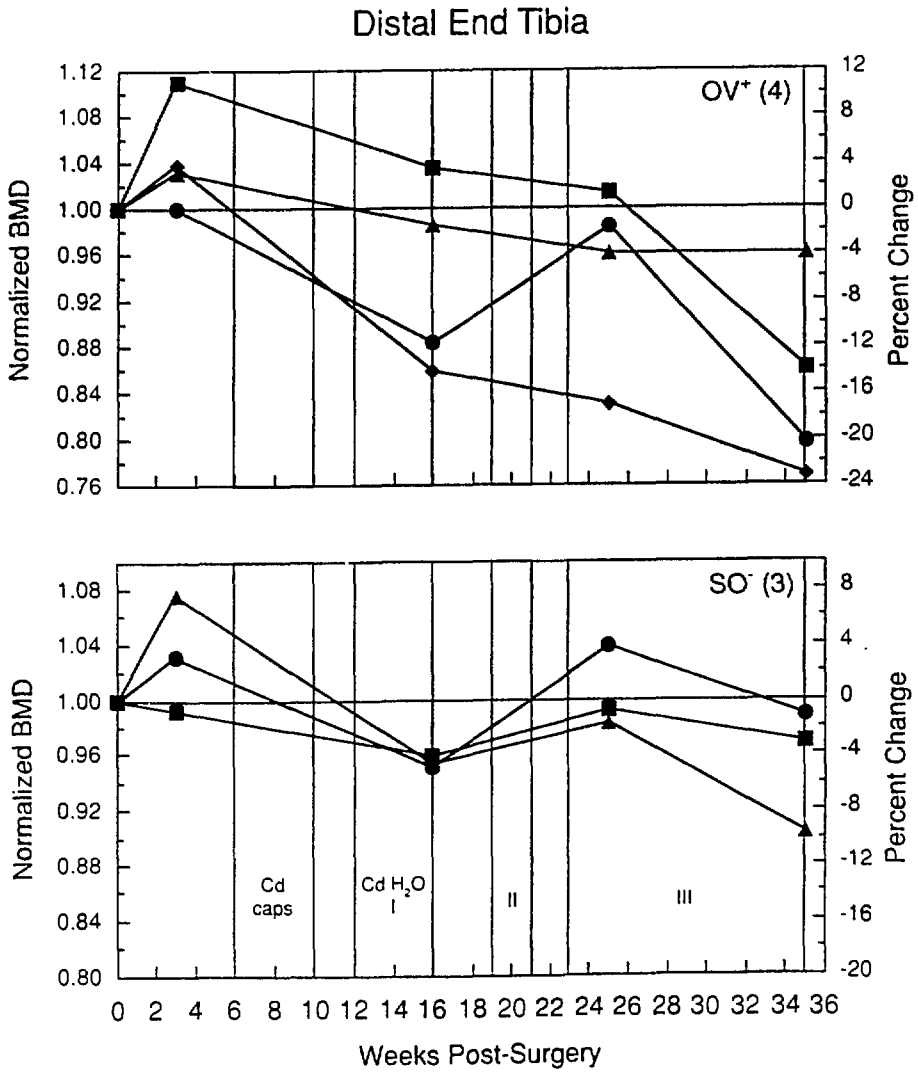
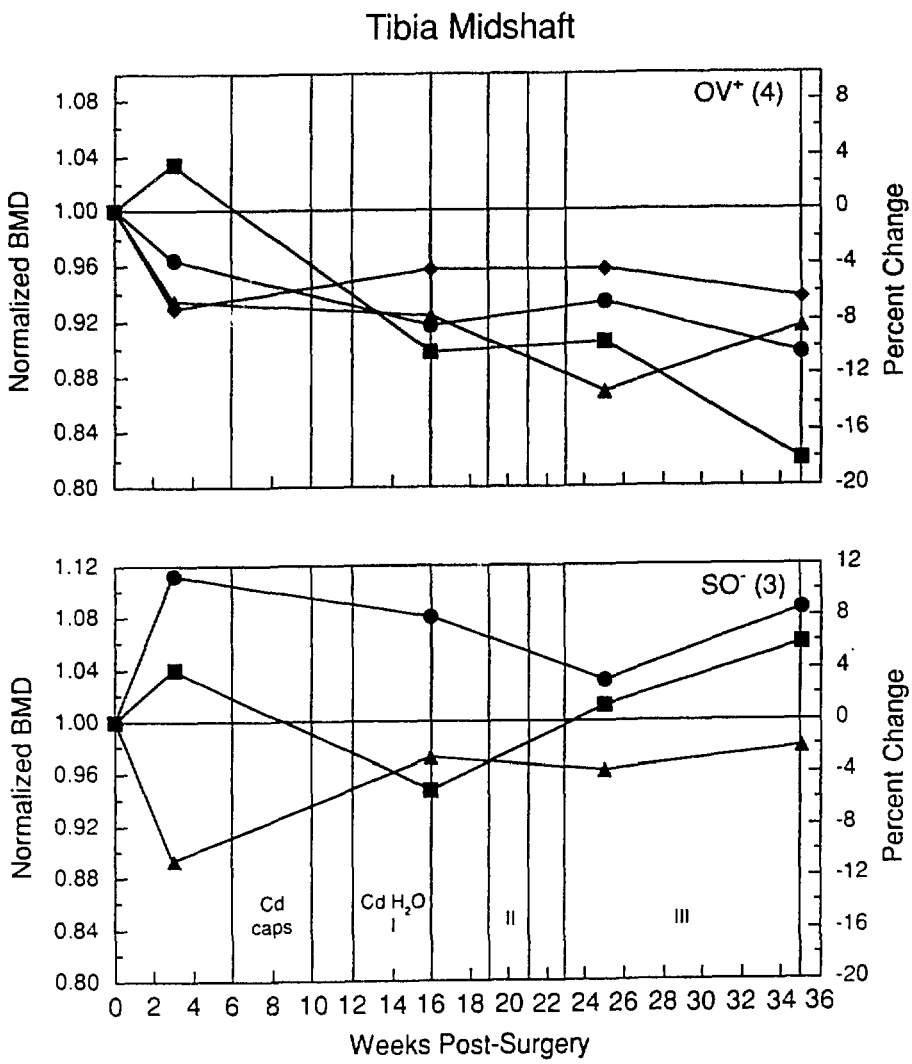
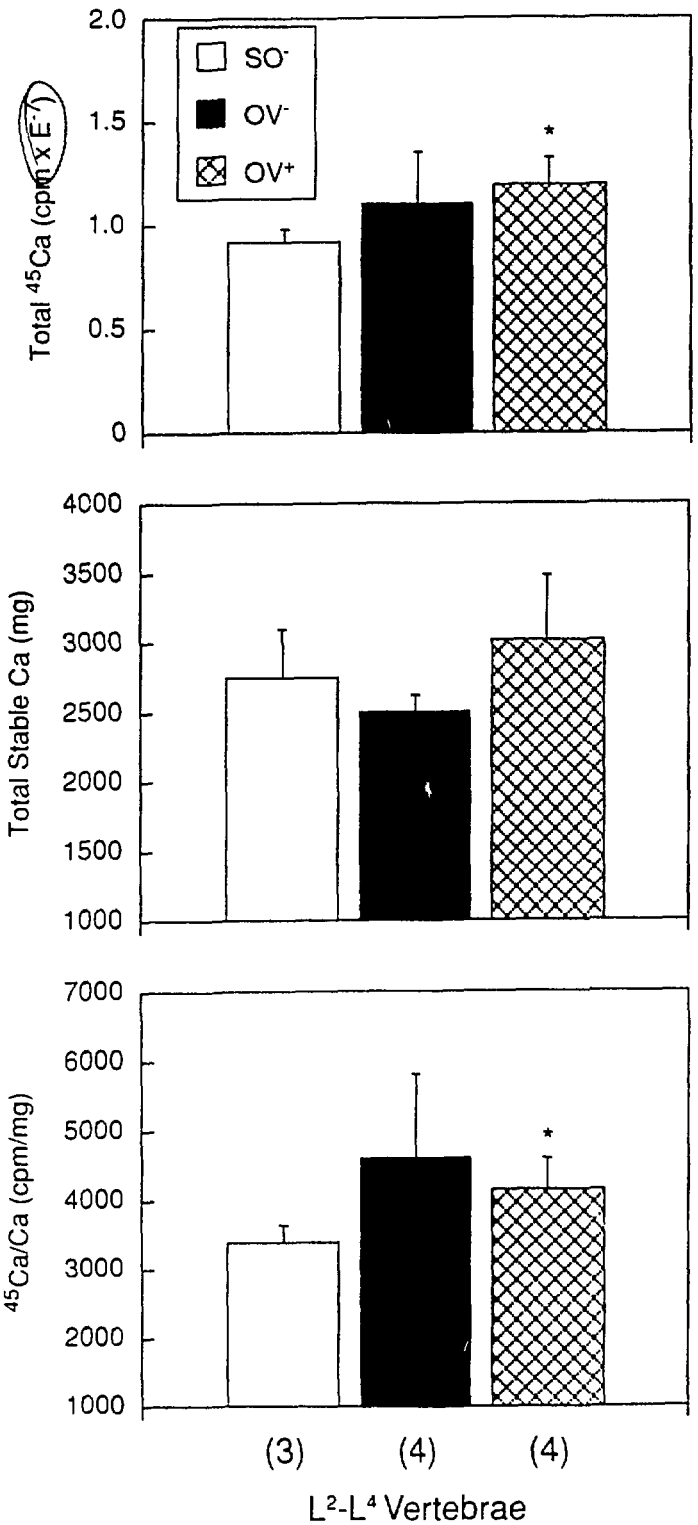

Distal End Tibia
OV+ (4)
SO- (3)
Normalized BMD
Percent Change
Cd caps
Cd H2O I
II
III
Weeks Post-Surgery

Figure 6c/6d


Tibia Midshaft
OV+ (4)
SO- (3)
Normalized BMD
Percent Change
1.08
1.04
1.00
0.96
0.92
0.88
0.84
0.80
8
4
0
-4
-8
-12
-16
-20
1.12
12
Cd caps
Cd H2O I
II
III
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Weeks Post-Surgery

9a/9b/9c


SO-
OV-
OV+
Total 45Ca (cpm x E-1)
2.0
1.5
1.0
0.5
0
*
Total Stable Ca (mg)
4000
3500
3000
2500
2000
1500
1000
45Ca/Ca (cpm/mg)
7000
6000
5000
4000
3000
2000
1000
*
(3)
(4)
(4)
L2-L4 Vertebrae

3a/8b/8c

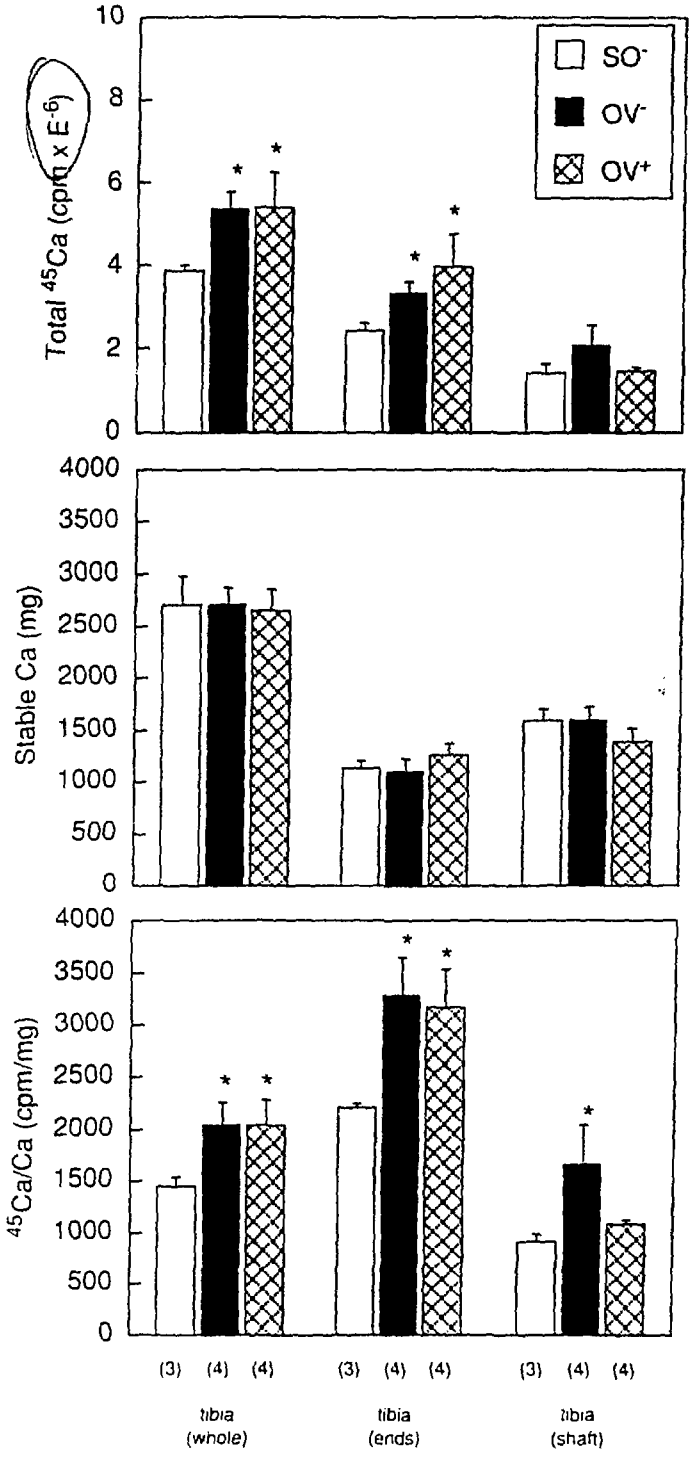
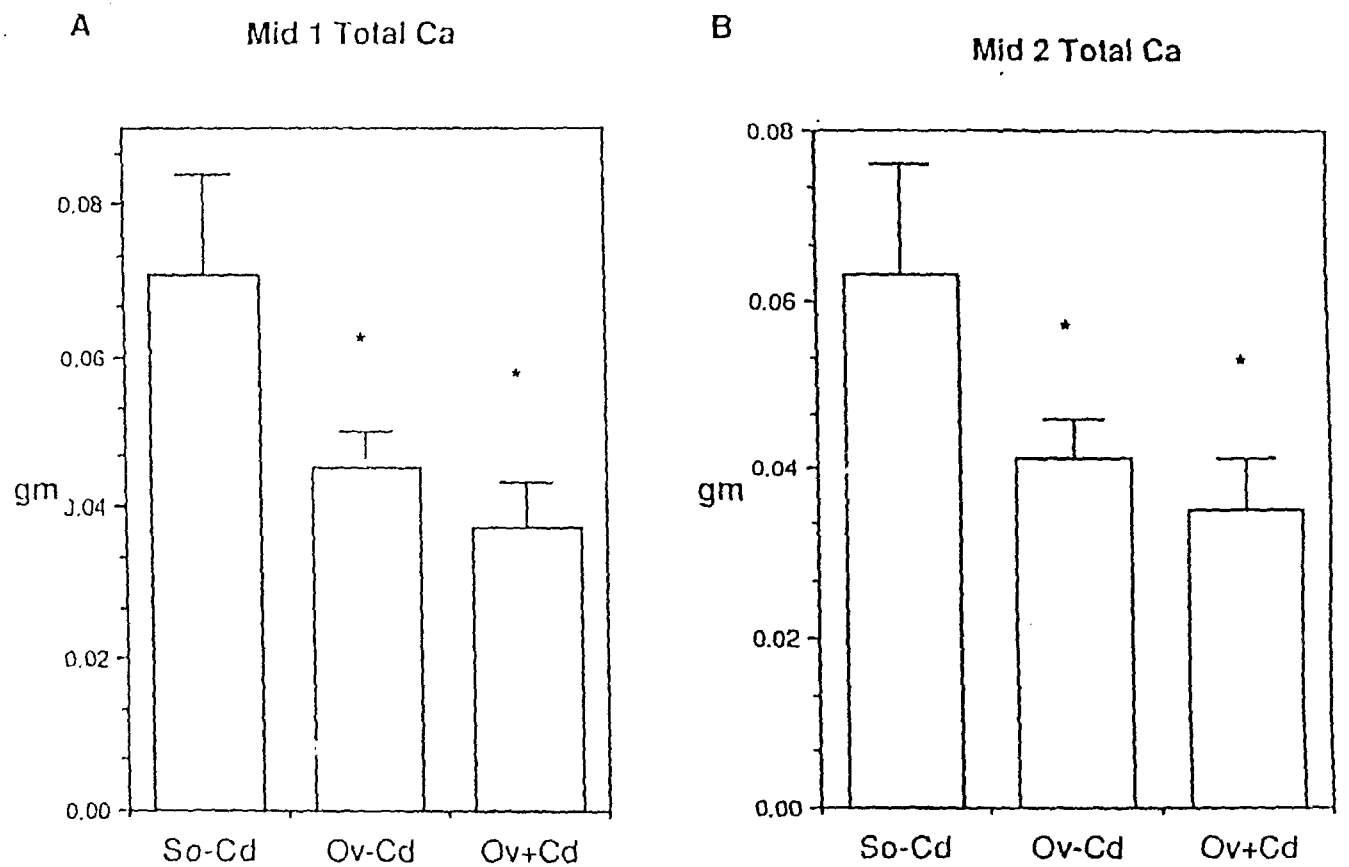

SO-
OV-
OV+
Total 45Ca (cpm x E-6)
Stable Ca (mg)
45Ca/Ca (cpm/mg)
0
500
1000
1500
2000
2500
3000
3500
4000
2
4
6
8
10
(3) (4) (4)
(3) (4) (4)
(3) (4) (4)
tibia (whole)
tibia (ends)
tibia (shaft)

Fig. 9


A
Mid 1 Total Ca
gm
0.08
0.06
0.04
0.02
0.00
So-Cd
Ov-Cd
Ov+Cd
B
Mid 2 Total Ca
gm
0.08
0.06
0.04
0.02
0.00
So-Cd
Ov-Cd
Ov+Cd