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# The Long Term Effect of Estrogen Administration on the Metabolism of Male Rat Bone[1] (40350)

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There is considerable information about the effect of estrogen upon bone metabolism in the female including recent work (1) outlining in some detail the long-term effects of the hormone on various parameters of bone metabolism. Because of well known sex differences in the incidence of metabolic bone disease and because it became apparent that there were some differences in the response of bones of male and female animals, the following experiments were carried out in order to determine the long term effect of estrogen on the bones of male rats.

*Materials and methods.* Hundred and fifty-gram male rats were divided into four groups. The animals of the first group were left intact and served as the control group. Group 2 included intact animals treated with 400 $\mu$g per 100 g body wt of 17-$\beta$-estradiol in sesame oil twice a week. The hormone was introduced directly into the gastric lumen. The rats in group 3 were surgically castrated and those in group 4 were castrated and treated with the same dosage schedule of 17-$\beta$-estradiol. The animals in the control groups received similar amounts of sesame oil without hormone. Five rats in each group were sacrificed by decapitation for each set of chemical determinations at 1, 3, 6, 9 and 12 months following the institution of therapy. Serum was collected for chemical determinations. The femora and tibiae were removed immediately and dissected free of soft tissues and periosteum. The epiphyses were discarded and the bone marrow was removed by flushing with a cold saline solution. The metaphysis was separated from the diaphyseal portion of the bone in a standard fashion and only metaphyseal bone was used for chemical analysis. Body weights were recorded monthly. Serum calcium and phosphorous determinations were carried out in an autoanalyser.

The following determinations were carried out on bone: The pooled metaphyses of a single animal were lyophylized and used for each set of determinations. The lipids were extracted and washed according to the method of Folch *et al.* (2). The ash content was determined after ashing a sample of dried defatted bone powder in a furnace at 680° for 20 hr. The hydroxyproline content was measured in an aliquot of fluid from a sample which had been hydrolyzed in 6 *N* HCl at 100° for 17 hr according to the method of Stegemann (3). Hexosamine was estimated after hydrolysis in 3 *N* HCl at 100° for 17 hr by a modification of the method of Boas (4) with omission of the resin treatment. Incubation studies were carried out according to the method of Deiss *et al.* (5). Minced methaphyseal fragments were incubated in buffered Krebs-Ringer bicarbonate medium at pH 7.4 in a Dubnoff incubator under 95% oxygen, 5% $CO_2$ at 37° for 4 hr. The incubation medium contained either 10 $\mu$Ci of L-proline $^{14}C$ with a specific activity of 232 mc/-mole or 10 $\mu$Ci of D-glucose-[$^{14}C$] with a specific activity of 4.06 mc/-mole. After incubation, the bones were washed with saline and cold water several times and hydrolyzed at 100° for 17 hr with 6 *N* HCl for hydroxyproline or with 3 *N* HCl for hexosamine. The $^{14}C$ hydroxyproline was isolated by paper chromatography and the specific activity of the hydroxyproline fraction determined according to methods previously described. In order to determine the specific activity of $^{14}C$ hexosamine the hydrolysate was applied to an ion exchange resin (Dowex 50W) according to Boas (4). An aliquot was dissolved in 15 ml of aquasol (New England Nuclear, Boston, MA) and the radioactivity determined in a liquid scintillation counter. The degree of quenching was estimated by internal standardization and the data corrected.

Collagenolytic activity was determined according to the method of Kaufmann (3). 50 mg of metaphyseal bone was cut into four

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pieces and placed in a tube containing 100 μl of purified neutral soluble rat skin collagen labelled with $^3H$ proline and $^3H$ hydroxyproline (approximately 5,000 cpm) with 400 μl of 0.05 *M* Tris–HCl buffer at pH 7.5. They were incubated at 35° for 3 days and the collagenolytic activity of the bone was determined by counting the release of radio-activity into the medium. Blank values were obtained by parallel incubation of metaphyseal bone boiled at 100° for 3 min.

In order to judge the uptake of mineral into bone, rats were injected intravenously with 10 μCi of calcium-45 containing 2 μ*M* CaC12. Five days after injection, the rats were sacrificed by decapitation and the tibiae and femorae were removed immediately. The bone marrow was removed as before and the metaphyseal region of the tibia was separated and ashed in a furnace at 680° for 20 hr. The ashed metaphysis was dissolved with 1 ml of 2 *N* and 100 μl of the solution was mixed with 10 ml of aquasol and counted in a liquid scintillation counter.

*Results.* Estrogen administration to the intact rat caused a consistent and sustained decrease in body weight. Castration also caused a decrease in body weight and estrogen administration appeared to have no significant effect upon this parameter, although when administered to the castrated animal, there was a suggestion of further decrease in weight.

There was no influence of either castration or estrogen administration on serum calcium or phosphorous.

Estrogen administration to the intact animal caused a small but significant increase in the bone ash content and this was sustained over the entire 12 month period (Fig. 1). Castration caused a small but significant decrease in the ash content which was still present at 12 months and estrogen administration returned this value to normal.

Estrogen administration to the intact animal caused a significant and sustained decrease in the total hydroxyproline content of bone (Fig. 2). Castration caused an initial

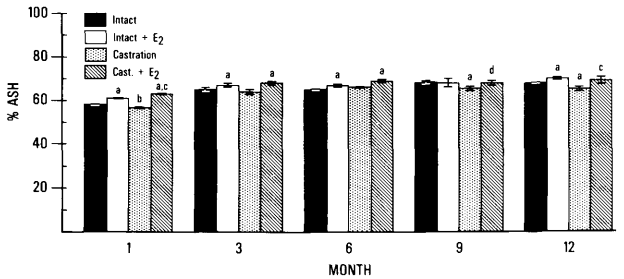


FIG. 1. *Percent ash of dry bone.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a) $P < 0.01$, or (b) $P < 0.05$. Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c) $P < 0.01$, or (d) $P < 0.05$. The same method of illustrating data is utilized in all figures.

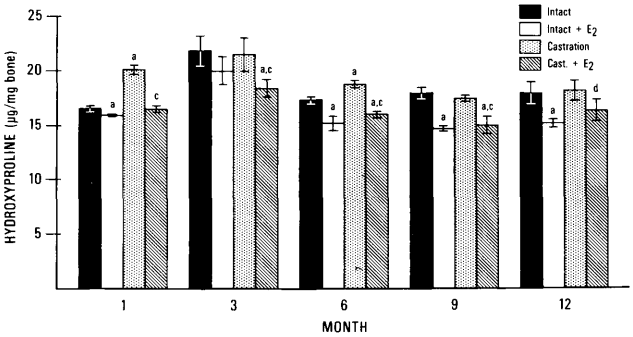


FIG. 2. *Hydroxyproline content of bone.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a) $P < 0.01$, or (b) $P < 0.05$. Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c) $P < 0.01$, or (d) $P < 0.05$. The same method of illustrating data is utilized in all figures.

increase in bone hydroxyproline content at 1 month but by 12 months there was no difference between the castrated and intact animals. Estrogen administration to the castrated animal did cause a sustained decrease in bone hydroxyproline. Hydroxyproline incorporation rates (Fig. 3) indicated that estrogen administration to the intact animal caused a decrease in the uptake of radioactive proline in bone. Castration caused no significant change and estrogen administration to the castrated animal also decreased the synthesis rates of bone collagen. Castration appeared to decrease the total bone hexosamine (Fig. 4) value at 6 months but at 12 months, the value had returned to normal. Estrogen administration to the intact animal appeared at 12 months to have increased the bone hexosamine content. The specific activity of bone hexosamine (Fig. 5) was decreased when estrogen was administered to the intact animal. Castration had no effect but estrogen administration to the castrated animal also caused a decrease in the value.

Estrogen administration to the intact animal caused a decrease in the uptake of radioactive calcium into bone (Fig. 6). Castration also appeared to cause a decrease and estrogen administration to the castrated animal caused a further decrease in this value.

There was no significant effect of estrogen administration on bone collagenolytic activity of male rat bone.

*Discussion.* There appear to be several significant differences when one compares this data with that derived from a similar study of the female rat (1). In the first place, removal of the ovaries in the female leads to a decrease in serum calcium and estrogen replacement returns this to normal. Secondly, data in the female indicated that removal of the ovaries causes an increase in bone turn-

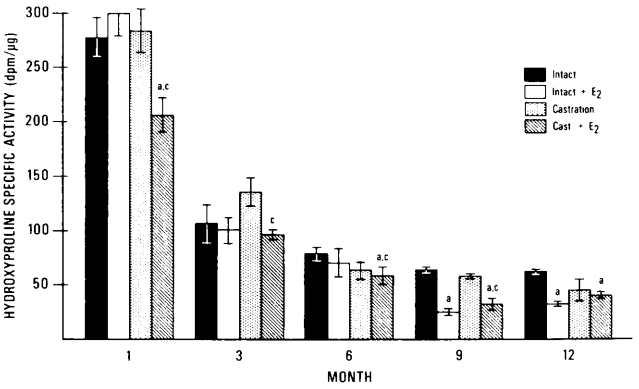


FIG. 3. *Hydroxyproline specific activity of bone.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a) $P < 0.01$, or (b) $P < 0.05$. Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c) $P < 0.01$, or (d) $P < 0.05$. The same method of illustrating data is utilized in all figures.

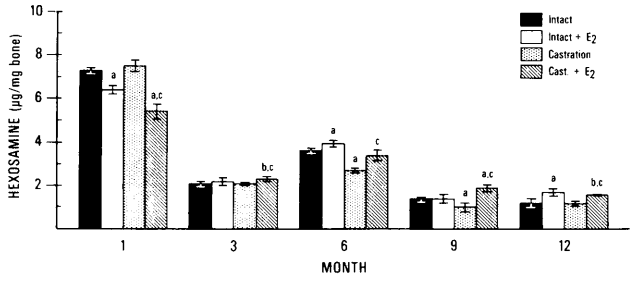


FIG. 4. *Hexosamine content of bone.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a) $P < 0.01$, or (b) $P < 0.05$. Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c) $P < 0.01$, or (d) $P < 0.05$. The same method of illustrating data is utilized in all figures.

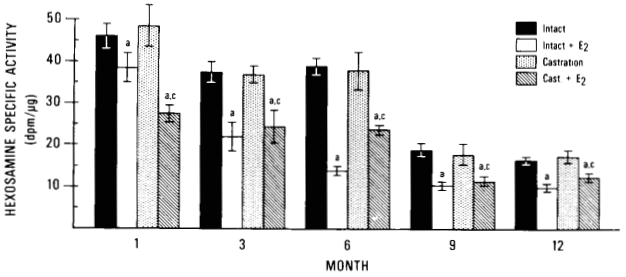


FIG. 5. *Hexosamine specific activity.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a) $P < 0.01$, or (b) $P < 0.05$. Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c) $P < 0.01$, or (d) $P < 0.05$. The same method of illustrating data is utilized in all figures.

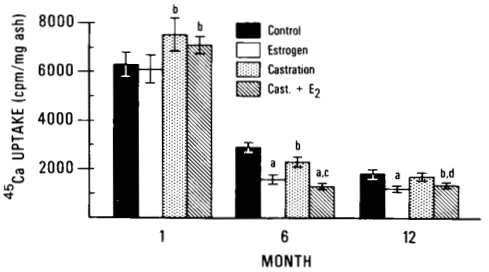


FIG. 6. *Uptake of calcium 45.* The bars represent the mean value and the SE are illustrated. Significant differences between all groups and the intact control animals are indicated by (a) $P < 0.01$, or (b) $P < 0.05$. Significant difference between the castrated animals and the estrogen treated castrated animals are indicated by (c) $P < 0.01$, or (d) $P < 0.05$. The same method of illustrating data is utilized in all figures.

over. Thus there was an increase in the uptake of radioactive calcium, an increase in the synthesis rates of collagen and glycosaminoglycan and an increase in collagenolytic activity. Estrogen administration returned the synthesis rates to normal and decreased collagenolytic activity to somewhat below normal, indicating decreased resorption. Oophorectomy led to a decrease in ash content and estrogen administration to the intact animal, and to the oophorectomized animal returned it to normal.

Several interpretations are possible to explain the data in the female. Decreased sensitivity to parathyroid hormone certainly is a possible explanation. The data could also be explained by postulating an estrogen mediated increase in calcium absorption from the gut leading to a decreased need for calcium mobilization from bone. Further work is necessary before this problem will be fully understood.

The failure of estrogen to alter the serum calcium in the male rat either indicates that the homeostatic mechanisms function better in the male or that there is a basic difference in response. The incubation studies as well as the calcium uptake indicate that estrogen given to the intact or castrated male rat causes a decrease in formation which appears to increase in magnitude until about 6 months and is still present at 12 months. The collagenolytic data demonstrates no significant change in resorption rates. This then is another difference between the male and female rat.

That estrogen has an effect on the male has been known for some time (7). Igarashi (8) demonstrated that estrogen protects the male animal against the loss of bone mineral brought about by a low calcium diet. In short term studies, Shai and Wallach (9) demonstrated once more the retardation of body and skeletal growth with an increase in skeletal mass relative to body weight brought about in male rats by estradiol. They also demonstrated a decrease in resorption and in mineral deposition as indicated by $^{85}Sr$ studies. Finally, they demonstrated an estrogen mediated decrease in the sensitivity of male animals to the effect of exogenous calcitonin. The sex of the animal as well as its age are apparently important in determining the effect of estrogen in mediating the effect of calcitonin. Kaplan (10) showed that before puberty, the response of the two sexes was equal. Following puberty, the male decreased in sensitivity only slightly with increasing age, while females diminished rapidly. In addition and perhaps more importantly, the castrated males treated with estrogens were much less

sensitive than were the intact controls.

The end result of long term estrogen administration to the male rat is a slight but significant increase in ash content which appears to be associated with a decrease in collagen content on a per weight basis and a slight increase in hexosamine content. However, all parameters demonstrate a decrease in the rate of synthesis of bone matrix. In contrast to the data from the female rat, collagenolytic activity showed no change. These facts are difficult to reconcile because if in the face of decreased formation rate, there is an increase in bone mass, a decrease in resorption should have been measured. Perhaps the changes in collagenolytic activity which occurred were exceedingly small and resulted over a prolonged period in a decrease in resorption which could not be measured by the method utilized. It also is possible that there is a discrepancy between the mobilization rates of mineral and matrix in the estrogen treated male animal, and that in fact, the increase in ash content associated with a decrease in the organic components of matrix is reflecting this. Finally, it is possible that there is a decreased ability of the collagenolytic enzyme to actually resorb matrix, with a resultant change in resorption.

The data here do not allow one to determine the mode of action of estrogen in the male. It has been reported (11) that there is no receptor protein for estrogen in the female rat bone. It is recognized that male animals do possess receptor proteins to estrogens (12) in some tissues but up to date no reports in the literature have reported the presence of these substances in male bone cells. In addition, there is no information on a possible direct effect of estrogen on male rat bone utilizing tissue culture methods. It does, however, seem important to record the fact that male animals respond in a different fashion from females.

*Summary.* Hundred and fifty-gram male rats were divided into four groups with the first containing intact controls, the second intact animals treated with 400 $\mu$g per 100 g body wt of 17-$\beta$-estradiol twice a week. The animals in the third group were castrated and those in the fourth were castrated and treated with the same dosage of estrogen. Animals were sacrificed at varying periods of time from one to 12 months. Estrogen administration caused a sustained decrease in body weight in the intact animal but did not change the body weight in castrated animals. Estrogen had no effect on either serum calcium or serum phosphorus. Estrogen administration to the intact animal caused a small but significant increase in ash content of bone. Castration caused a small decrease in this value which was still present at 12 months and estrogen administration returned the value to normal. Estrogen administration caused a decrease in total hydroxyproline content of bones of intact animals. Castration did not alter this value but estrogen administration to the castrated animal decreased the bone hydroxyproline content. Hydroxyproline incorporation rates were decreased in bones of both the intact and castrated animals. Castration did not alter the total hexosamine content of bones but estrogen administration to both the intact and castrated animals caused an increase in bone hexosamine content. Estrogen administration caused a decrease in the synthesis rate of proteoglycans in bones of both the intact and castrated animals. Estrogen administration caused a decrease in the uptake of radioactive calcium into bones of both the intact and castrated animals. There was no significant effect of estrogen on collagenolytic activity in male rat bone. It is concluded that estrogen administration to the male rat, causes changes which are different from those found in the female. There appeared to be no change in serum calcium or phosphorus values. A decreased synthesis of bone matrix and decreased uptake of radioactive calcium brought about no measurable change in the resorption of bone matrix.

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