Biphasic Effects of Genistein on Bone Tissue in the Ovariectomized, Lactating Rat Model (44243)

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Abstract. **The ovariectomized (OVX), lactating rat model has been used to investigate the skeletal effects of the plant estrogen, genistein, over a 14-day period. The OVX, lactating rat on a low-calcium diet loses slightly more than 50% of its bone mineral mass during the first 2 weeks of lactation, and we have demonstrated that estrogen treatment can significantly reduce the loss of femoral mass (ash weight). Following OVX, the rats were assigned to treatment or control groups (both placebo and positive control with estrogen replacement). The treatment groups received one of three doses of a genistein-rich preparation each day *via* the feed for 2 weeks, after which time the pups began to have an interest in solid feed. A positive control group received conjugated estrogen in the feed. The genistein doses were: low (0.5 mg/d); intermediate (1.6 mg/d); and high (5.0 mg/d). Measurements included ash weights of the femur, scanning electron microscopy (SEM) of the proximal tibia, and uterine weights. SEM results were as follows: (1) at the low dose genistein was approximately equally effective to estrogen in the retention of cancellous bone tissue, as reflected in the number and density of trabeculae in hemisections of the tibial subepiphyseal region, but at high doses genistein was less effective; and (2) rats treated with low-dose genistein, like estradiol, had rougher endosteal surfaces and smaller pores on these surfaces than untreated control rats. Mean ash weights of the entire femur were highest in the rats treated with the low dose compared to control rats ($P < 0.05$), and they were higher than ash weights of rats administered the intermediate or high doses of genistein. The mean ash weights of the femurs were consistent with the genistein effects on the tibias observed by SEM. In summary, a biphasic response to the genistein preparation was found in this OVX rat model. Interpretation of the results suggests that, at the low dose, genistein appears to be an agonist at the estrogen receptor locus, whereas at higher doses the genistein is less effective and may even have adverse effects on bone cells. These findings of a biphasic effect of genistein (i.e., an inverted U effect) are consistent with those of other recent reports in the literature on isolated bone cells and on reproductive tissues. In summary, lower doses of genistein from soy foods would be expected to act similarly to estrogens with a beneficial effect on bone tissue, but at high doses that are unlikely to be consumed in human diets, this soy derivative may have potentially adverse effects on bone cell functions and thereby on bone tissue.** [P.S.E.B.M. 1998, Vol 217]

The potential importance of phytoestrogens, especially those steroid-like molecules derived from soybeans that have a high level of consumption, to human health is now being actively investigated. Several investigators, notably Adlercreutz *et al.* (1) and Setchell *et al.* (2), have established the basic physiology and biochemistry of genistein and related soy derivatives in human subjects over the past 2 decades. What has emerged is a beginning understanding that genistein may have beneficial effects on several organ systems and thereby reduce the risks of cardiovascular diseases and selected cancers. More recent research has suggested that genistein and related molecules may also improve bone mass or density and possibly prevent the loss of bone mass in rat models following ovariectomy (3). These animal findings suggest that genistein

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could lower the chance of adult and elderly women of developing osteoporosis, and possibly reduce postmenopausal fractures related to low bone mass.

The rationale for these studies is based on the early observation that sheep feeding on plants containing molecules with estrogen-like activities experienced stimulation of estrogen-responsive reproductive tissues (4). If women in their child-bearing years consumed so much of these molecules, primarily genistein and related molecules (genistin and biochanin A), that their reproductive organs were affected, this would be an undesirable effect. Such an event in adult females, however, is virtually impossible because food "doses" would not be that high in women consuming soy-based diets (5). It is entirely possible, however, that lower doses of genistein might interact with estrogen receptors within osteoblasts and thereby indirectly prevent or inhibit bone resorption through an inhibitory action on osteoclasts (6). If treatment with genistein at low doses can be shown to maintain normal bone structure and mass, this molecule may have important uses in human foods as an additive for the control of osteoporotic fractures. If the anticipated results obtained from experiments using rodent models are judged to add significantly to our knowledge of the potential beneficial effects of phytoestrogens on bone mass or quality, then extended human trials using soy phytoestrogens, longer than previously reported studies (7–9), should be explored.

The present research was undertaken to explore the effects of a genistein-rich preparation on cancellous bone tissue in an ovariectomized (OVX), lactating rat model previously exploited by our research group because of the much greater stress placed on its skeleton to provide calcium for suckling pups (10). In the experiment reported herein our objectives were: (1) to determine the optimal dose of genistein for the retention of bone mass and for the protection of the microarchitecture of cancellous bone tissue in a long bone; and (2) to identify any gross effects of genistein on uterine tissue in this model.

Methods

The lactating, OVX rat model was used in this experiment. (OVX *per se* has little effect on bone loss in this model, but removal of endogenous estrogens eliminates any potential interactions between ovarian hormones and exogenously administered molecules.) Body weight and food consumption of the dams were monitored throughout the experiment, and body weights of the litters of pups were also determined at the end of the experiment. The pups grew well despite the fact that their mothers were kept on a low-calcium diet throughout the experiment to show an effect (Table I).

Rats were fed a low-calcium (0.1% Ca) diet, formulated by Dyets, Inc. (Bethlehem, PA) according to the published composition of Boass and coworkers (11). Both genistein and conjugated estrogens (Premarin, a gift of Wyeth-Ayerst Research, Princeton, NJ) were mixed in the feed. Food consumption by the lactating rats was approximately 25 g/day.

Table I. Weights of Dams and Litters at 16 Days of Lactation

Treatment group	*n*	Body weight of dams, g	Litter weight, g
L, OVX + Vehicle	5	320.8 ± 7.0	370.2 ± 11.6
L, OVX + Conj. E	5	307.6 ± 9.0	379.8 ± 11.2
L, OVX + Low G	5	338.4 ± 19.0	375.2 ± 15.9
L, OVX + Mid G	5	324.0 ± 9.1	386.4 ± 11.5
L, OVX + High G	5	328.7 ± 8.3	366.3 ± 10.1
Nonmated	5	302.5 ± 9.2	—

Note. Dams, nonmated control females, and litters (11–12 pups per dam) were weighed on Day 16 of lactation, the last day of the treatment period. No significant differences in either mean body weights or litter weights were found among groups by ANOVA. Means ± SE. ANOVA = analysis of variance; L = lactating; OVX = ovariectomized; Conj. E = conjugated estrogens (Premarin); G = genistein; SE = standard error of the mean; *n* = number of rats per group.

Timed-pregnant and nonmated (control) Sprague-Dawley rats were obtained from Charles River Inc. (Raleigh, NC). Following parturition, the dams were ovariectomized (OVX) and then given Premarin or various doses of genistein (see below). The genistein preparation was a gift of Protein Technologies Inc. (St. Louis, MO).

Day 0—Parturition (Pups Continue Nursing Throughout)
Day 2—OVX, and Start Treatments (Mixed in Feed); 5 Rats per Group
- Group 1—OVX + Vehicle (Corn Oil in Diet)
- Group 2—OVX + Conjugated Estrogens or Premarin (Mixed in Feed, 16 μg/d)
- Group 3—OVX + G (Low Dose, 1.0 mg/d of the protein preparation per day, or 0.5 mg/d of genistein)
- Group 4—OVX + G (Intermediate Dose, 3.2 mg/d or 1.6 mg/d of genistein)
- Group 5—OVX + G (High Dose, 10.0 mg/d or 5.0 mg/d of genistein)
- Group 6—Nonmated Controls (Control Diet)

Days 3 to 15—Continue Treatments in Feed
Day 16—Sacrifice Rats and Remove Tissues

The uterine assay involved drying (100°C) for 24 hr and weighing of both horns and the remainder of the uterus. The bone assays performed were ash weights and scanning electron microscopy (SEM). Both femurs and tibias were collected for analysis. One femur was cleaned by blunt dissection and dried at 100°C for 24 hr. After weighing the dried bone, it was ashed in a porcelain crucible at 600°C for 24 hr in a muffle furnace, and the ash was weighed.

Tibias were removed and cleaned of soft tissue in preparation for SEM. The bones were fixed in 10% buffered formalin several days and then rinsed in 0.1 *M* cacodylate buffer, pH 7.4. The bones were then cross-sectioned at midshaft with a Buehler diamond saw cooled with running water. The proximal half was cut sagitally to obtain hemisec-

tions. The samples were placed in 2% sodium hypochlorite overnight, dehydrated with ethanol, and vacuum-dried. The hemisections were mounted on specimen stubs, gold/palladium coated, and viewed by secondary electron imaging using an ETEC scanning electron microscope at 20 kV (12).

Statistical differences among group means were determined by analysis of variance and *t*-tests.

The Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina, Chapel Hill has approved the use of rats in this experiment.

Results

The combination of lactation and a low-calcium diet resulted in extensive loss of bone mineral in this study, as well as in a previous one (10). The femur ash weights of the genistein-treated rats were decreased by approximately 45% from the age-matched, nonmated control mean, but the control (vehicle) OVX lactating rats lost almost 53% of their bone mineral mass over the 2 weeks of study (Fig. 1). This approximate 8% improvement in bone mineral retention, which represents 20–25 mg of calcium phosphate, was significant at < 0.05. Although a tendency was found for genistein-treated rats to retain bone mass better than the untreated controls and of rats treated with conjugated estrogens (Premarin), the low-dose genistein group produced the greatest numerical protective effect.

SEM photographs of representative tibias removed from rats of each group, all of which had been fed a low-calcium diet, are given in Figure 2 (A to F). Tibias from nonmated control rats (Fig. 2A) on a low calcium diet for the 2-week duration of this experiment had dense, well formed trabeculae filling the metaphyseal space. This appearance was similar to the surface morphology of bones from rats fed a normal calcium diet (10, 13). The primary and secondary spongiosa were fully intact and well differentiated and the endosteal surface was smooth. Ovariectomy at the start of lactation (Fig. 2B) caused a marked reduction in the number and density of trabeculae. The primary spongiosa was reduced and the endosteum appeared roughened, suggesting bone erosion. In bones of ovariectomized dams treated with estrogen during lactation (Fig. 2C), the degree of trabecular loss was similar to that found in the OVX control (vehicle) rats (Fig. 2B), but the endosteal surface was much smoother. The addition of graded amounts of genistein in OVX treated dams, produced varying morphology in hemisectioned tibias. Tibias from OVX treated rats given a low dose of genistein (Fig. 2D) seemed to have lost less trabeculae than the OVX control (vehicle) rats. Although the primary spongiosa showed a heavy loss, the endosteal surface was only moderately roughened. Bones of OVX rats administered an intermediate dose of genistein (Fig. 2E), seemed to have been protected from the heaviest loss of trabeculae, as well as, if not better than those bones treated with the low dose genistein. The endosteal surface showed variable roughness. A high dose of genistein in the OVX dams (Fig. 2F) seemed not to have protected the metaphysis from heavy loss of trabeculae, although the endosteal surface appeared similar to those in the other two treatment groups.

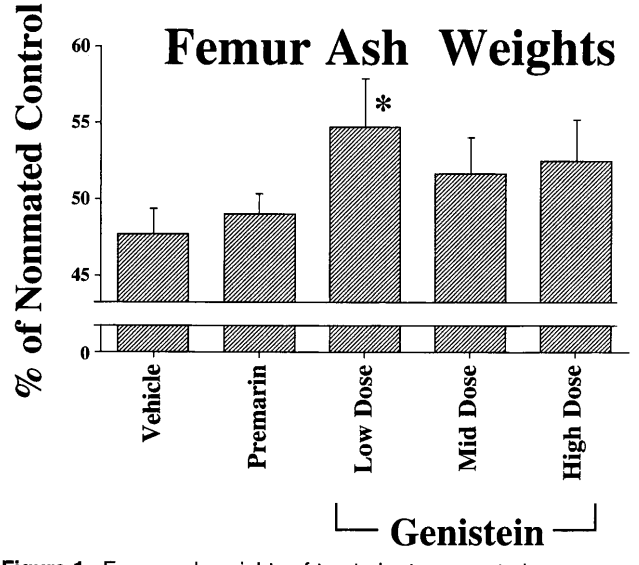

Figure 1. Femur ash weights of treated rats presented as percentage of nonmated control females. Values are expressed as means (± SE) compared to age-matched, nonmated control rats (mean = 100%). Rats treated with low-dose genistein had significantly more bone mass than vehicle-treated OVX rats. $^*P < 0.05$.

Although a visible thinning of the cortical bone in the OVX control (vehicle) group was found (Fig. 2B), this was not demonstrated in the other groups treated with conjugated estrogen or genistein. The lack of change in the other groups could indicate that they were all protected from the effects of the ovariectomy by their specific treatment.

Uterine weights (Fig. 3) were decreased by greater than 65% in the OVX control (vehicle) group compared to nonmated control rats, and genistein-treated rat groups showed similar declines. The group treated with conjugated estrogen (Premarin) had a slightly, but significantly (< 0.05) greater mean uterine weight than of the groups of vehicle- or genistein-treated rats.

The pup litters gained at almost the same rate, independent of treatment, over the 14-day period of study (Table I), and the final body weights of dams and nonmated control rats were also very similar. No statistical difference was found among any of the OVX, lactating groups (ANOVA).

Discussion

This experiment provides both architectural visualization of cancellous bone tissue and quantitative bone mass data following treatment with the phytoestrogen, genistein, at different dose levels in the OVX lactating rat model compared to animals treated with conjugated estrogens or vehicle. Uterine weights of genistein-treated OVX rats, even at the highest dose, were always significantly below those of estrogen-treated OVX and sham-OVX control uterine weights. Uterine weights of conjugated estrogen-treated rats

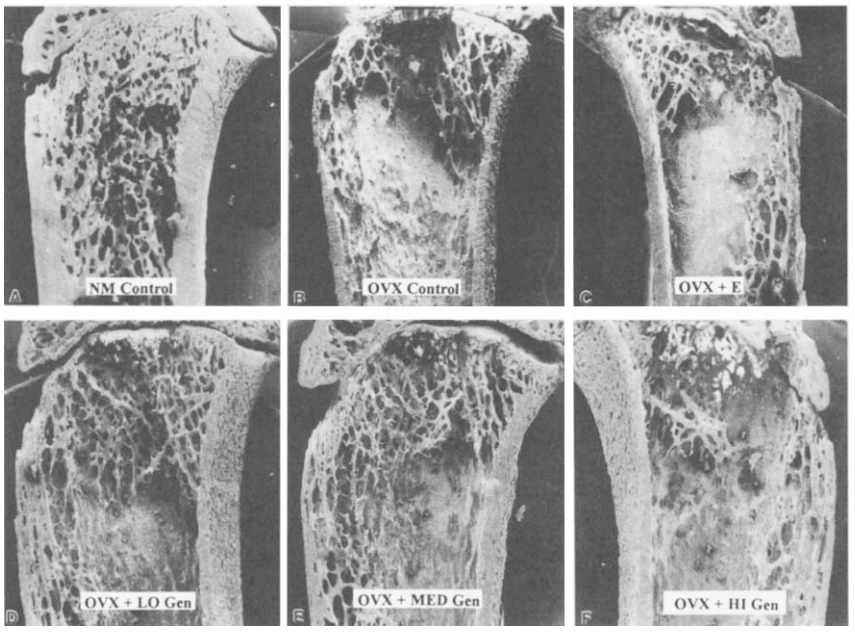

Figure 2. Scanning electron micrographs of proximal tibial hemisections. Panel A is a nonmated control showing normal trabecular architecture. Panel B is an OVX only control (Con) treated with vehicle showing maximum bone loss. Panel C is an OVX + conjugated estrogen (E) (positive control) showing partial protection against trabecular and cortical bone loss. The effect of genistein (G) treatment in OVX dams at low (Lo) (Panel D), intermediate (Med) (Panel E), and high (Hi) (Panel F) daily doses seems to be biphasic, in that the low and intermediate doses provide modest protection from bone loss, whereas the high dose provided much less preservation of trabecular bone. Magnifications are 10X.

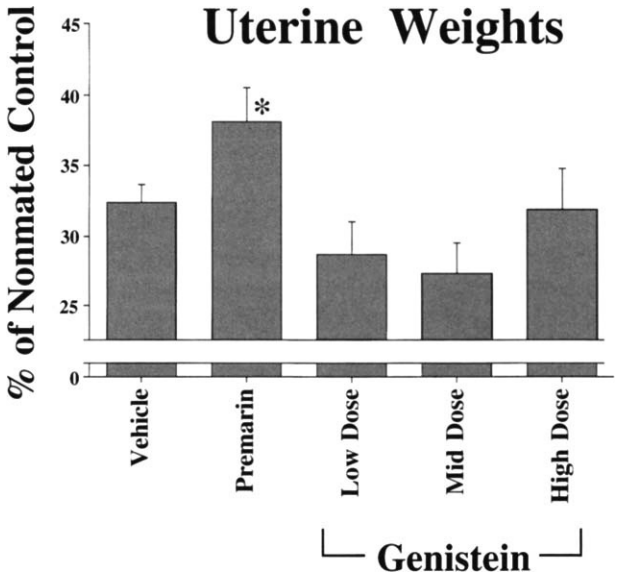

Figure 3. The effect of genistein on uterine weights. Mean weights (± SE) were determined for each treatment group and compared to untreated, nonmated control rats. Note that the group treated with conjugated estrogen (Premarin) had a small but significant effect on the mean uterine weight. $^*P < 0.05$.

were lower than the nonmated control rats, but significantly greater than of control OVX rats that received no treatment (vehicle).

A tendency was found for genistein-treated OVX rats to retain bone mass better than the untreated control (vehicle) animals, but the low-dose genistein treatment groups in this experiment produced the greatest quantitative effect on the retention of bone compared to the high-dose treatment groups (see Fig. 1). This bone-protective effect of genistein is similar to that of estradiol, as shown by our research group using the same model (13), but better than that of conjugated estrogens in the present study. This biphasic response is consistent with reports of other groups (14–16) of a differential dose effect of phytoestrogens on osteoblast-like cells or reproductive tissue cells both *in vivo* and *in vitro*. These results suggest that genistein has a modest bone-conserving effect at low doses, but little or no effect on skeletal retention at high doses. This finding is consistent with the report by Brandi's group (14) of a biphasic dose effect of ipriflavone on osteoblast-like cells *in vitro*.

The beneficial action of genistein in bone cells has been reasonably established to occur in osteoblast cells in mammals, but the precise biochemical mechanism, like that of estradiol, has not been determined (6). The research findings of Barnes *et al.* (17), Setchell *et al.* (2), and other investigators (18) have suggested that genistein acts *via* estrogen receptors, but these and other workers have more recently reported that higher doses of genistein have multiple cellular effects, such as inhibition of protein kinases (19), that are not specifically associated with estrogen receptor activation. Part of the difficulty of establishing cellular mechanisms of action of genistein is the typical use of high doses by investigators that result in pharmacologic effects and that do not pinpoint estrogen receptor-mediated events. Future studies need to pay careful attention to the use of nonpharmacologic doses of genistein.

The major findings of this experiment are anticipated to broaden our understanding of the agonistic effects of an important phytoestrogen found in soy products, now being consumed in increasing amounts by North American women during their child-bearing years (5). If genistein can be shown to have positive benefits to skeletal tissue of human subjects at low intake dosages, the recommendation can be made to this population to increase their consumption of soy products on a more frequent basis. If these phytoestrogens are demonstrated to have adverse effects on human skeletal or estrogen metabolism, including estrogen

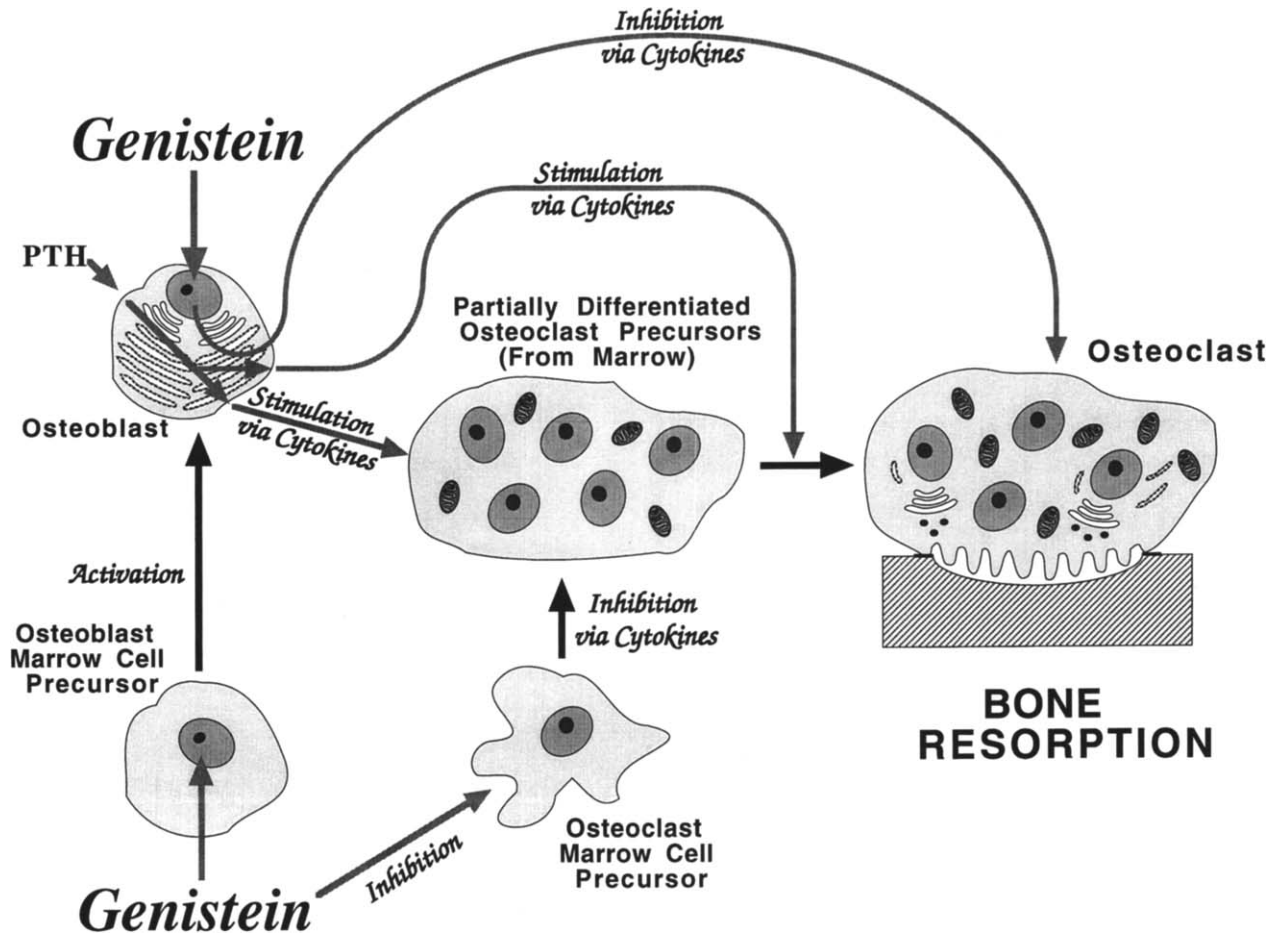

Figure 4. Speculative illustration of the potential actions of genistein on osteoblast and osteoclast cells and their precursors. The bone-retentive effects of genistein involve agonistic roles of this phytoestrogen at low doses on estrogen receptors in osteoblasts. Cytokines with either inhibitory or stimulatory effects on osteoblasts may be secreted by osteoblasts or precursors of osteoblasts in response to genistein or parathyroid hormone (PTH). The solid lines represent developmental changes in bone cells, whereas the lighter lines represent hormonal effects and cross-talk among cells *via* cytokines. The net effect of genistein in this model is to suppress osteoclastic activity.

production by the ovaries, estrogen receptor activity in diverse tissues, or gonadotropin regulation, then both public health agencies and women should be advised about potential deleterious consequences of excessive consumption patterns.

In summary, our results suggest that genistein has a modest bone-conserving effect at low doses, but results in less effective skeletal retention at high doses, and that genistein has only a small effect on uterine tissue at any dosage used. We have not found any uterotrophic effect of this genistein-rich soy extract in young, growing OVX rats (unpublished data). A possible mechanism of action of genistein, as an estrogen agonist at low doses, is illustrated in Figure 4. Genistein acts through osteoblastic cells to inhibit osteoclastic bone resorption. The effects of genistein may be mediated by binding to estrogen receptors (18) or by altering cellular activity of tyrosine kinase (19) or other nonreceptor molecules. Genistein may both stimulate the maturation of osteoblast precursors and act on mature osteoblasts to increase secretion of cytokines. The most important effect of cytokines in this model is the inhibition of osteoclastic activity which results in a reduction in bone resorption.

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