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Effect of Naproxen on Cancellous Bone in Ovariectomized Rats

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) affect bone metabolism in vitro and in vivo. They delay but do not alter the outcome of healing processes in bone. In some bone loss models, they block bone resorption and slow the rate of loss. We studied the effect of naproxen, a potent NSAID, on cancellous bone of the proximal tibial metaphysis of 6-month-old adult female ovariectomized rats.

Animals were ovariectomized, divided into groups, and fed standard diets differing only in naproxen content for 42 days. The rats of the groups ate 2.0, 5.5, 12.7, and 32 mg naproxen per kg body weight per day, respectively. Serum levels of naproxen were determined. Bone volume, mineralizing surface, osteoblast activity, osteoclast surface, and bone resorption rate were determined by bone histomorphometric techniques.

The rats' dose-related serum naproxen levels ranged from 4 to 28 μg/ml. Naproxen inhibited up to 70% of the bone loss occurring after ovariectomy at a serum level of 4 μg/ml. We deduced that naproxen blocked bone resorption in ovariectomized rats by slowing osteoclast activity at all doses. In contrast, naproxen slowed bone formation only at serum levels > 20 μg/ml in ovariectomized rats. These findings may have clinical relevance in helping to prevent postmenopausal bone loss in women.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) block inflammation by inhibiting the production of prostaglandin E_2 (PGE_2).[1] PGE_2 stimulates bone resorption and formation both in vitro[2-4] and in vivo.[5,6] When PGE_2-related acceleration of bone resorption and formation exists, NSAIDs could reduce it. When bone loss accompanies the accelerated turnover, NSAIDs may also reduce the rate of bone loss.

Past studies are consistent with this idea. NSAIDs cause no changes in normal bone in vivo.[7-11] This seems consistent because normal tissue does not have excessive PGE_2 levels. With no elevated PGE_2 production to block, naproxen's most likely mode of action is missing. However, when bone tissue itself has been surgically manipulated, resulting in inflammation and bone wounding, concurrent treatment with NSAIDs delays but does not alter the ultimate success of healing.[12-21] In inflamed tissues with high PGE_2 levels, NSAIDs are likely to act by lowering PGE_2 levels.

The purpose of this investigation was to study the effect of an NSAID, naproxen, in a high-turnover bone model lacking inflammation and osseous surgery. Ovariectomy in the 6-month-old female rat induces high bone turnover and accelerated bone loss. Although bone PGE_2 levels after ovariectomy have not been measured in vivo, bone PGE_2 production is higher than controls in calvariae explanted from young ovariectomized rats.[22] Estrogen depletion bone loss in the adult female rat also resembles postmenopausal osteopenia development in adult women.[23-33]

MATERIALS AND METHODS

Animal procedures

A total of 41 female Sprague-Dawley rats aged 180 days and weighing roughly 290 g (SASCO Co., Omaha, NE)

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were caged individually and given free access to food and water. The rats were treated according to USDA animal care guidelines and with the approval of the Creighton University Animal Research Committee. After 7 days on site, 6 rats were killed. On that day, ovariectomy by dorsal approach,[34] under Ketaset-xylazine anesthesia, was performed on the 35 remaining rats. Immediately after surgery they were weight randomized to five groups and caged individually. From the time of surgery the animals had free access to water and were fed diets containing the following naproxen concentrations: 0, 37, 100, 230, and 580 mg/kg food. Before sacrifice, the animals received double calcein (10 mg/kg; Sigma, St. Louis, MO) labels by IP injection (1 ml/kg; 2 days on, 10 days off, 2 days on, and 2 days off before sacrifice). After 42 days, the 35 ovariectomized rats that had consumed naproxen were killed (Table 1).

Necropsy was done between 9 a.m. and 1 p.m. We anesthetized each rat as for surgery and drew 6–8 ml blood through the inferior vena cava, causing death by exsanguination. Terminal serum naproxen levels were measured by high-performance liquid chromatography (HPLC, mg/ml).[35] The right tibia was removed, and the anterior eminence of bone was shaved with a razor blade, barely exposing the bone marrow. The shaved tibia was placed in 10% phosphate-buffered formalin (pH 7.2) for 24 h. Next, the proximal centimeter was sawed off and transferred to 70% ethanol. During a 2 week period this bone sample was dehydrated in graded ethanols, defatted in acetone, and embedded in modified methyl methacrylate.[36]

Section preparation and quantitation

Pairs of 5 μm frontal sections were prepared from the anterior aspect of the tibia with a Jung Model K microtome. The first was stained by the Goldner method,[37] and the second was left unstained. Coverslips were affixed to all with Permount. Each slide was given a random number to obscure its identity from the observer.

Bone elongation rate was measured by finding the distance between the two fluorochrome labels in newly formed primary spongiosa[38]; 12 days was not a sufficient time for the labels to separate. We concluded that since we could have identified labels separated by 50 μm or more, these rats were growing less than 5 μm/day. This rate of bone elongation in this age of rat was also found by other authors.[39] Since we could not detect bone elongation, we also assumed that this age of rat is an imperfect but acceptable approximation of steady-state conditions of the mature skeleton.

On each slide a standardized trapezoidal data collection area was outlined with a felt-tip pen (Fig. 1). It measured 10–12 mm², included no primary spongiosa, and extended 4 mm distally. It contained only cancellous bone and marrow. The entire trapezoid was viewed under a light/epifluorescent microscope with ×10 oculars and a camera lucida. The camera lucida projected onto a graphics pad interfaced to an IBM PC XT computer. BIOQUANT II Software (R&M Biometrics, Nashville, TN) was used to collect the raw data. With a ×1 objective, the area of the trapezoid was outlined [total tissue area (TtT.Ar)]. With a ×4 objective, the area of each bone island was outlined to find cancellous bone area (B.Ar). This same movement also determined the bone surface (B.Pm). With a ×16 objective, double-labeled surface (dL.Pm) was measured. With a ×40 objective, interlabel thickness of double labels (IrL.Wi) and osteoclast surface (Oc.Pm) were measured.[40]

We judged that identifying single-labeled surface in the secondary spongiosa was much less reliable than identifying double label. Our values for MS/BS, which use only double-labeled surface, are likely to underestimate the true mineralizing surface.

The five bone histomorphometric variables that best describe bone volume, forming cell number, resorbing cell number, individual forming cell activity, and individual resorbing cell activity were calculated. From TtT.Ar and B.Ar, total cancellous bone volume (BV/TV) was calculated. From B.Pm, dL.Pm, and Oc.Pm, the percentage of mineralizing surfaces (based on double label, MS/BS) and percentage of osteoclast surface (Oc.S/BS) were calculated. From IrL.Wi and the interlabel time period, the mineral apposition rate (MAR), a measure of individual osteoblast activity, was calculated. Finally, we calculated surface-based bone formation rate (BFR/BS) and bone resorption rate (BRsR/BS) by the method described previously.[41]

Statistics

The Kruskal-Wallis nonparametric test was applied to analyze differences among the groups. The Wilcoxon test was also used to compare groups for differences in bone histomorphometric parameters.[42]

TABLE 1. EXPERIMENTAL DESIGN AND TERMINAL SERUM NAPROXEN

Group	*Rats per group (N)*	*Naproxen in food (mg/kg)*	*Naproxen consumed (mg/kg per day)*	*Duration (days)*	*Serum naproxen (μg/ml) concentration (x ± SD)*
0	6	0	0.0	0	0.0 0.0
1	6	0	0.0	42	0.0 0.0
2	8	37	2.0	42	3.6 0.8
3	6	100	5.5	42	10.0 1.6
4	8	230	12.7	42	20.2 5.1
5	7	580	32.0	42	27.8 9.9

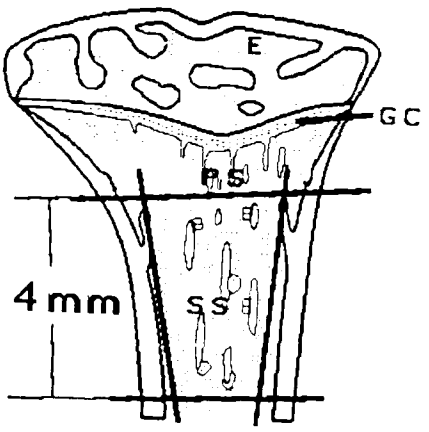

FIG. 1. Frontal section of proximal tibia. The epiphysis (E), epiphyseal growth cartilage (GC), primary spongiosa (PS), and secondary spongiosa (SS) are noted. Bone islands are clear and marrow is stippled. We analyzed a 10 mm^2 trapezoidal area of the secondary spongiosa. It never included the primary spongiosa or bone attached to the cortex.

RESULTS

The rats sustained both surgery and drug treatment without complications. They ate about 18 g food per day, thus consuming, respectively, 0.66, 1.8, 4.1, or 10.4 mg naproxen per day (2.1, 5.6, 12.8, or 32.5 mg per day). The level of naproxen consumption resulted in correspondingly increased serum naproxen levels from 3.6 to 27.8 μg/ml (Table 1). The rats initially weighed 298 ± 29 g. Their final weight was 342 ± 28 g, with no intergroup differences.

In untreated ovariectomized rats, bone volume (BV/TV) declined from 12.1% at baseline to 3.9% at day 42 ($p < 0.005$, Fig. 2). All rats treated with naproxen had significantly higher BV/TV than untreated ovariectomized rats ($p < 0.05$ to $p < 0.01$, Fig. 2). Animals with naproxen levels >20 μg/ml had higher BV/TV than untreated ovariectomized rats ($p < 0.05$) but tended to have less than those with <10 μg/ml. Rats with serum naproxen >20 μg/ml had significantly higher BV/TV than untreated OX rats ($p < 0.05$) but somewhat lower BV/TV than baseline rats (Fig. 2). Although rats with serum naproxen < 10 μg/ml had somewhat lower BV/TV than baseline rats, the changes were not statistically significant (Fig. 2).

In untreated ovariectomized rats, mineralizing surface (MS/BS) rose from 3.4% at baseline to 15.6% in untreated OX rats ($p < 0.001$, Fig. 3). Animals with naproxen levels < 20 μg/ml had no significant differences from untreated ovariectomized rats. However, animals with 28 μg/ml had lower MS/BS than all other day 42 rats ($p < 0.05$). No significant differences existed among the groups in mineral apposition rate (MAR).

Osteoclast surface (Oc.S/BS) rose from 2.4% at baseline to 3.4% in untreated ovariectomized rats (Fig. 4); this difference was not statistically significant. No significant differences existed among the naproxen-treated ovariecto-

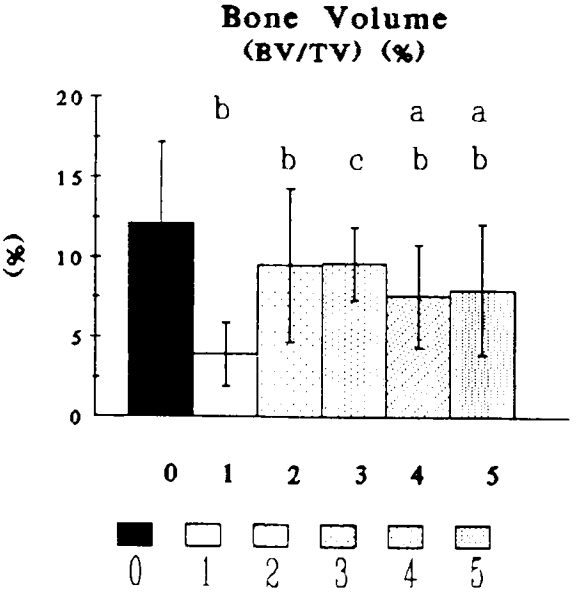

FIG. 2. Bone volume by group. The mean ± standard deviation is plotted for each group. Group 0 is baseline. Group 1 is untreated ovariectomized animals. Group 2 had 3.7 μg/ml of serum naproxen. Group 3 had 10.0 μg/ml of serum naproxen. Group 4 had 20 μg/ml of serum naproxen. Group 5 had 28 μg/ml of serum naproxen. Comparisons to baseline group are on upper level; comparisons to untreated ovariectomized group are on lower level. Differences are expressed as (a) $p < 0.05$; (b) $p < 0.01$; (c) $p < 0.001$.

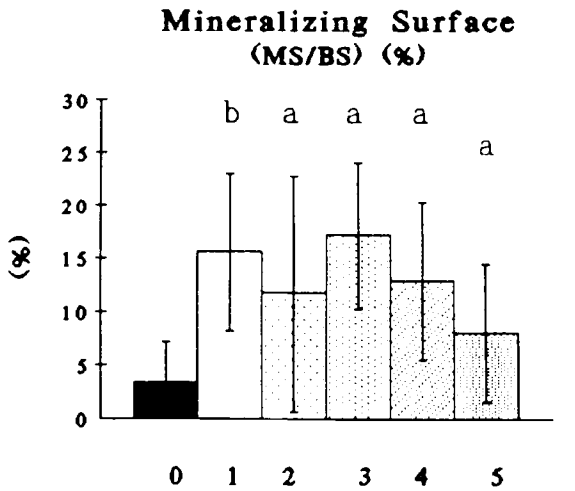

FIG. 3. Mineralizing surface by group., The mean ± standard deviation is plotted for each group. Comparisons to baseline group are on upper level; comparisons to untreated ovariectomized group are on lower level. Differences are expressed as (a) $p < 0.05$; (b) $p < 0.01$; (c) $p < 0.001$.

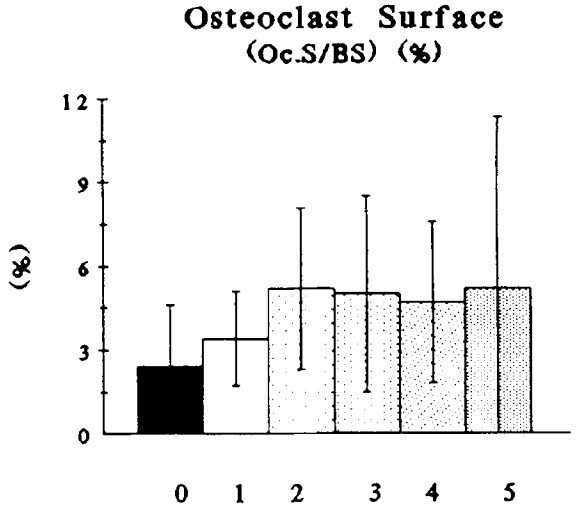

FIG. 4. Osteoclast surface by group. The mean ± standard deviation is plotted for each group. Comparisons to baseline group are on upper level; comparisons to untreated ovariectomized group are on lower level. Differences are expressed as (a) $p < 0.05$; (b) $p < 0.01$; (c) $p < 0.001$.

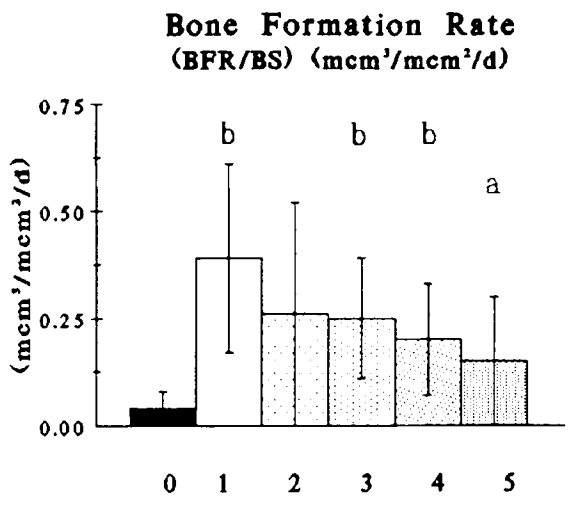

FIG. 5. Bone formation rate by group. The mean ± standard deviation is plotted for each group. Comparisons to baseline group are on upper level; comparisons to untreated ovariectomized group are on lower level. Differences are expressed as (a) $p < 0.05$; (b) $p < 0.01$; (c) $p < 0.001$.

mized rats. The data suggest a trend toward increasing osteoclast surface with naproxen treatment.

Bone formation rate (BFR/BS) was 0.05 $\mu m^3/\mu m^2$ per day at baseline. In untreated ovariectomized rats, BFR/BS rose significantly to 0.4 $\mu m^3/\mu m^2$ per day (Fig. 5). In naproxen-treated rats, it ranged from 0.13 to 0.25 $\mu m^3/\mu m^2$ per day, generally above that in baseline rats. However, at the serum level of 28 μg/ml, naproxen significantly reduced bone formation rate ($p < 0.05$), below that in untreated ovariectomized rats.

Bone resorption rate (BRsR/BS), an index of osteoclast activity, was 0.04 $\mu m^3/\mu m^2$ per day at baseline (by definition the same as BFR/BS, when BV/TV is constant). In untreated ovariectomized rats, bone resorption rate (BRsR/BS) rose to 1.36 $\mu m^3/\mu m^2$ per day (Fig. 6). In naproxen-treated rats, it ranged from 0.17 to 0.78 $\mu m^3/\mu m^2$ per day. At the serum level of 10 μg/ml, naproxen significantly lowered bone resorption rate ($p < 0.05$) below that in untreated ovariectomized rats.

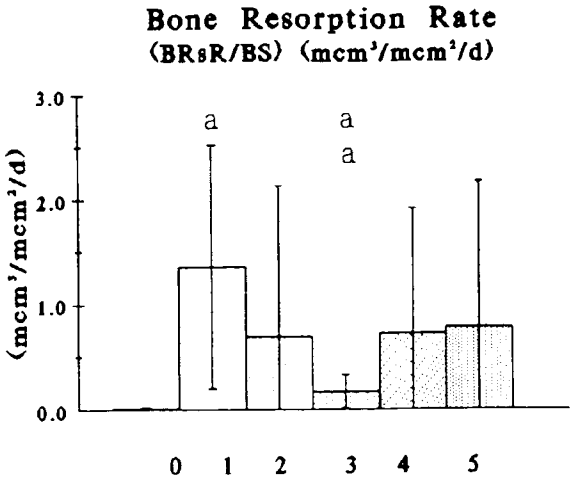

FIG. 6. Bone resorption rate by group. The mean ± standard deviation is plotted for each group. Comparisons to baseline group are on upper level; comparisons to untreated ovariectomized group are on lower level. Differences are expressed as (a) $p < 0.05$; (b) $p < 0.01$; (c) $p < 0.001$.

DISCUSSION

Adult female rats given naproxen for the first 42 days after ovariectomy have more cancellous bone in the proximal tibial metaphysis than similar untreated rats. We deduce that some doses of naproxen inhibit bone loss by slowing bone resorption without affecting bone formation. The minimum serum level of naproxen that depresses resorption is one order of magnitude below the level necessary for therapeutic anti-inflammatory effects.[43] Near anti-inflammatory levels of naproxen are less effective at preserving bone mass because they slow bone formation activity by decreasing osteoblast numbers.

Cancellous bone of the proximal tibial metaphyseal secondary spongiosa of an older ovariectomized rat is a reasonable model for vertebral cancellous bone of a woman in her early postmenopausal years with declining estrogen production.[24,33] It has remodeling activity similar to that reported in the rat tail vertebra.[24,44] We confirm an earlier observation that the elongation rate at the nearby epiphyseal growth cartilage is about 5 μm/day.[24,39] In this study, 65–70% of the proximal tibial cancellous bone dis-

appeared during 6 weeks after ovariectomy. At 6 weeks, rats treated with naproxen lost less than half the bone lost by untreated ovariectomized rats. These data suggest that naproxen may slow cancellous bone loss in humans after acute estrogen depletion. Although animals treated with lower doses of naproxen showed no statistically significant decline in bone volume, the possibility of encountering a type II error still exists. We need data from longer term studies with larger groups to know the time and extent of this bone-saving effect.

The anti-PGE_2 actions of naproxen may be responsible for its ability to slow bone loss in ovariectomized rats. The ability of naproxen to partially preserve bone mass in ovariectomized rats by slowing resorption suggests that some of the accelerated bone loss after estrogen depletion is due to elevated PGE_2. Bone PGE_2 production is higher than controls in calvariae explanted from young ovariectomized rats.[22] Furthermore, in calvariae from rats pretreated with estrogen, bone PGE_2 production rate is the same as in controls.[22] In vivo, estrogen treatment provides complete protection against postovariectomy bone loss in rats.[45,46] Naproxen, a cyclooxygenase inhibitor, lowers PGE_2 levels in other tissues. We did not measure bone PGE_2 levels. However, it seems reasonable to believe that naproxen, like estrogen, lowers bone production of PGE_2,[22] thereby reducing the rate of bone loss.

Estrogen, bisphosphonates, and parathyroid hormone also slow estrogen-depletion bone loss in rats.[45-47] Naproxen seems less effective than these agents. Since naproxen is likely to work by inhibiting PGE_2 production, it probably inhibits that portion of the bone loss related to elevated PGE_2. This suggests that estrogen-depletion bone loss has facets unrelated to elevated PGE_2 production.

Low doses of naproxen appeared to inhibit bone resorption without changing osteoclast numbers. From this we infer that osteoclast activity may have been reduced. Although the mechanism for this effect is unknown, naproxen may inhibit collagenase and other metalloproteinase activity of osteoclasts, as it does in chondrocytes in vitro.[48] Studies of osteoclast ultrastructure would define the organelles altered by naproxen.[49,50] In vitro studies of enzyme production by osteoclasts during naproxen treatment would further clarify this mechanism.

Agents that inhibit osteoclast activity without depressing cell numbers are valuable when the aim is to transiently slow bone resorption without other effects, as in coherence therapy.[51] Estrogen, acetazolamide, gallium, mithramycin, and calcitonin[45,46,52-55] also inhibit osteoclast activity. However, these compounds also depress osteoclast numbers. Bisphosphonates also decrease osteoclast activity while paradoxically increasing osteoclast numbers.[56] The chemical binding of bisphosphonates to bone surfaces also plays a role in their antiresorptive effects. The ability of naproxen to transiently slow resorption without apparent interaction with bone crystalline structure deserves further investigation.

Exogenous parathyroid hormone also prevents bone loss in newly ovariectomized rats.[47] In young male rats, PTH increases bone resorption, bone formation, and cancellous bone mass.,[57] However, indomethacin does not block this action of PTH,[58] suggesting that PTH's effects are PGE_2 independent. This raises the possibility that some combination of a PGE_2 inhibitor to slow osteoclast activity and PTH to stimulate formation could be effective for building bone.

Low doses of naproxen are more effective than high doses at preserving cancellous bone mass after ovariectomy. Others have described a similar biphasic effect in which NSAIDs have their most positive effect at intermediate doses.[59] In explanted neonatal rat calvariae, indomethacin, flurbiprofen, and piroxicam stimulate PGE_2 production at 10^{-9}–10^{-11} M but inhibit it at 10^{-8}–10^{-6} M. In both growing and adult rats, intermediate doses of flurbiprofen stimulate bone elongation rate and periosteal bone accumulation but higher doses inhibit both.[11]

In surgical bone wound healing, acute inflammation is followed by woven bone production. NSAIDs extend the duration but do not prevent healing when given at and shortly after surgery. Models tested include fracture healing,[12-15] osteotomy,[17-19] tooth extraction,[16] bone ingrowth,[21] and heterotopic bone formation.[9,18] NSAIDs seem able to interfere with the inflammatory phases of healing and, thus, with the timely production of woven bone. In these models of bone healing it seems possible that PGE_2 affects the outcome through its interaction with the inflammatory phase. The ability of NSAIDs to delay healing and slow accelerated bone processes is most likely through their anti-PGE_2 effects.

Our data suggest that naproxen provides incomplete protection against estrogen-depletion bone loss after 6 weeks. As in wound healing, we suggest that naproxen extends the time for estrogen-depletion bone loss to occur without altering the final bone mass. The incomplete protection probably means that estrogen corrects defects other than the obvious defects related to turnover and elevated osteoclast activity. Longer term studies of naproxen treatment in both ovariectomized and intact rats would clarify this point.

Research to prevent postmenopausal bone loss by means other than estrogen replacement therapy may have to consider that there is an estrogen-dependent quantum of cancellous bone. This compartment may resond to estrogen more efficiently than to any other compound. The narrowing of the marrow cavity at puberty suggests the appearance of an estrogen-dependent quantum of bone in menstruating human females.[60] Likewise, the self-limited decline in bone mass in estrogen-depleted subjects[61,62] suggests its disappearance. The self-correcting negative calcium balance of the first year or two after menopause[63] suggests that a new equilibrium of near neutral balance follows the disappearance of that compartment of bone. A quantum of cancellous bone of similar behavior appears to exist in female rats. Regardless of the involvement of "remodeling" or "modeling," its disappearance appears to be relevant to the estrogen-depleted woman.

In summary, ovariectomized rats treated with naproxen from the time of surgery for 42 days have more cancellous bone in their proximal tibial metaphysis than nontreated ovariectomized rats. We deduce that this effect is due to a suppression of osteoclast activity at naproxen serum levels

of 4-28 μg/ml. Above 20 μg/ml some suppression of bone formation activity was also observed, which resulted in a somewhat less effective preservation of bone mass. These levels of naproxen are considerably below those necessary for therapeutic anti-inflammatory activity. Further studies now underway will ascertain the time for which estrogen-depletion bone loss inhibition attributable to naproxen may be maintained.

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