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*Report*

# Effects of anti-estrogens on bone in castrated and intact female rats

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**Summary**

The effects of the antiestrogens tamoxifen and keoxifene on the bone density of intact and ovariectomized female rats were determined after 4 months of therapy. The antiestrogens did not cause a decrease in bone density in intact animals, although uterine wet weight did decrease. Ovariectomy caused an increase in body weight (25%) and a significant decrease in femur density ($P<0.01$). Antiestrogens did not further decrease the bone density of ovariectomized rats but rather helped to maintain bone density. Antiestrogens as well as estrogen (oral estradiol benzoate 25 $\mu$g daily) helped to maintain bone density in the range observed for the intact rats, but inhibited estrogen stimulation of uterine weight. These contrasting pharmacological actions of antiestrogens suggest that patients receiving long-term adjuvant tamoxifen therapy for breast cancer should be evaluated to determine whether tamoxifen can retard the development of osteoporosis.

## Introduction

Tamoxifen, a non-steroidal antiestrogen, is used extensively for the treatment of breast cancer [1]. Its initial application for the treatment of advanced breast cancer [2] has been extended to successful use as an adjuvant therapy following mastectomy [3–5]. Currently, long-term adjuvant therapy with tamoxifen is being evaluated [6, 7], and an application as a chemosuppressive agent to prevent the occurrence of breast cancer is projected [8].

The extended duration of tamoxifen therapy raises an important toxicological question. Estrogen is implicated in the maintenance of bone density [9]. Prolonged antiestrogen therapy might therefore precipitate an early osteoporosis, thereby limiting the usefulness of the drug in treating younger women. If this is the case, the drug would be unlikely to be used as a preventative agent in women only at risk for breast cancer.

A recent report demonstrated that the antiestrogen clomiphene could actually protect ovariectomized rats from a decrease in bone density [10]. Clomiphene is, however, a mixture of geometric isomers [11]. The *trans* isomer enclomiphene is a partial estrogen with antiestrogenic properties, whereas the *cis* isomer zuclomiphene is an estrogen. It is, therefore, unclear whether the overall estrogen-like properties of the mixed isomers of clomiphene are responsible for the observed effect on bone.

In this study, we have focused our attention on tamoxifen, a pure *trans* isomer of a substituted triphenylethylene related to clomiphene [1], and keoxifene, an antiestrogen with a high affinity for the estrogen receptor but weaker estrogenic properties than tamoxifen [12]. These antiestrogens have been studied to determine their effects upon intact or ovariectomized rat bone density.

## Materials and methods

Female rats of the Sprague-Dawley strain were obtained from King Rats, Oregon, WI. Tamoxifen ((Z)-2-[4-(1,2-diphenyl-1-butenyl) phenoxy]-N,N dimethylethanamine) was obtained from Imperial Chemical Industries PLC, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, England. Keoxifene (6-hydroxy-2-(4-hydroxyphenyl) benzo [b] thiene-3-yl-4-[2-(1-piperidine) ethoxyphenyl] methane hydrochloride) was obtained from Eli Lilly Laboratories, Indianapolis, IN. Estradiol-3-benzoate was obtained from Sigma Chemicals, St. Louis, MO.

Seventy-nine 9-month-old retired, female breeder rats were randomly allocated to 10 treatment groups. Seven rats were used as baseline controls, and the remaining animals were either ovariectomized or underwent a sham operation. Rats were treated daily (per os in 0.2 ml peanut oil) with tamoxifen (100 $\mu$g), keoxifene (100 $\mu$g), or estradiol-3-benzoate (25 $\mu$g), or with a combination of either tamoxifen or keoxifene and estradiol-3-benzoate. These doses were selected based upon their known pharmacology [1] and prior experiments with these compounds in this laboratory [1, 13]. One group of eight ovariectomized and eight sham-operated rats received only the vehicle. The experiment was continued for four months. All rats were housed in individual cages, and received distilled water and a laboratory diet of 0.5% Ca and 0.3% P *ad libitum* [14].

The rats were killed by exsanguination under pentobarbital anesthesia. The femurs were harvested and immediately frozen; later they were thawed and dissected from the soft tissues. The length of the femur and the mid-diaphyseal width was measured. The bones were put in distilled water for 6 hours, then weighed in and out of distilled water. The difference between these measurements, expressed in grams, equals the bone volume in cubic centimeters. Fat and water were removed from the bones with six 48-hours changes of acetone. The bones were dried at 50° C for 24 hours, and their dry weights were recorded. Then the bones were put in a 500° C furnace for 48 hours and reduced to ash. The ash weight was then determined with standard procedures. Statistical comparisons were (when indicated) made with Student's t-test.

## Result

The body weight increased more rapidly for ovariectomized rats than for intact rats (Table 1).

There was a significant decrease in bone density when these 9 month old rats were ovariectomized. After 4 months, the mean dry weight and total ash of the femur were significantly lower for the ovariectomized rats compared to the intact controls (Table 1).

Two types of experiments were conducted to evaluate the effects of antiestrogen. The body weights of the animals were measured throughout the 4 month therapeutic period. The first therapeu-

*Table 1.* Effects of ovariectomy in old rats after four months. Results are mean ± standard deviations. Eight per group.

| 13-Month-old-rats | Body weight (g) | Dry weight of femurs (g) | Total ash of femurs (g) | Ash/Volume of femur (g/cm$^3$) |
|---|---|---|---|---|
| Normal | 306 ± 25 | 0.608 ± 0.041 | 0.404 ± 0.028 | 0.703 ± 0.034 |
| | b | c | c | c |
| Ovariectomized | 351 ± 42 | 0.535 ± 0.045 | 0.349 ± 0.026 | 0.618 ± 0.038 |
| | c | a | a | b |
| (Baseline | 259 ± 20 | 0.492 ± 0.040 | 0.315 ± 0.027 | 0.664 ± 0.024) |

Probability of no difference between groups:
a 0.05>P>0.01
b 0.01>P>0.001
c P<0.001.

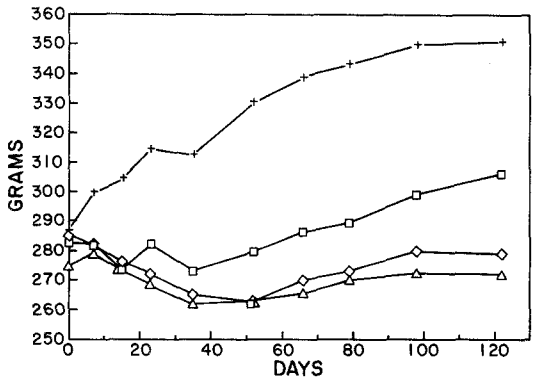


*Fig. 1.* The change in body weights of female rats (8 per group) following ovariectomy (+), intact control (□), tamoxifen (100 μg daily, ◇), or keoxifene (100 μg daily, △).

tic study compared the effect of antiestrogens in intact animals with ovariectomy. Tamoxifen and keoxifene both caused a stabilization of body weight whereas ovariectomy caused a 25% increase in body weight over the 4 month period (Fig. 1). The oral administration of the antiestrogens produced a profound decrease in intact uterine weight but this was not as complete as the decrease produced by ovariectomy (Fig. 2). Interestingly, the antiestrogens had no effect upon the ash density of the intact rat femur (Fig. 3). In contrast, ovariectomy produced a profound decrease in the ash density ($P<0.001$).

The oral administration of estradiol benzoate to ovariectomized rats produced a stabilization of body weight increase which after 4 months was not significantly different than normal, intact animals (Fig. 4). Tamoxifen caused a greater effect upon body weight than estradiol benzoate, whereas keoxifene was equivalent to estradiol benzoate (data not shown). These estrogen-like effects of the anti-

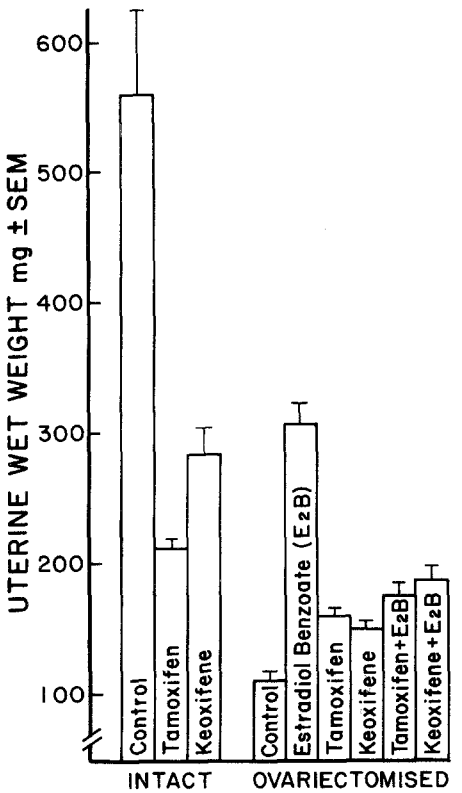


*Fig. 2.* The effect of four months of treatment with antiestrogens or estrogen (estradiol benzoate $E_2B$) to intact or ovariectomized rats (8 per group) on uterine wet weights. See Materials and methods for treatment regimens.

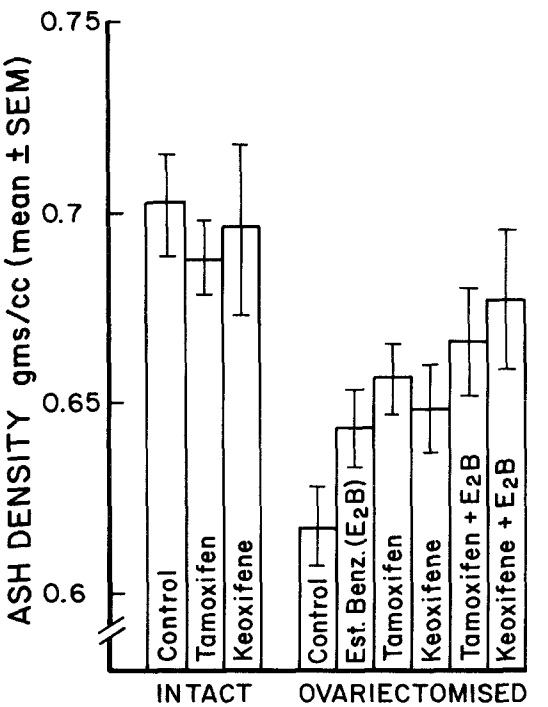


*Fig. 3.* The effect of four months of treatment with antiestrogens or estrogen (estradiol benzoate $E_2B$) to intact or ovariectomized rats (8 per group) on femur ash density. See Materials and methods for treatment regimens.

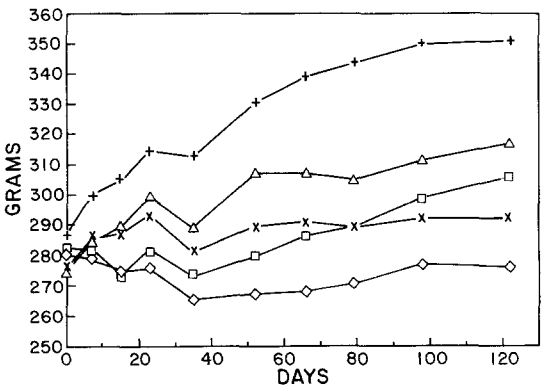


*Fig. 4.* The change in body weights of female rats (8 per group) following ovariectomy (+), intact control (□), or ovariectomy plus estradiol benzoate (△), estradiol benzoate and keoxifene (×), or estradiol benzoate and tamoxifen (◇). See Materials and methods for treatment regimens.

estrogens on body weight were not reflected in an increase in uterine weight; estradiol benzoate produced a two-fold increase in ovariectomized rat uterine wet weight whereas tamoxifen and keoxifene only produced small increases in uterine wet weight (Fig. 2). The combination of estradiol benzoate and the antiestrogens appeared to produce an additive estrogenic effect on the body weight (Fig. 4) but both antiestrogens inhibited estradiol benzoate stimulation of uterine wet weight (Fig. 2).

Estradiol benzoate slowed the decrease in ash density produced by ovariectomy, but this was not statistically significant. In contrast, both tamoxifen and keoxifene produced a significant retardation of the decrease in ash density produced by ovariectomy ($P<0.05$), and a combination of estradiol benzoate and the antiestrogens was at least equally effective. In fact, the combination of estradiol and the antiestrogens was not significantly different than the respective intact controls treated with the antiestrogens alone (Fig. 3).

## Discussion

These studies were designed to determine the effect of antiestrogens and/or ovariectomy upon the bone density of old rats. Old rats showed osteoporotic changes upon ovariectomy as previously described [14, 15], but pharmacologically active oral doses of antiestrogens did not alter bone density in intact animals. The antiestrogen-induced decrease in uterine wet weight was observed in intact rats, but the antiestrogens did not produce the increase in body weight that is associated with ovariectomy. The estrogenic effect of antiestrogens on body weight has been observed previously [16, 17], and was readily demonstrated here in the ovariectomized animal when estrogen-deprived weight gain was prevented by the antiestrogens.

Estrogen can reverse the osteoporosis produced in the female rat [18]; we selected a low dose of estradiol benzoate that would control the weight gain observed upon ovariectomy. The antiestrogens caused a stabilization of bone loss in the ovariectomized rats and, surprisingly, a combination of antiestrogens plus the estradiol maintained the bone density virtually at the level of the intact rat. The results illustrate the target site specificity of the antiestrogens, with a complete inhibition of estrogen-stimulated uterine wet weight while, simultaneously, a positive estrogenic effect on both body weight and bone density.

The mechanism of the disparate pharmacology is unknown, but these results may have important implications for the clinical applications of antiestrogens. Estrogen is used for the prevention of osteoporosis in post menopausal women. Early concerns about an increased risk of developing endometrial carcinoma [19] have been ameliorated by the sequential use of oral progestational agents followed by steroid withdrawal to precipitate menses. It is possible, however, that in the future, tamoxifen could be considered to be used as a substitute for estrogen in this setting. This could serve a dual purpose: to further reduce the risk of endometrial carcinoma because the drug has been used to treat the disease [20] and potentially to reduce the risk of developing breast cancer, while still preventing bone density loss. However, before these clinical applications could be considered, the use of tamoxifen as an effective chemo-suppressive agent in stage I breast cancer must be carefully evaluated; longitudinal determinations of bone

density of such patients during long-term tamoxifen therapy will confirm whether the estrogen-like effects observed in this animal study also occur in patients.

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