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Genistein aglycone effect on bone loss is not enhanced by supplemental calcium and vitamin D_3: A dose ranging experimental study ☆

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ABSTRACT

Genistein aglycone (GEN) has a favorable effect on bone loss. We investigated the effects of GEN alone or in combination with supplemental calcium and vitamin D_3 in an animal model of bone loss to evaluate if there was additional benefit. Ovariectomized (OVX) and SHAM-OVX rats were used. OVX were divided into 12 groups and randomized to receive: GEN at 27, 54, 200, 500 or 1000 mg (human equivalent dose (HED)/day/ip injection alone or with calcium carbonate (Ca) (360 mg/kg/day/gavages) and vitamin D_3 (D_3) (50 IU/kg/day/gavages) or Ca/D_3 without GEN or untreated for 6 weeks. SHAM-OVX were randomized into 7 groups and treated with: Ca and D_3 alone or in combination with GEN (same doses as OVX), or left untreated. Bone mineral density (BMD), bone-alkaline phosphatase (b-ALP), collagen C-telopeptides (CTX), osteoprotegerin (OPG) and soluble receptor activator of NFκB ligand (sRANKL) were assessed. Femurs were excised and tested for breaking strength and histology. Uterine weight was analyzed to assess GEN's estrogenic effects on the SHAM-OVX.

The most effective dose of GEN, independent of Ca/D_3 supplementation, was 54 mg/day. Higher doses yielded no further improvement in bone biomarkers, histology or strength. Only 1000 mg/day HED of genistein produced statistically significant changes in uterine weight of the SHAM-OVX. This study suggests that 54 mg/day of GEN is the threshold dose for efficacy. In addition, supplemental calcium and vitamin D_3, beyond normal dietary intake do not enhance the effects of genistein on improving measures of bone loss. This observation has implications regarding the use of calcium and vitamin D_3 supplementation.

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Introduction

At first glance, it is paradoxical that Asian countries with some of the lowest calcium intakes worldwide have a relatively low rate of osteoporotic hip fractures (Schwartz et al. 1999; Xu et al. 1996; Ross et al. 1991). Although genetic, morphological, and other factors may account, in part, for this finding, high soy consumption, containing high isoflavone content, has been posited as a key factor underlying the low rate of fractures among Asians (Usui 2006; Cassidy et al. 2006). Although it is difficult to precisely identify the specific isoflavone(s) most responsible for preventing or restoring bone loss, current evidence suggests that the aglycone genistein, derived from dietary genistin, may be the most active isoflavone for the prevention of bone loss in postmenopausal women (Zhang et al. 2008). Food based soy trials using mixed and glucoside forms of isoflavones have failed to produce meaningful gains in BMD (Vupadhyayula et al. 2009; Kenny et al. 2009; Alekel et al. 2010). Well-controlled clinical trials, however, have demonstrated that pure genistein aglycone (54 mg/day) increased BMD at the lumbar spine and femoral neck and caused favorable changes in serum and urinary bone markers in osteopenic, postmenopausal women with no clinically significant adverse effects on the breast and uterus noted (Marini et al. 2007, 2008a,b; Bitto et al. 2010). The mechanism of action for genistein induced inhibition of bone resorption and induction of bone formation is not completely known, but binding to ER-β is implicated in its novel effect on bone formation (Bitto et al. 2010).

ER-β is robustly expressed by developing human bone, especially the cancellous bone compartment that is most subject to loss following gonadal hormone deprivation (Bord et al. 2001). There

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is a greater than 9-fold increase in ERβ expression in cultured human osteoblasts during bone mineralization, whereas ERα levels remain unchanged during this process (Arts et al. 1997). These data strongly suggest the important involvement of ER-β in bone formation and, by extension, the use of selective ER-β agonists such as genistein to treat bone loss. The question of whether calcium and vitamin D_3 supplementation beyond normal dietary intake is advantageous in combination with genistein has never been determined.

In the presence of adequate dietary calcium, vitamin D_3 promotes absorption of 30% of ingested calcium, compared to 10–15% calcium absorption in cases of vitamin D_3 deficiency (Holick 2004; Holick et al. 2005). Calcium intake has not necessarily been associated with a reduction in fracture risk (Bischoff-Ferrari et al. 2007), though it must be noted that fracture prevention is tightly linked to adequate calcium plus vitamin D_3 consumption (DIPART [vitamin D Individual Patient Analysis of Randomized Trials] Group 2010). Interestingly, compliance may be a limiting factor in the efficacy of calcium for fracture prevention as Prince et al. (2006) found that only 57% of patients took 80% or more of their prescribed 1200 mg daily calcium dose over a 5 year period. Moreover, the group of compliant women were the only ones to show a significant reduction in fracture risk. Other nutrients beyond calcium and vitamin D_3 are also known to play a role in bone maintenance and remodeling, including isoflavones (Nieves 2009).

There is no data available to assess the contributions of genistein alone or in combination with supplemental calcium and vitamin D_3 on bone. In order to clarify this issue, we investigated the effects of different HEDs of genistein aglycone alone or in combination with supplemental calcium carbonate and vitamin D_3 on bone markers and functional and histological bone parameters in ovariectomized rats.

Materials and methods

Animals and randomization

All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals. Three month old Sprague–Dawley OVX and age matched SHAM-OVX rats, weighing about 220–250 g, were purchased from Charles River Laboratories. Animals were maintained under standard environmental conditions with water and food *ad libitum*. After 1 week of stabilization, animals were randomly assigned to different treatments that lasted for 6 weeks (Fig. 1).

Dose calculation

Genistein aglycone doses are presented as human equivalent daily doses (HEDs) according to the following formula: (human dose in mg/m^2) = (km) × (dose in mg/kg) where human m^2 for a woman of 60 kg (considered as a medium weight from our cohort of post-menopausal women) is 1.62 and km is the conversion factor (for a rat of 250 g is 7.0) as previously stated (Freireich et al. 1966). To calculate Ca and D_3 doses we referred to the normal intake rat standard diets which contained 1260 IU of D_3 per kg of chow and 9163 mg of calcium per kg of chow. A daily intake of 20 g of chow was considered the standard for each animal of the diet yielding 183.26 mg/day of calcium and 25.2 IU/day of D_3 per animal per day. Considering that Marini et al. (2007, 2008a) supplemented tablets with half of the normal recommended intake for post-menopausal women, 50% more (with respect to standard dietary intake) of Ca and D_3 was added to the rat normal intake. GEN HED 27 mg/day for each rat received 0.595 mg/day/ip of GEN (2.38 mg/kg); GEN HED 54 mg/day for each rat received 1.19 mg/day/ip of GEN (4.76 mg/kg); GEN HED 200 mg/day for each rat received 4.40 mg/day/ip of GEN (17.63 mg/kg); GEN HED 500 mg/day for each rat received 11.02 mg/day/ip of GEN (44.09 mg/kg); GEN HED 1000 mg/day for each rat received 22.04 mg/day/ip of GEN (88.18 mg/kg). Genistein doses were adjusted weekly according to body weight. Each rat received an additional 91.63 mg/day (366.52 mg/kg) of Ca; vitamin D_3 daily dose given by oral gavages in combination with Ca. Each rat received 12.6 IU/day (50.4 IU/kg).

Treatments

Both OVX and SHAM-OVX animals were treated in one of the following ways: (1) no treatment; (2) Ca and D_3; (3) Ca and D_3 in combination with 27 mg/day HED GEN; (4) Ca and D_3 in combination with 54 mg/day HED GEN; (5) Ca and D_3 in combination with 200 mg/day HED GEN; (6) Ca and D_3 in combination with 500 mg/day HED GEN; (6) Ca and D_3 in combination with 1000 mg/day HED GEN. To test the effect of GEN alone, OVX animals were also treated in the following manner: (1) 27 mg/day HED GEN; (2) 54 mg/day HED GEN; (3) 200 mg/day HED GEN; (4) 500 mg/day HED GEN; and (5) 1000 mg/day HED GEN.

At the end of the treatment blood was drawn for ELISA then animals were sacrificed, uteri were weighed and femurs were removed for breaking strength measurements and histological examination.

Bone mineral density

Bone mineral density of the right femoral head was measured using dual-energy X-ray absorptiometry (Hologic QDR-4500A, Waltham, MA). For basal (day 1) and final (day 42) measurements, animals were kept anesthetized with sodium pentobarbital (50 mg/kg/ip), as previously described (Bitto et al. 2008, 2009a,b).

Biochemical analysis

Animals were sacrificed under general anesthesia (pentobarbital 50 mg/kg/ip) after blood collection by cardiac puncture. Blood was centrifuged and serum stored immediately at −20 °C for analysis. Sera were evaluated in duplicate by commercially available ELISA kits for the following: bone-alkaline phosphatase (b-ALP; IDS Ltd., UK), collagen C-telopeptide (CTX, Nordic Bioscience Diagnostics, Nordic, Herlev, Denmark), osteoprotegerin (OPG, IDS Ltd.) and soluble receptor activator of NFκB ligand (sRANKL; IDS Ltd.).

Femur breaking strength

Immediately after death, the maximum load (breaking strength) tolerated by excised femurs, expressed in Newton (N), was measured on coded samples by using a calibrated tensometer (Sans, China), as previously stated (Bitto et al. 2008, 2009a,b).

Histology

Histological analysis was performed by an investigator who was blinded to the treatment groups. Femurs were removed, fixed in 10% neutral buffered formalin, placed in decalcifying solution for about 24 h at 37 °C, dehydrated in 95% (v/v) ethanol and then embedded in paraffin. Three, 5-μm-thick paraffin-embedded horizontal bone sections were cut from the proximal end of the diaphysis and stained with haematoxylin and eosin. Femoral heads, the area between hip-joint cartilage and metaphyseal cartilage, were scored for general bone quality and trabecular density according to the scoring system in Fig. 4B and according to published reports (Bitto et al. 2008, 2009a,b).

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