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Evaluation of bone quality and quantity in osteoporotic mice – The effects of genistein and equol

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ABSTRACT

The technology of gene manipulation is often used in mice. A crucial point for osteoporosis research is the evaluation of biomechanical and morphologic parameters. These parameters, however, are difficult to measure in mice. Nevertheless, this study demonstrates the capability of using techniques for the evaluation of bone quality and quantity after various treatments in osteopenic mice.

After ovariectomy, 60 C57BL/6J mice were divided into 4 groups and were fed a soy-free diet (C) supplemented with estradiol, genistein or equol for 3 months. To analyze the osteoprotective effects of the tested supplements, we evaluated the bone biomechanical properties, histomorphometric changes and bone mineral density of the proximal tibiae metaphysis.

The biomechanical parameters of genistein (GEN) were shown to be similar to those levels observed with estradiol (E). The biomechanical parameters of both GEN and E were significantly superior to those observed with C. Supplementation with equol (EQO) demonstrated higher mean biomechanical values than those observed with C. The histomorphometric evaluation demonstrated an increased number of nodes in mice treated with GEN and E as compared to the mice treated with EQO and C. Treatment with E and EQO led to improved cortical bone, which was only partly seen with the mice treated with GEN. The analysis of the bone mineral density (BMD) demonstrated that treatment with GEN and E resulted in a significant improvement as compared to the mice treated with C, while the cancellous density was significantly increased in all of the supplementation groups.

This study conclusively demonstrated that bone quality and quantity parameters can be measured in mice. Furthermore, biomechanical and morphologic evaluations were shown to be reliable for use in mice. Further studies may combine these techniques with gene manipulation technology to better understand osteoporosis. Treatment with GEN resulted in improved biomechanical results and enhancement of morphologic parameters.

Introduction

Postmenopausal osteoporosis and the resulting morbidity and increased mortality from osteoporotic fractures are major health problems in elderly women. Postmenopausal osteoporosis is a heterogeneous disorder that result from both estrogen deficiency and aging and leads to an increased risk of fracture. Estrogen deficiency is one of the most common reasons for increased bone resorption and accelerated bone loss (Pacifici 1998).

The incidence of osteoporosis in homogenous populations correlates inversely with the calcium intake, and it appears that the highest incidence of fractures occurs in populations with the highest calcium intake (Hegsted 1986). Therefore, it is possible that other macro- and micronutrients may contribute to the varying incidence of fractures between populations. Hormone replacement therapy (HRT) has been used to prevent hip fractures, spine fractures, and osteoporosis (Rossouw et al. 2007). After stopping the WHI study because of life-threatening side effects of the HRT, the search for alternatives for the HRT and for treatment of osteoporosis became necessary. Attention has been focused on phytoestrogens, especially isoflavones, as a potential and safe alternative for pharmaceutical hormone replacement therapy (Holmes et al. 2004). Phytoestrogens are a heterogeneous group of plant-derived compounds that exhibit estrogen-like effects in estrogen-dependent tissues. Phytoestrogens belong to the phenol group of chemicals. All phytoestrogens have weak estrogen-like

Abbreviations: BMD, bone mineral density; Cn.Dn., cancellous bone density; C, control; Ct.Ar., cortical bone area; Ct.Dn., cortical density; EQO, equol; E, estradiol; fL, failure load; GEN, genistein; Fmax, maximum load; N.Nd., number of nodes; Tb.Ar., trabecular bone area; yL, yield load

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activity (Li et al. 2004); however, a significant serum level can be reached upon exposure to high doses.

Analysis of the anti-osteoporotic efficiency of phytoestrogens has given contrasting results. A mild osteoprotective effect of phytoestrogens was observed by Sirtori (2001), although soy was not considered as a source of phytoestrogens.

Phytoestrogens are classified as naturally occurring selective estrogen receptor modulators (SERMs), and have a direct interaction with the estrogen receptors (ER) α and β. ER-α is involved in bone maturation and maintenance of bone mineral density in both genders. Various classes of phytoestrogens and their compounds interfere with estrogen-mediated biological pathways in different ways (Humpel et al. 2005).

Beside daidzein, the isoflavonoid genistein (GEN) (Fig. 1) is one of the major phytoestrogens of soybeans and soy-based products. GEN possesses some structural similarities to estrogen, and binds to the estrogen receptor in several tissues such as bone and uterus. GEN inhibits the expansion of osteoclasts in cell culture, whereas it stimulates the growth of osteoblasts (Hertrampf et al. 2006; Rickard et al. 2003; Yamagishi et al. 2001).

Equol (EQO) (Fig. 2) is the active metabolite of the isoflavonoid daidzein or formononetin, which is produced by the gut microflora *Streptococcus intermedius* and, Ruminococcus. This metabolization of daidaen and formononetin are individually different, therefore, its active metabolite EQO is used in studies (Rowland et al. 2000). EQO is a chiral molecule existing as both R and S enantiomeric forms. EQU is structurally similar to 17β-estradiol, and has affinity for both estrogen receptors (ER α and β). EQO has a 100 fold higher affinity to ER as compared to daidzein (Sathyamoorthy and Wang 1997). The affinity of EQO to ER is similar to that observed for GEN; however, the observed transcriptional activity in the effector cells is the strongest with EQO among all isoflavones. Fujioka et al. (2004) demonstrated a significant increase in the BMD of osteopenic mice using EQO.

Although the rat osteopenia model has been extremely useful for modeling the clinical syndrome of postmenopausal osteoporosis, the underlying molecular mechanisms for bone disorders, including osteoporosis, have been explored predominantly in mouse (Bellino 2000). Because of the advanced state of gene manipulation technology in this species, and its numerous inbred strains varying in peak bone mass, the mouse has the potential of being an even more useful preclinical model of postmenopausal bone loss than the rat. Therefore, it is necessary to establish evaluation procedures besides indirect bone parameters like BMD or bone turnover parameters. Because fracture is the crucial point in osteoporosis, all treatment options have to be evaluated by biomechanical tests.

The purpose of this study was to demonstrate that biomechanical and histomorphometric evaluations commonly performed in rat models are able to be measured in the mouse model of osteoporosis. Furthermore, we have studied and analyzed the effects of genistein and equol on bone quality and quantity.

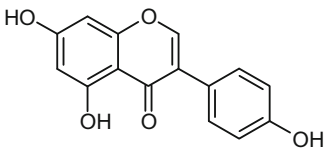

Fig. 1. Structure of genistein.

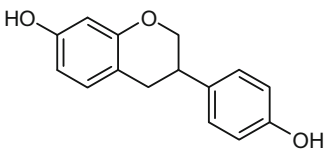

Fig. 2. Structure of equol.

Material and methods

Animals and substances

Experiments were performed with 60 female two-month-old C57BL/6J mice obtained from Fa. Winkelmann (Borchen, Germany). Animals were bilaterally ovariectomized at the age of 2 months. They were briefly exposed to CO_2 until they fell unconscious and then anaesthetized via i.p. injection of Ketamin (Hostaket®, Hoechst) and Xylazin (Rompun®, Bayer). Each mouse was given 7.5 mg xylazin and 62.5 mg ketamine per kg of body weight. Immediately after ovariectomy, the mice were separated into 4 different treatment groups (15 mice per group) and treatment proceeded for 3 months.

Group 1 (C), the control, received phytoestrogen-free pelleted food with potato proteins added (Ssniff SM R/M, 10 mm, Ssniff Special Diet, Soest, Germany). Group 2 (E) received the control diet supplemented with estradiolbenzoate (17β-Estradiol-3-benzoate – 98.5% purity). E was purchased from Sigma. The concentration of E in the chow was 4.3 μg/g. On the basis of ingested food, it was calculated after termination of the experiments that the animals had received daily doses of 12.9 μg E.

Groups 3 (GEN) received supplementation with genistein (4',5,7-trihydroxyisoflavone – >98.5% purity), supplied by Changzhou Dahua Import and Export Corp., Ltd, China. The concentration of genistein in the chow was 1 mg/g. The animals received daily doses of 3 mg GEN.

Group 4 (EQO) received soyfree-free food with the addition of a racemic mixture of equol (7-hydroxy-3-(4′-hydroxyphenyl)-chroman, 98% purity, Changzhou Dahua Import and Export Corp., Ltd, China) at a concentration of 4 mg/g of chow. On the basis of ingested food, it was calculated after termination of the experiments that the animals had received daily doses of EQO of 12 mg.

All batches of chow were prepared 1 week prior to the start of the experiment. The doses of substances used in this study were determined in preliminary studies and by references given in the literature (Fujioka et al. 2004; Sehmisch et al. 2008; Yamagishi et al. 2001).

The study protocol was approved by the District Government and conforms to German animal protection laws (Az: 33.42502/01-30.05. Bezirksregierung Braunschweig).

After 12 weeks, the animals were sacrificed under anesthesia and the wet weight of the uterus was determined. The tibiae were freed from the skin, muscles and tendons. The fibulae were separated at the synostosis. The tibiae were stored in tubes at −20 °C until measurements were performed.

Bending and breaking test

A ZWICK machine, type 145660 Z020/TND (Zwick/Roell, Ulm, Germany), was used according to the procedure standardized by Stürmer et al. (2006). The base and stamp of the device were modified according the smaller proportions of mice tibiae. Because of the modifications of the breaking device a right left comparison with 20 mice of the same size was performed. In the comparative bioassay the thawed tibiae, moistened with isotonic saline solution, were placed with the 3-point contact on the aluminum base so that the base was fixed in the ZWICK-testing set with a distance of exactly 1 mm between the end of the proximal tibia and the centre of the roller stamp. The stamp was driven down with the primary force of 1 N to fix the tibia and the