Comparison of osteopenia after gastrectomy, ovariectomy and prednisolone treatment in the young female rat

Vikas V Surve[1], Niklas Andersson[1], Daisy Lehto-Axtelius[2] and Rolf Håkanson[1]

Departments of [1]Pharmacology, Institute of Physiological Sciences, University of Lund, Sölvegatan 10, SE-223 62 Lund, Sweden. E-mail: Rolf.Hakanson@farm.lu.se; [2]Radiology, Centre for Oral Health Sciences, Malmö University, Malmö, Sweden. Correspondence: Professor Rolf Håkanson
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ABSTRACT – Rat models of osteopenia include ovariectomy and long-term glucocorticoid treatment. Although ovariectomy produces significant trabecular bone loss after 2 weeks, long-term glucocorticoid treatment has been reported to cause osteopenia in some studies but not in others. In the present 8-week-study, we compared the osteopenia associated with gastrectomy (GX) to that induced by ovariectomy (OVX) or prednisolone (PRE) treatment. Female Sprague-Dawley rats (10 weeks old) were subjected to GX, OVX, PRE treatment or SHAM operation. At the end of the study, calvariae, femurs and fifth lumbar vertebrae (L5) were collected and subjected to bone density measurement (femur and L5), transillumination (calvaria) and histomorphometry (calvaria and femur). Bone density was reduced in L5 and the distal femur in the OVX and GX groups, but not in the PRE group. Transillumination of the calvaria showed marked bone loss in the GX rats, but not in the other groups. Morphometric analysis of the femur revealed reduced trabecular bone volume, trabecular thickness, trabecular number and osteoclast number, but increased osteoclast surface (expressed as per cent of the trabecular bone surface covered by osteoclasts) in the GX and OVX rats. The PRE rats seemed unaffected. Cortical thickness was reduced in the GX rats, but not in the other groups. The findings indicate that GX induces osteopenia in, e.g., femur and vertebra of a magnitude similar to or greater than that induced by OVX, while at the same time inducing osteopenia in the calvaria. Although osteoclast activation seems to contribute, the precise mechanism underlying the GX-evoked osteopenia remains obscure.

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Osteopenia is common in experimental animals after ovariectomy (OVX) (Aitken et al. 1972, Wronski et al. 1985, 1986, Yamazaki and Yamaguchi 1989, Kalu 1991, Miller and Wronski 1993), glucocorticoid (prednisolone) treatment (PRE) (Lindgren et al. 1982, 1983, Baylink 1983, Rickers et al. 1984, Yamazaki et al. 1986, Goulding and Gold 1988, Ørtoft et al. 1995, Turner et al. 1995) and gastrectomy (GX), i.e., surgical removal of the stomach (Bussaberger et al. 1938, Ivy 1940, Persson et al. 1993, Klinge et al. 1995, Lehto-Axtelius et al. 1998). GX is also associated with generalized osteopenia in man (for reviews on the subject, see Editorial 1986, Tovey et al. 1991, 1992). OVX induces significant trabecular bone loss already after 2 weeks, as shown by histomorphometry (Dempster et al. 1995, Wronski et al. 1998). From past experience we know that GX induces trabecular bone loss in the young rat 2 weeks after the operation (Persson et al. 1993, Klinge et al. 1995, Lehto-Axtelius et al. 1998). Although large animals, like rabbit and dog, seem to become osteopenic after treatment with glucocorticoids, this does not appear to happen in rats. Some reports describe bone loss, others an increased amount of bone tissue after PRE treatment (Lindgren et al. 1982, 1983, Baylink 1983, Rickers et al. 1984, Yamazaki et al. 1986, Goulding and Gold 1988, Turner et al. 1995), while others have not detected bone loss (Shen et al. 1997, Okazaki et al. 1998). The mechanism underlying the GX-evoked osteopathy is unknown. The present study is the first to evaluate and compare the magnitude of the osteopenia induced in young female rats by OVX, PRE or GX.

Animals

Female Sprague-Dawley rats, 10 weeks old (weighing 215–220 g) at the start of the experiments, were fed a diet of commercial rat food pellets and tap water ad libitum. The duration of the study was 8 weeks. The rats were killed by exsanguination from the abdominal aorta under chloral hydrate anesthesia. GX rats were given intramuscular injections, once every other week (beginning the first week after surgery), of 0.1 mL vitamin B_{12} (1 mg/mL) (since gastrectomy causes loss of the intrinsic factor which is essential for absorption of vitamin B_{12}) and 0.1 mL iron sorbitol (50 mg Fe^{3+} sorbitol/mL) (to compensate for the anticipated poor absorption of iron due to the loss of gastric acid). These supplements had no effect on the body weight of unoperated rats (Wojtyczka et al. 1998). The experiments described below were approved of by the local Animal Welfare Committee, Lund, Sweden.

Experimental design

Operations were performed under chloral hydrate anesthesia (300 mg/kg body weight). After surgery, all rats were housed in groups of 3–4.

Ovariectomy

6 rats were bilaterally ovariectomized (OVX), i.e., surgical removal of both ovaries via an incision in the lower abdomen. The ovaries were gently removed to avoid damaging the glands, thereby causing reimplantation of minute residues. Success of OVX was shown by an acceleration in body weight gain (Figure 1) and confirmed at necropsy by uterine atrophy (data not shown).

Gastrectomy

6 rats were gastrectomized (GX), i.e., resection of the glandular part of the stomach followed by joining of the duodenum to the nonglandular part of the stomach end-to-end (Lehto-Axtelius et al. 1998). One rat died during the first week and was replaced by another, killed one week later than the others. Success of GX was suggested by a low concentration of gastrin in the circulation (data not shown).

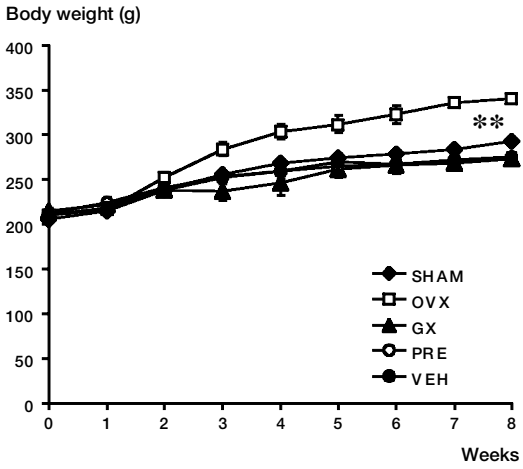

Figure 1. Body weights of SHAM, OVX, GX, VEH- and PRE-treated rats plotted against weeks of treatment. Mean values ± SEM (vertical bars, n 6 rats in each group). Fisher PLSD test for the difference between Sham and OVX rats, ** $p < 0.01$.

Sham operation

6 rats underwent SHAM operation by an abdominal mid-line incision, followed by gentle manipulation of the viscera.

Prednisolone treatment

6 rats received prednisolone (PRE) (80 mg/kg body weight). Prednisolone sodium succinate, dissolved in sterile water at the time of injection, was injected into one of the rump muscles twice a week for the duration of the study. The dose was based on the study by Yamazaki et al. (1986), who described reductions in bone density and cortical thickness index after 12 weeks of treatment. 6 control rats received the same injection of vehicle (VEH), i.e., sterile water.

Blood and tissue collection

Serum was collected at the end of the study. The gastrin concentration was determined by radioimmunoassay (Stadil and Rehfeld 1973). Both femurs, the calvaria, and the fifth lumbar vertebra (L_5) were removed from each rat, cleaned and examined, as described below. Fresh (unfixed) calvariae were cleaned and the periosteum removed. Drying was avoided by covering the calvaria with gauze soaked in saline and storing them in an airtight container at +4 °C, pending examination.

Each calvaria was placed on a glass plate on top of a commercial light source, emitting fluorescent light of constant intensity (Klinge et al. 1995). The resulting transillumination images were photographed with a camera connected to an operation microscope, magnification ×16.

Femur length

The femurs were cleaned and the joint removed. The distance between the most prominent parts of the distal and proximal femur was measured with an electronic digital caliper (0–150 mm, resolution 0.01 mm).

Bone density

Distal femur specimens were collected by dividing the right distal femur, 10 mm from the distal epiphyseal end, with the help of calipers and a circular saw. The specimen represented the distal epiphysis together with a part of the metaphysis. The L5 vertebra and distal femur specimens were cleaned of soft tissue and weighed. Bone volumes were determined with Archimedes' principle and the bones then incinerated in an oven at 800 °C for 18 h. The resulting ash was weighed. The bone ash weight divided by the bone volume gave the bone mineral density, g/cm^3.

Histomorphometry

The bone specimens to be analyzed consisted of the left distal femur, sawed off 10 mm from the epiphysis with the help of calipers, described above) and calvaria from each rat (see below). The specimens were fixed in buffered formalin (4%) and decalcified in formic acid (4N, 15%) for 2 days. Each femur was embedded in methyl methacrylate while the calvaria was embedded in paraffin. Longitudinal sections of the femur specimens (7 μm thick) were cut (frontal plane). Specimens of the calvariae were collected 4 mm from the coronal suture towards the parietal suture and sectioned (7 μm thick). The femur sections were subjected to tartrate-resistant acid phosphatase (TRAP) staining to show osteoclasts (Minkin 1982), while the calvaria sections were stained with hematoxylin/eosin and phloxin. The sections of the femur were subjected to histomorphometric analysis.

The histomorphometric parameters were defined according to the report by the American Society for Bone and Mineral Research committee (Parfitt et al. 1987). Trabecular bone volume (BV/TV %), trabecular thickness (Tb.Th μm), trabecular number (Tb.N/mm) and osteoclast number (N.Oc/mm^2) were determined in mid-longitudinal sections of the metaphyseal area. Measurements were made 0.05 mm from the lowest point of the growth plate (the first level) and further down into the trabecular bone. The second and third levels were half a field of vision and a whole field of vision, respectively, below the first level. BV/TV% was measured (magnification ×200), using a 42-point Weibel 2 grid in the ocular. The BV/TV% was calculated by dividing the number of hits over the trabeculae with the total number of hits per graticule within the area between the two endosteal bone surfaces. For the measurement of Tb.Th, we used an ocular micrometer and the thickness of each trabecula (μm) within the area from one endosteal bone surface to the other was measured (magnification ×200). The value for the Tb.N/mm was obtained by using a micrometer to measure the distance from one endosteal bone surface to the other (mm), after which the number of trabeculae crossing the line, represented by the micrometer scale, was counted (magnification ×100). The cortical thickness (Ct.Th μm) was determined at 5 levels (0.5 mm apart), starting 0.2 mm below the lowest point of the growth plate (magnification ×40). The number of osteoclasts per unit area (N.Oc/mm^2) was determined, using indexed squares from Graticules (Tonbridge, Kent, U.K.); all TRAP-positive cells with at least one visible nucleus were counted (magnification ×200). The osteoclast-covered trabecular bone surface, expressed as per cent of the trabecular bone surface (osteoclast surface, Oc.S/BS%), was measured (magnification ×200), using a 42-point Weibel 2 grid; the number of hits over nucleated osteoclasts at the interface between bone and bone marrow was divided with the total number of hits over the bone-bone marrow interface.

The calvaria sections were examined in a Leitz Aristoplan light microscope. Calvaria thickness measurements and morphometric analysis were made with image analysis, performed at an objective magnification ×4. The images were recorded, using a video camera, and transferred to a PC with custom-designed software (PC-Image: application

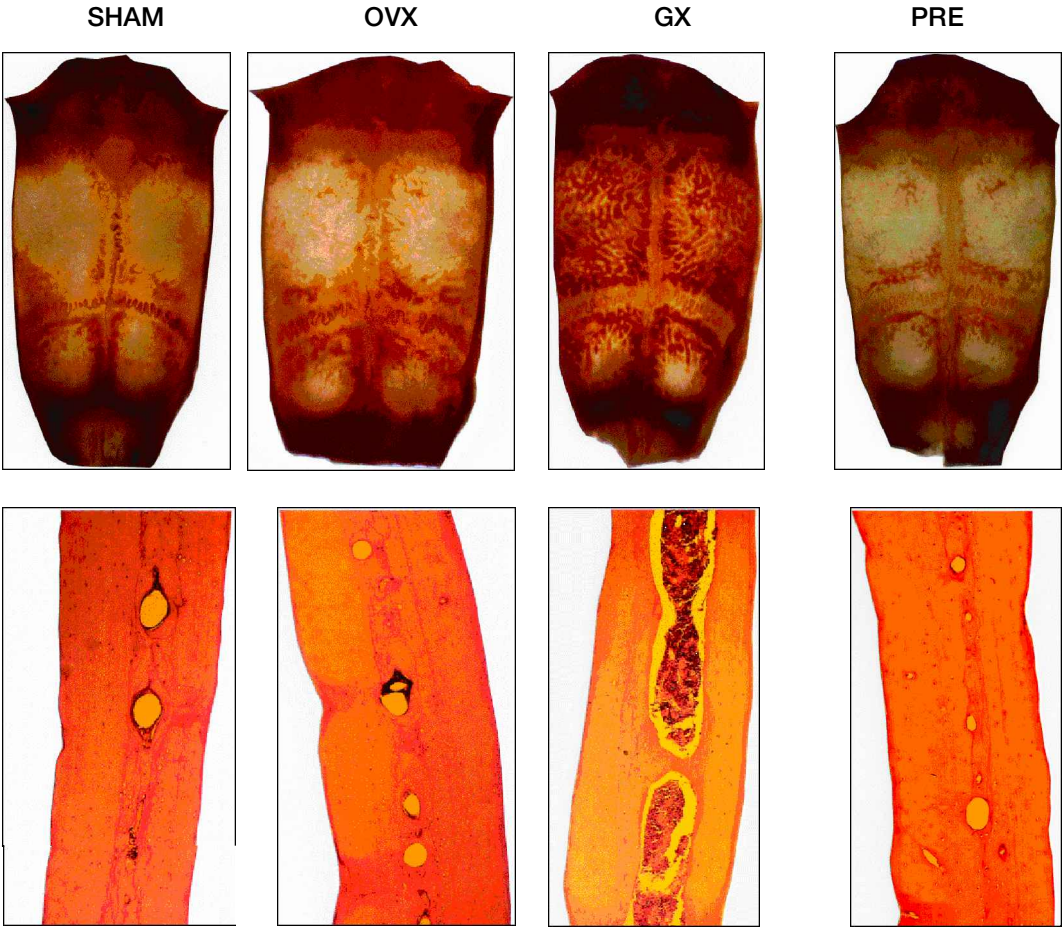

Figure 2. Transillumination photographs of calvariae (above) and microphotographs of transverse sections of calvariae (below) from SHAM, OVX, GX and PRE-treated rats, stained with hematoxylin/eosin and phloxine, magnification ×20. Note the enlarged lacunae/sinuses in the Gx rats and the lack of such changes in the other groups.

program for image analysis and measurements). This system provides information on % bone tissue (Rooryck and Klinge 1995). For measuring the thickness of the calvaria, we placed a computerized ruler on the image of the sections and the distance between the two periosteal surfaces was determined. The sections were evaluated without prior knowledge of the type of experiment that had been performed.

Statistics

Data were compared with one-factor factorial analysis of variance (ANOVA) (multiple comparisons). We used Fisher's PLSD test and the Student's t-test for unpaired observations. $P < 0.05$ was considered statistically significant.

Results

Postoperative body weight and femur length

The OVX rats gained weight more rapidly than the other groups ($p = 0.006$). The GX rats tended to gain weight more slowly than the SHAM-operated rats ($p = 0.08$) and the PRE rats showed a slight ($p = 0.06$) reduction in body weight (Figure 1). No change in femur length occurred in any group (data not shown).

Calvaria

Measurement of thickness and transillumination analysis. The thicknesses of the calvariae were similar in all the groups (results not shown). With the naked eye, we observed lacunae/sinuses using the transillumination technique in the GX group,

Table 1. Effect of OVX, GX, or PRE treatment on percent bone tissue in calvaria. Mean values (SEM), 6 rats in each group

Groups	Bone tissue (%)
SHAM	97 (0.5)
OVX	91 (1.6)
GX	71 (0.6) [a]
VEH	96 (0.6)
PRE	94 (0.8)

[a] $p < 0.01$ (GX versus SHAM; Fisher PLSD test)

Table 2. Effect of OVX, GX or PRE treatment on bone mineral density (ash weight/volume, g/cm^3) in femur and vertebra (L_5). Mean values (SEM), 6 rats in each group

Groups	Right distal femur (g/cm^3)	L_5 (g/cm^3)
SHAM	0.70 (0.02)	0.75 (0.04)
OVX	0.59 (0.04) [a]	0.63 (0.04) [a]
GX	0.54 (0.02) [a]	0.62 (0.04) [a]
VEH	0.76 (0.03)	0.73 (0.04)
PRE	0.71 (0.02)	0.68 (0.05)

[a] $p < 0.05$ (GX and OVX versus SHAM; Fisher PLSD test)

but not in the OVX or PRE groups (Figure 2).

Histomorphometry. The part of the calvaria selected for examination has no major blood vessels, and in SHAM-operated/VEH-treated rats, bone tissue relative to total tissue was 96–98%. After 8 weeks, we found large cavities/sinuses in the calvaria of the GX rats containing hematopoietic tissue, sometimes occupying the entire space of the lacunae (Figure 2). Thus, the bone surface area, expressed as a percentage of the total biopsy surface area, was reduced by GX ($p = 0.006$). No such change was observed in the OVX or PRE rats (Table 1).

Distal femur and L_5

Bone density (ash weight/volume). The bone density of the right distal femur and L5 was lower in GX ($p = 0.02$ and 0.04) and OVX ($p = 0.03$ and 0.04) rats, but not in the PRE rats (Table 2).

Histomorphometry. In the OVX and GX rats, but not in the PRE rats, the BV/TV%, Tb.Th, Tb.N/mm and N.Oc/mm^2 in the distal femur were reduced while the Oc.S/BS% was increased, more so in the GX than in the OVX rats. Ct.Th was significantly reduced in the GX rats but not in the other groups (Figure 3).

Discussion

Previous studies of rats have shown that OVX (Aitken et al. 1972, Wronski et al. 1985, 1986, Yamazaki and Yamaguchi 1989, Kalu 1991, Miller and Wronski 1993) and long-term PRE treatment (Lindgren 1982, Baylink 1983, Lindgren et al. 1983, Rickers et al. 1984, Yamazaki et al. 1986, Goulding and Gold 1988, Turner et al. 1995) cause osteopenia as does GX (Bussaberger et al. 1938, Ivy 1940, Persson et al. 1993, Klinge et al. 1995, Lehto-Axtelius et al. 1998). The osteopenia seen after OVX is characterized more by trabecular than by cortical bone loss (Kalu 1991, Miller and Wronski 1993, Turner et al. 1995) while GX rats show both trabecular and cortical bone loss (Persson et al. 1993, Klinge et al. 1995, Mühlbauer 1998). In the present study, treatment with PRE failed to affect the bone; in fact, PRE treatment in rats does not routinely cause osteopenia (Yamazaki et al. 1986, Shen et al. 1997, Okazaki et al. 1998). The increase in weight of OVX rats in this study has been shown in the past to have no effect on the bone structure (Roudebush et al. 1993) or to offset the loss of trabecular bone very transiently (Wronski et al. 1987).

Transillumination and histomorphometric examination of the calvaria showed a reduction in bone volume in percentage of total tissue volume after GX, but no change after OVX or treatment with PRE. This indicates that the mechanisms underlying GX-evoked and OVX-evoked osteopenia differ. Bone density (ash weight/volume) was lowered in L5 and the distal femur in the OVX and GX rats (but not in the PRE rats), indicating that the bone mineral content is negatively affected in OVX and GX rats. Low trabecular bone volume and trabecular number as well as reduced trabecular thickness in the GX and OVX rats accoard with osteopenia of trabecular type. Cortical thickness was the same in all groups, except in the GX rats, which had reduced thickness, again indicating a difference in the mechanism underlying GX- and OVX-evoked osteopenia. The reduced amount of bone after OVX and GX suggests a reduced rate of formation or accelerated degradation. The

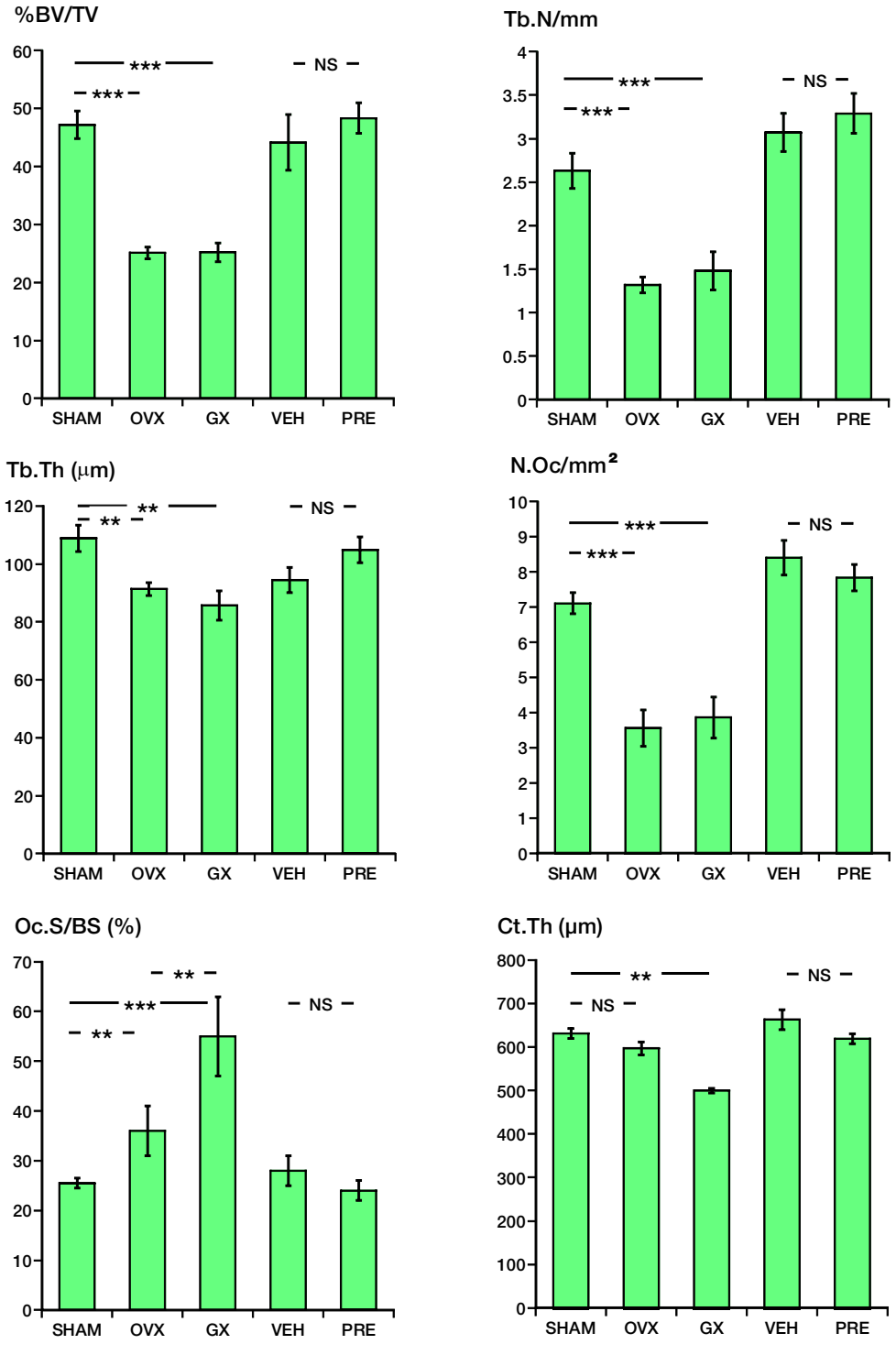

Figure 3. Trabecular bone volume (BV/TV%) (A), trabecular number (Tb.N/mm) (B), trabecular thickness (Tb.Th. μm) (C), osteoclast number (N.Oc/mm^2) (D), osteoclast surface (Oc.S/BS%) (E) and cortical thickness (Ct.Th μm) (F) in the left distal femur of SHAM, OVX, GX, VEH-, and PRE-treated rats. Mean values ± SEM (vertical bars, n 6 rats in each group). Fisher PLSD test, **, $p < 0.01$ ***, $p < 0.001$, NS, not significant.

reduction in osteoclast number in the OVX and GX groups as well as the increase in osteoclast surface (percentage of trabecular bone surface covered by osteoclasts) in the OVX and GX groups, more so in the GX than in the OVX group, favors the view that accelerated degradation is responsible for the bone loss. Conceivably, the reduction in osteoclast number reflects the reduced trabecular bone volume while the increase in osteoclast surface accords with the accumulation of osteoclasts on the remaining bone. The PRE-treated rats showed no statistically significant bone loss. Indeed, several recent reports of experiments on rats have failed to show much bone loss after PRE treatment compared to OVX (Yamazaki et al. 1986, King et al. 1996, Li et al. 1996, Shen et al. 1997, Okazaki et al. 1998), while others have suggested beneficial effects of PRE with respect to several parameters that evaluate bone (Lindgren et al. 1982, 1983, Baylink 1983, Rickers et al. 1984, Yamazaki et al. 1986, Goulding and Gold 1988, Turner et al. 1995). In a parallel study, we monitored circulating tartrate-resistant acid phosphatase (TRAP) and osteocalcin as markers of bone resorption and bone turnover (formation), respectively (Surve et al. 2001). The levels of TRAP and osteocalcin were elevated in the first week after OVX and GX, and they remained higher in GX rats than in SHAM rats throughout the study.

Our findings show that osteopenia after GX was similar to (femur, vertebra) or greater (calvaria) than that after OVX and that the effects of PRE treatment were insignificant in comparison. Osteoclast activation seemed to contribute to the osteopenia after GX and OVX. GX-evoked osteopenia is a recognized clinical problem (Editorial 1986, Tovey et al. 1991, 1992), which suggests that gastric dysfunction may contribute to the development of osteoporosis in the elderly. The rat is said to be a useful model for studying human osteopenia (Frost and Jee 1992), and although the precise mechanism underlying GX-evoked osteopenia remains obscure, GX rats may serve as an alternative experimental model for study of osteoporosis in humans.

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