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Daidzein but Not Other Phytoestrogens Preserves Bone Architecture in Ovariectomized Female Rats In Vivo

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Abstract Ovariectomy of immature female rats, results in significant decrease of trabecular bone volume and in cortical bone thickness. Previously, we found that estradiol-17β (E_2) restored bone structure of ovariectomized (Ovx) female rats to values obtained in intact sham-operated female rats. E_2 also selectively stimulated creatine kinase (CK) specific activity a hormonal-genomic activity marker. In the present study, we compared the effects of E_2 and the phytoestrogens: daidzein (D), biochainin A (BA), genistein (G), carboxy-derivative of BA (cBA), and the SERM raloxifene (Ral) in Ovx, on both histological changes of bones and CK, when administered in multiple daily injections for 2.5 months. Bone from Ovx rats, showed significant disrupted architecture of the growth plate, with fewer proliferative cells and less chondroblasts. The metaphysis underneath the growth plate, contained less trabeculae but a significant increased number of adipocytes in the bone marrow. D like E_2 and Ral but not G, BA, or cBA, restored the morphology of the tibiae, similar to that of control sham-operated animals; the bony trabeculeae observed in the primary spongiosa was thicker, with almost no adipocytes in bone marrow. Ovariectomy resulted also in reduced CK, which in both epiphysis and diaphysis was stimulated by all estrogenic compounds tested. In summary, only D stimulated skeletal tissues growth and differentiation as effectively as E_2 or Ral, suggesting that under our experimental conditions, D is more effective in reversing menopausal changes than any of the other isolated phytoestrogens which cannot be considered as one entity. J. Cell. Biochem. 103: 1826–1832, 2008.

Key words: daidzein; estradiol-17β; raloxifene; adipocytes; trabeculae

Phytoestrogens are found in many edible plants, and are a diverse group of biologically active compounds, with structural similarity to that of estrogens. The two principle components are isoflavones and lignans. The parent estrogenic isoflavones daidzein (D), genistein (G), and biochainin A (BA) have been demonstrated to have important role in reducing symptoms associated with estrogen deficiency disorders. They may be protective against osteoporosis due to their ability to exert osteogenic actions on bone, particularly on bone turnover and growth [Anderson and Garner, 1998].

Studies on phytoestrogens and their effects on bone vary, from observations on the whole soy protein [Somekawa et al., 2001; Gallagher et al., 2004] to research of different known formula of mixed isoflavones and/or lignans [Kreijkamp-Kaspers et al., 2004]. Epidemiological studies among Asian women showed that a higher consumption of phytoestrogens was associated with higher values of bone mineral density (BMD) and a lower incidence of osteoporotic fractures despite the lower calcium intakes in these populations [Somekawa et al., 2001]. Furthermore, numerous clinical human studies on the effects of isoflavone-rich diet on bone have shown significant relationships between phytoestrogens and surrogate markers for bone turnover such as femoral neck, lumbar, and/or radial BMD [Mei et al., 2001; Cook et al., 2003; Dodin et al., 2005], as well as positive effects on menopausal symptoms like lipid profile or cardiovascular disease, suggesting alternative compounds for promotion of menopausal health, as a

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replacement for the harmful endpoints in hormonal-treated women. However, a number of studies did not find a significant positive effect of isoflavones [Chen et al., 1985] or lignans [Wuttke et al., 2003] on BMD. Also, studies have shown that the effects of isoflavones are more marked in women in later menopause [Chen et al., 1985; Kreijkamp-Kaspers et al., 2005] and no significant positive effects in early postmenopausal women [Gallagher et al., 2004] or in healthy menstruating women [Anderson and Garner, 1998]. Studies in animal models, however, have demonstrated the beneficial effects of phytoestrogens, mostly isoflavones, on postmenopausal estrogen deficiency in ovariectomized (Ovx) rats, measuring bone mass of trabecular bone and/or cortical bone, BMD, mechanical stress, and several markers of bone turnover such as serum osteocalcin and urinary deoxypyridinoline [Arjmandi et al., 1996; Ishida et al., 1998; Uesugi et al., 2001; Atkinson et al., 2004]. Treatment of Ovx rats with silymarin, a standardized mixture of flavono-lignans, which binds exclusively to the estrogen receptor β (ERβ), had estrogenic effects in the femoral metaphysis, osteoblastic parameters, and on the osteoclastic activity, but was inactive or antagonist in the uterus [Wuttke et al., 2003]. In vitro studies using cultured bone cells had also shown that isoflavones modulated different cellular activities [Heim et al., 2004; Ge et al., 2006; Somjen et al., 2006a,b].

In newborn humans, bone marrow contains few adipocytes and is characterized as erythropoietic. With advancing age, the number and size of adipocytes increases in a linear manner [Gimble, 1990] till fat occupies 50% of the human marrow cavity [Gimble et al., 1996]. Clinical observations documented an inverse relationship between adipocytes and osteoblasts. In osteoporotic patients, increased bone marrow adipose tissue correlates with decreased trabecular bone volume [Martin and Zissimos, 1991]. Osteoblasts and adipocytes originate from common mesenchymal precursor in bone marrow [Gimble et al., 1996]. Also, the adipocyte is the most abundant stromal cell phenotype in adult human bone marrow [Martin and Zissimos, 1991] possibly due to a cellular stress response pathway activation with aging [Minaire et al., 1984]. Since, ovariectomy causes aging of bone, one of the age signs may also be adipogenesis, which replaces osteogenesis, leading to osteoporosis.

In the present study, we compared the effects of long-term daily treatment with estradiol-17β (E_2), Raloxifene (Ral), or isolated phytoestrogens and their synthetic carboxy-derivatives or vehicle on bone histology as well as on creatine kinase (CK) specific activity in the skeletal tissues of Ovx rats.

Our study showed that the adipogenesis caused by ovariectomy is a reversible process and can be corrected and rejuvenate the bone marrow to its normal accurate chronological age by the addition of E_2 or Ral. However, the phytoestrogens analyzed had no effect on bone histology or bone marrow restoration, except D, which was the only phytoestrogen with the ability to restore bone of Ovx rats.

MATERIALS AND METHODS

Animals

Wistar-derived, locally bred female rats, aged 25 days and weighing 60 g at the start of the experiment, were maintained, on a 14 h light/10 h dark schedule at 23°C, and provided with food pellets and water ad libidum. They were divided into eight groups, each containing seven animals: intact vehicle treated (sham operated, control 1), Ovx vehicle treated (control 2), and six groups of Ovx animals, treated with E_2, Ral, G, D, BA, or carboxy BA (cBA) [Somjen et al., 2003]. All rats were handled according to the NIH guidelines and the regulations of the Committee on Experimental Animals of the Tel-Aviv Sourasky Medical Center and the NIH guidelines.

Hormonal Treatment

Starting 2 weeks post-surgery, Ovx rats were injected 5 days per week for 2.5 months with 166 μg/kg E_2 (n = 7), 1.66 mg/kg Ral (n = 7), 16.6 mg/kg G (n = 7), D (n = 7), cBA (n = 7), or BA (n = 7) or with vehicle: 0.1% ethanol in saline (n = 7; Ovx control 2). Non-Ovx animals (sham operated) were also injected with the same vehicle (n = 7; non-Ovx, control 1).

Histology and Histomorphometry

After 2.5 months of treatment, 24 h after the last injection, rats were sacrificed by cervical dislocation and organs were removed for histology and biochemical analysis. Samples of whole tibiae from each group of Ovx and non-Ovx female rats were fixed for 48 h in neutral buffered 4% formaldehyde in 0.1 M sodium phosphate buffer pH 7.4, decalcified (2–3 weeks

at room temperature) in 10% ethylene diamine tetraacetic acid (EDTA), dehydrated in graded alcohols, and embedded in paraffin. Sections (6-μm thick) were stained with hematoxylin eosin for general morphology.

Creatine Kinase Specific Activity Preparation and Assay

Rat bones were collected in cold isotonic extraction buffer [Somjen et al., 2006a,b], the epiphyseal cartilage and diaphyseal bone were obtained and homogenized using a Polytron homogenizer (Kinematica A. G., Littau, Switzerland). Enzyme extracts were obtained by centrifugation of homogenates at 14,000*g* for 5 min at 4°C in an Eppendorf micro centrifuge. CK specific activity was measured in a Kontron Model 922 Uvicon Spectrophotometer using a Sigma coupled assay kit (UV-47). Protein was assayed by Coomassie brilliant blue dye binding. Results are means ± SEM of $n = 7$ and are expressed as % of control of the specific activities of CK in hormone-treated compared to vehicle-treated, control animals.

Reagents

E_2 and all phytoestrogens were purchased from Sigma-Aldrich (Rehovot, Israel). cBA was synthesized by us [Somjen et al., 2003] and Ral was the gift of Dr. Brigitte Fournier from Ciba–Geigi, Basel, Switzerland. All other reagents were of analytical grade.

Statistical Analysis

The significance of differences between mean values obtained from the different experimental and control groups were evaluated by ANOVA. A value of $P < 0.05$ was considered significant.

RESULTS

Morphology of Rat Tibiae Treated With Various Phytoestrogens

All specimens were analyzed at the metaphysis including the growth plate and the primary spongiosa adjacent to the growth plate. Examination of the vehicle-injected control sham-operated intact animals revealed that the growth plate contained the typical arrangement of cartilage cells, including proliferative, chondroblastic, and hypertrophic chondrocytes. Numerous trabecular spicules were observed underneath and adjacent to the lower aspect of the growth plate (Fig. 1).

After 3 months of ovariectomy, vehicle-injected control animals demonstrated changes in the growth plate structure. The overall growth plate architecture was disrupted with fewer proliferative and chondroblastic cells. The metaphysis underneath the growth plate contained only few trabeculae as compared to the intact control tibiae. Numerous adipocytes were observed in the bone marrow (Fig. 1). A disrupted organization of the growth plate and trabeculae was observed after ovariectomy with no proliferative chondrocytes. Most of the growth plate contained chnodroblastic and hypertrophic chondrocytes. Although the mineralized zone appeared to be resorbed, it was not accompanied with bone trabecules formation. Numerous adipocytes were observed in the bone marrow.

After 2.5 months treatment with E_2, the tibiae morphology was similar to the one observed in intact sham-operated bone. The growth plate contained the typical arrangement of proliferative, chondroblastic, and hypertrophic cellular populations. The bony trabeculae observed in the primary spongiosa were thicker compared to those from intact control animals. The bone marrow contained less adipocytes (Fig. 1). After treatment with E_2, the tibiae morphological appearance was similar to the one observed in the intact sham-operated bone, but with fewer adipocytes.

Treatment of 2.5 months with Ral led to an almost complete recovery of the bone architecture. The growth plate arrangement was typical and normal. The main feature was the appearance of thin elongated bone spicules at the lower aspect of the growth plate, indicating a more synchronized growth of the bone as compared to the Ovx and E_2 treatments. No adypocytes were observed in bone marrow in this region (Fig. 1).

Similar to E_2 and to Ral, the growth plate architecture has been almost fully restored also after 2.5 months treatment with D. The primary spongiosa contained thick bone trabeculae and almost no adipocytes were observed in the bone marrow. The metaphysis underneath the growth plate contained only few trabeculae compared to the intact sham-operated control tibiae. Numerous adipocytes were observed in the bone marrow underneath and adjacent to the lower aspect of the growth plate (Fig. 1).

No change was observed after treatment with G, BA, or cBA for 2.5 months. The growth plate contained fewer cells and almost no trabeculae were observed. However, the bone marrow contained large number of adipocytes (Fig. 2).

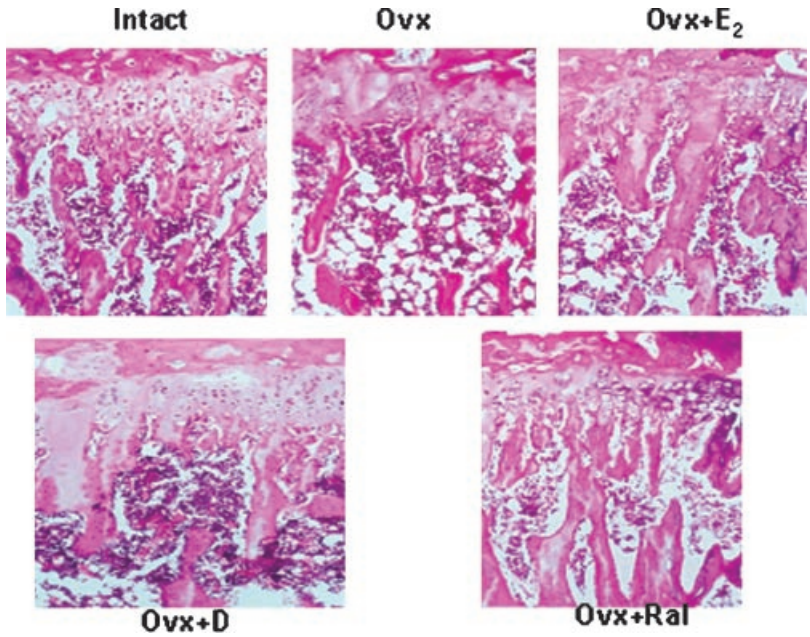

Fig. 1. The effect of ovariectomy (Ovx) compared to sham-operated intact female rats and the effects of 2.5 months daily injections with either E_2, D, or Ral on bone structure in ovariectomized female rat. Details are described in Materials and Methods.

Stimulation of CK Specific Activity in Rat Skeletal Tissues Treated With Various Phytoestrogens

After treatment of 2.5 months with the different hormones, CK specific activity was stimulated by all estrogenic compounds used, in epiphysis and diaphysis (Fig. 3). In uterus, only E_2, G, and D were stimulatory (data not shown). In all organs tested, ovariectomy resulted in decreased levels of constitutive enzymatic specific activity levels (Fig. 3).

DISCUSSION

Phytoestrogens are defined as naturally occurring compounds, found in plants, that are structurally and functionally related to E_2 or

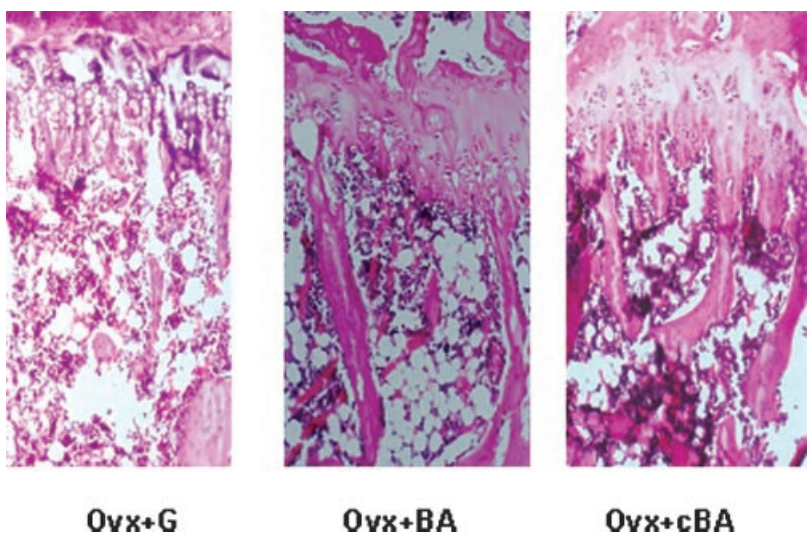

Fig. 2. The effects of 2.5 months daily injections with either G, BA, or cBA on bone structure in ovariectomized female rat. Details are described in Materials and Methods.

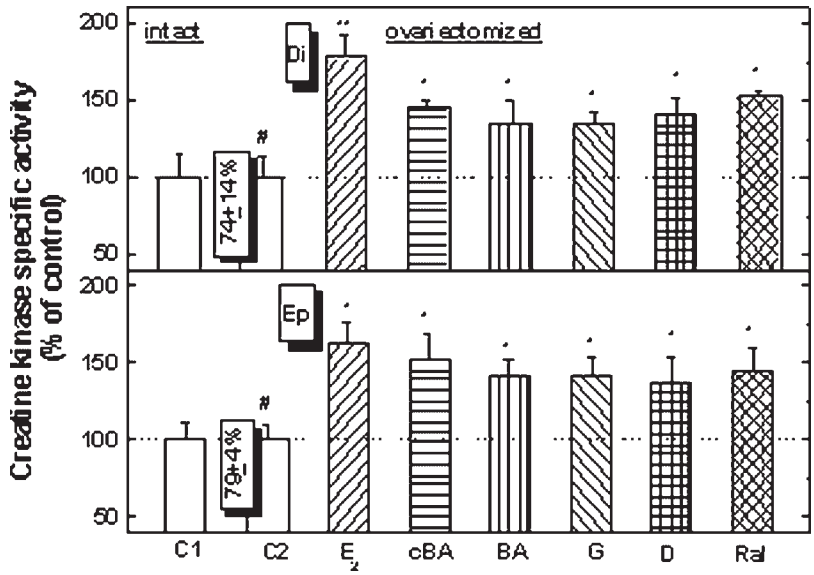

Fig. 3. The effects of 2.5 months daily injections with either E_2, G, D, BA, cBA, or Ral on creatine kinase specific activity (CK) in epiphyseal cartilage (Ep) and in diaphyseal bone (Di) of sham-operated intact (C1) (non-Ovx) or ovariectomized (C2) (Ovx) female rats. Results are means ± SEM.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The basal activity of CK in sham-operated intact (non-Ovx) rat organs were in Ep 0.58 ± 0.10 and in Di 0.68 ± 0.08 μmols/min/mg protein. The numbers in brackets is the difference between C2 and C1.

that produce estrogenic effects. A number of classes have been identified, such as lignans, isoflavones, coumestans, and resorcylic acid lactones [Knight and Eden, 1996]. They were first noted in 1926 to have estrogenic activity [Setchell, 1998]. Because they possess a phenolic ring, this enables them to bind to both types of estrogen receptor, ERα and the more preferable ERβ [Murkies et al., 1998; Ishimi et al., 1999].

Ovariectomy caused an almost complete cessation of bone growth, as was demonstrated by the disruption of the growth plate cell organization, and the complete disappearance of thin bone spicules, previously reported [Uesugi et al., 2001; Picherit et al., 2000].

The increased number of adipocytes in the bone marrow adjacent to the growth plate could indicate that ovariectomy affected the overall metabolism of bone marrow. Such change reflects a more mature stage of the bone as observed in aged animals. In new-born humans, the marrow contains few adipocytes and is characterized as "red" or erythropoietic. With advancing age, the number and size of adipocytes increase in a linear manner [Gimble, 1990; Gimble et al., 1996]. In osteoporotic patients, increased bone marrow adipose tissue correlates with decreased trabecular bone volume [Somekawa et al., 2001]. Osteoblasts and adipocytes originate from common mesenchymal precursor in bone marrow [Peck and Rifas, 1982; Gimble et al., 1996; Kirkland et al., 2002] and the trabecular bone and adipose tissue content in bone marrow are inversely related in human disuse osteoporosis [Minaire et al., 1984].

Many different plant-derived products are used for the treatment menopausal symptoms and post-menopausal osteoporosis. Usually, all the products are discussed "as one," although being totally different. It is not unusual to find sentences such as that there is insufficient evidence to recommend the use of phytoestrogens in place of traditional estrogen replacement therapy (ERT) [Glazier et al., 2001]. Even in the NIH statement on the treatment of menopausal symptoms, these compounds, which are soy extracts are suggested to probably have some mitigating effect on hot flashes as was reported in the NIH conference in 2005.

In the Practice Bulletin published by the American College of Obstetricians and Gynecologists (ACOG) entitled: "The Use of Botanicals for Management of Menopausal Symptoms," the authors emphasize that the effects of soy protein found in whole foods, soy protein isolates, and those of isoflavones isolates made into powders or

pills may not all be the same and therefore each one's effect should be examined and judged by itself.

In the present study, we compared the effects of long-term daily treatment with E_2, the SERM Ral or isolated phytoestrogens and their synthetic carboxy-derivatives or vehicle on bone histology regarding their ability to prevent damage to bones caused by Ovx, leading to decreased trabecular mass and increased adipogenesis in bone marrow, as well as on a biochemical marker the CK specific activity in skeletal tissues of female rats. Ovariectomy caused almost a complete cessation of bone growth as demonstrated in the disruption of the growth plate cell organization, and the complete disappearance of thin bone spicules, as previously reported [Somjen et al., 2007]. The increased number of adipocytes in the bone marrow adjacent to the growth plate indicated that this treatment might affect the overall metabolism of bone marrow. Such change reflects a more mature stage of the bone as observed in aged animals. E_2 by decreasing the lipid content of the cells in bone marrow appeared to reverse the aging process of bone marrow as well as repairing the morphological changes in the growth plate. G, BA, cBA had almost no effect on the Ovx bone as revealed by the facts that the structure of the growth plate was not replaced and increased amounts of adipocytes were observed in the bone marrow.

Treatment with D restored the damage in bone including disappearance of the adipocytes, indicating that in addition to its effect on bone growth and maturation, this substance may also have an effect on bone marrow metabolism. This suggests that it is better than the treatment with any other single phytoestrogenic compounds.

Since, ovariectomy causes accelerated maturation of the bone, the adipogenesis may also be one of the signs of aging. However, our study showed that the adipogenesis caused by ovariectomy is a reversible process and can be corrected leading to rejuvenation of the bone marrow to its normal accurate chronological age by the addition of E_2, Ral, or D. On the other hand, other different phytoestrogens such as G, BA, cBA had no effect on bone architecture or bone marrow restoration. The exact molecular mechanism(s) of action of D compared to E_2 or Ral is currently under investigation.

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