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Soy Affects Trabecular Microarchitecture and Favorably Alters Select Bone-Specific Gene Expressions in a Male Rat Model of Osteoporosis

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Abstract. We have recently reported that soy isoflavones particularly when provided in the context of soy protein are capable of preventing loss of bone mineral density due to orchidectomy in F344 rats. We hypothesize, that soy isoflavones also exert beneficial effects on bone microstructural properties, in part, by enhancing bone formation. Therefore, in the present study, we examined the dose-dependent effects of soy isoflavones on femoral bone microarchitectural properties and select bone-specific gene expressions in the same rat model. Seventy-two, 13-month old rats were either orchidectomized (ORX; 5 groups) or sham-operated (Sham; 1 group) and immediately placed on dietary treatments for 180 days. Four of the ORX groups were fed either casein- or soy protein-based diets each with one of two doses of isoflavones either 600 or 1200 mg/kg diet. Rats in the remaining ORX control and Sham groups were fed a control casein-based diet. Soy protein at the high isoflavone dose, and to a lesser extent with the lower dose, reduced the magnitude of the ORX-induced decreases in trabecular bone volume (BV/TV) and trabecular number (Th.N) and increase in trabecular separation (Tb.Sp) at the femoral neck site. These modulations of trabecular microstructural properties by isoflavones may be due to increased mRNA levels of alkaline phosphatase (ALP), collagen type I (COL), and osteocalcin (OC), which are associated with enhanced bone formation. These findings confirm our earlier observations that the modest bone protective effects of soy isoflavones are due to increased rate of bone formation.

Key words: Orchidectomy — Isoflavone — Microarchitecture — Northern Blot

Osteoporosis-related fractures are common among elderly women, however, the risk also increases in men due to aging with one-fourth to one-fifth of all osteoporosis-related fractures occurring in men [1, 2]. Gehlbach et al. [1] have reported that the medical costs of osteoporosis-related hip factures in the US exceed $5.5 billion annually in men older than 45 years [1]. Mortality rate after hip fracture has been reported to be twice as high in men compared to age-matched women [2]. Hospital charges due to hip fractures are also more costly in men than women because of longer average length of stay [1]. For a number of years bone mineral density (BMD) has been used to predict fracture risk [3, 4]. However, Melton and his colleagues [4] estimate that low bone mass might attribute to 30 to 85% of hip fractures in American men older than 45 year of age, indicating that BMD alone may not be the sole predictor of fractures. Thus, it is necessary to measure not only bone mass but also bone structural qualities, i.e. microarchitecture, for the prediction of fractures.

The loss of bone mass and the deterioration of bone microstructure have been linked to an imbalance between bone formation and bone resorption [5, 6]. For bone formation, mature osteoblasts synthesize bone matrix proteins including bone-specific alkaline phosphatase (ALP), collagen type I (COL), osteocalcin (OC), osteonectin (ON), and osteopontin (OP) [7]. As a part of the process of bone resorption, mature osteoclasts synthesize tartrate-resistant acid phosphatase (TRAP) which is released with degradation products of bone matrix [8].

Our earlier report [9] indicated that soy protein with isoflavones exerts modest bone protective effects in an aged orchidectomized (ORX) rat model of osteoporosis. However, to our knowledge there are no reports on the effects of soy protein with soy isoflavones on bone microarchitectural properties of male rats. Moreover, the mechanism by which soy protein with isoflavones could modulate bone mass and microstructure in preventing male osteoporosis has not been elucidated. Therefore, the purpose of the present study was to examine the dose-dependent effects of isoflavones alone or with soy protein on trabecular and cortical microarchitectural properties of the femur using microcomputerized tomography (μCT). Additionally, the effects on select mRNA expression levels of proteins indicative of bone formation and bone resorption using Northern blot techniques were explored.

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Materials and Methods

Animals and Diets

Seventy-two male Fisher 344 rats (National Institute on Aging, Bethesda, MD), aged 13 months, were used in this study. Rats were either sham-operated (Sham; 1 group) or orchidectomized (ORX; 5 groups) with 12 rats per group. Rats in the Sham and one ORX groups were fed a casein-based control diet (AIN-93M) and the remaining four ORX groups were fed one of the two doses of isoflavones in a casein-based diet (Iso1: 600 or Iso2: 1200 mg isoflavones/kg diet) or a soy protein-based diet (Soy: 600 and Soy+: 1200 mg isoflavones/kg diet) for 180 days. Rats were pair-fed to the average food intake of the group with the least food intake and adjustments were made every three days. Rats had free access to deionized water and were housed in an environmentally controlled animal laboratory. At the end of treatment period, rats were necropsied and right and left femurs were collected for analyses. Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee at Oklahoma State University were strictly followed.

Bone Area, Mineral Content (BMC), and BMD Aassessment

The right femurs were scanned using dual energy x-ray absorptiometry (DXA, QDR-4500C, Waltham, MA, USA) equipped with appropriate software for assessing bone area, BMC, and BMD in small animals as described elsewhere [9].

X-ray Microcomputed Tomography (μCT) Aanalysis

The treatment effects on trabecular structure of the femoral neck were evaluated using a μCT (μCT 40, Scanco Medical, Switzerland). For analyzing the femoral neck, the femur was cut below the intertrochanteric region and the head of the femur was placed vertically in the sample holder. The neck region was then scanned from the lateral border of the femoral head to include the trochanteric fossa. This region (16 μm/slice) was obtained from each femur using 1024×1024 matrix resulting in an isotropic voxel resolution of 22 μm^3 [10]. An integration time of 70 milliseconds per projection was used, with a rotational step of 0.36 degrees resulting in a total acquisition time of 150 minutes/sample. The volume of interest (VOI) was selected to include the entire trabecular region of the femoral neck. Bone morphometric parameters including bone volume over total volume (BV/TV), trabecular number (Tb.N), separation (Tb.Sp), thickness (Tb.Th), connective density (Conn.D), and structure model index (SMI) were obtained by analyzing the VOI.

For the cortical bone analysis, the midpoint of the femoral neck were evaluated. The regions of interest were selected and 45 slices were scanned, and semiautomated contours were drawn to include the cortical region of the bone. Cortical thickness (Co.Th) and porosity (Co.P) were analyzed to assess the effects of treatments on cortical bone.

RNA Extraction and Northern Blot Analysis

The left distal femurs were pulverized using Spex 6700 freezer mill (NJ, USA) and total RNA was extracted by the modified method of Chomczynski and Sacchi [11] and quantified by UV absorbance. The yield of total RNA was approximately 1.5 μg/mg of bone.

For Northern blot analysis, 10 μg of total RNA of each sample was denatured by incubation for 1 hour at 50°C in a solution of 0.01 mole/L of sodium phosphate buffer (pH 7.0), 50% of dimethyl sulfoxide, 6.7% of deionized glyoxal, and 0.3% of RNA dye. All loaded RNA samples were electrophoretically fractionated on 1% agarose gel with 0.01 mole/L of sodium phosphate buffer at 80V for 3 hours. After electrophoresis, the RNA in the gel was transferred overnight to membrane (Hybond™-N+, Amersham Biosciences, NJ, USA) by capillary action in 20X SSC (3 mole/L of sodium chloride and 0.3 mole/L of sodium citrate, pH 7.0). Transferred total RNAs to the membrane were stained with 30 μg/ml of methylene blue and visualized using a VersaDoc™ imaging system (Bio-Rad, Hercules, CA, USA) to confirm the integrity of RNA and ensure equal loading. After autocross-linking and washing with 3X SSC and 0.1% SDS for 15 mins, the membrane was prehybridized for 1 h at 65°C in Rapid-hyb buffer (Amersham Biosciences, NJ, USA) and for another 1 hour at 65°C in Rapid-hyb buffer with 1 mg of sonicated salmon sperm DNA (Stratagene, CA, USA) to remove non-specific background. Hybridizations were separately performed with [^{32}P]-labeled cDNA probes of ALP, COL, OC, ON, OP, TRAP, and 18S at 65°C for 2 hr. [^{32}P]-Labeled cDNAs were prepared with [^{32}P]dCTP (Amersham Biosciences, NJ, USA) by Megaprimer DNA labeling system, (Amersham Biosciences, NJ, USA) and purified by Nick™ column (Amersham Biosciences, NJ, USA). The blots were washed twice with 3X SSC and 0.1% SDS at 65°C for 15 min per washing. The membranes were placed on a phosphor screen (Bio-Rad, Hercules, CA, USA) in cassette and kept at room temperature overnight. The levels of ALP, COL, OC, ON, OP, TRAP, and 18S mRNAs were detected using Molecular Image® FX (Bio-Rad, Hercules, CA, USA) and quantified using a Quantity One® soft program (Bio-Rad, Hercules, CA, USA). cDNA of 18S ribosomal RNA (Ambion, Austin, TX, USA) was used as an internal control to normalize gene expression. cDNA probes of ALP (2.5kb), COL (1.6kb), OC (0.5kb), ON (1.2kb), and OP (1.2kb) were generously provided by Dr. Tobias (University of Bristol Division of Medicine, UK) [12].

TRAP cDNA probe was obtained using the methods of Battaglino et al. [13]. Briefly, total RNA was extracted from RAW 264.7 mouse monocytes which were incubated with 50 ng/ml of receptor activator of nuclear factor kappa B ligand (RANKL) for 48 hours. Two micrograms of total RNA was denatured by heating at 65°C and reverse transcribed in presence of random hexamers (pdN_6; 100 pmol/L; Pharmacia, Piscataway, NJ, USA), dATP, dTTP, dCTP, and dGTP (dNTPs; 250 μmol/L; Pharmacia, Piscataway, NJ, USA), RNase inhibitor (20 U per reaction; Promega, Madison, WI, USA), and reverse transcriptase (Superscript™ 200 U per reaction; Gibco BRL, Grand Island, NY, USA) at 37°C for 75 min. The reaction was stopped by heating at 70°C. Aliquots of reverse-transcribed cDNA (2 μl) were denatured by heating at 95°C and subjected to polymerase chain-reaction in the presence of 75 pmol TRAP primers, 1.5 mmol/L of $MgCl_2$, 25 μmol/L of dNTPs, and Amplitaq™ DNA polymerase (2 U per reaction; Perkin-Elmer, Foster City, CA, USA) in 50 μl reaction. In the primer-pair of TRAP (size, 465 bp), sense was 5′-ACACAGTGATGCTGTGTGGCAACTC-3′ and antisense was 5′-CCAGAGGCTTCCACATATATGATGG-3′. cDNA of TRAP were then amplified by 94°C denaturation for 30s, 58°C annealing for 30s, and 72°C elongation for 45s with 30 cycles and then were resolved on 1.2% agarose-TAE [Tris-acetate (40 mmol/L), EDTA (1 mmol/L)] gels. After electrophoresis, the gels were stained with ethidium bromide for visualization using VersaDoc™ imaging system (Bio-Rad, Hercules, CA, USA) to confirm the size of cDNA of TRAP. cDNA of TRAP cut from the gel was used for the probes of Northern blot.

Statistical Analysis

Analysis of variance (ANOVA) and least square means were calculated using PROC GLM groups via SAS (version 8.2, SAS Institute, Cary, NC, USA). When ANOVA indicated any significant difference among the means, Fisher's Protected Least Significant Difference Procedure was used to determine which means were significantly different ($P < 0.05$).

Table 1. Effects of orchidectomy (ORX) and isoflavones in conjunction with soy protein or casein on femoral DXA parameters in aged male rats

Measures	Sham	ORX	Iso1*	Iso2**	Soy*	Soy+**
Area (cm^2)	2.21 ± 0.02[a]	2.15 ± 0.02[b]	2.11 ± 0.02[b]	2.16 ± 0.02[ab]	2.15 ± 0.02[b]	2.14 ± 0.02[b]
BMC (g)	0.529 ± 0.008[a]	0.453 ± 0.007[b]	0.453 ± 0.008[b]	0.466 ± 0.008[b]	0.466 ± 0.009[b]	0.462 ± 0.009[b]
BMD (g/cm^2)	0.239 ± 0.003[a]	0.210 ± 0.002[b]	0.214 ± 0.003[b]	0.216 ± 0.003[b]	0.217 ± 0.003[b]	0.216 ± 0.003[b]

Values are mean ± SE, $n = 12$. In each row, means that do not share the same letters are different at $P < 0.05$
* Isoflavone content is 600 mg/kg diet; ** Isoflavone content is 1200 mg/kg diet. Protein source is either casein or soy protein for the Iso and Soy, respectively
BMC, bone mineral content; BMD, bone mineral density

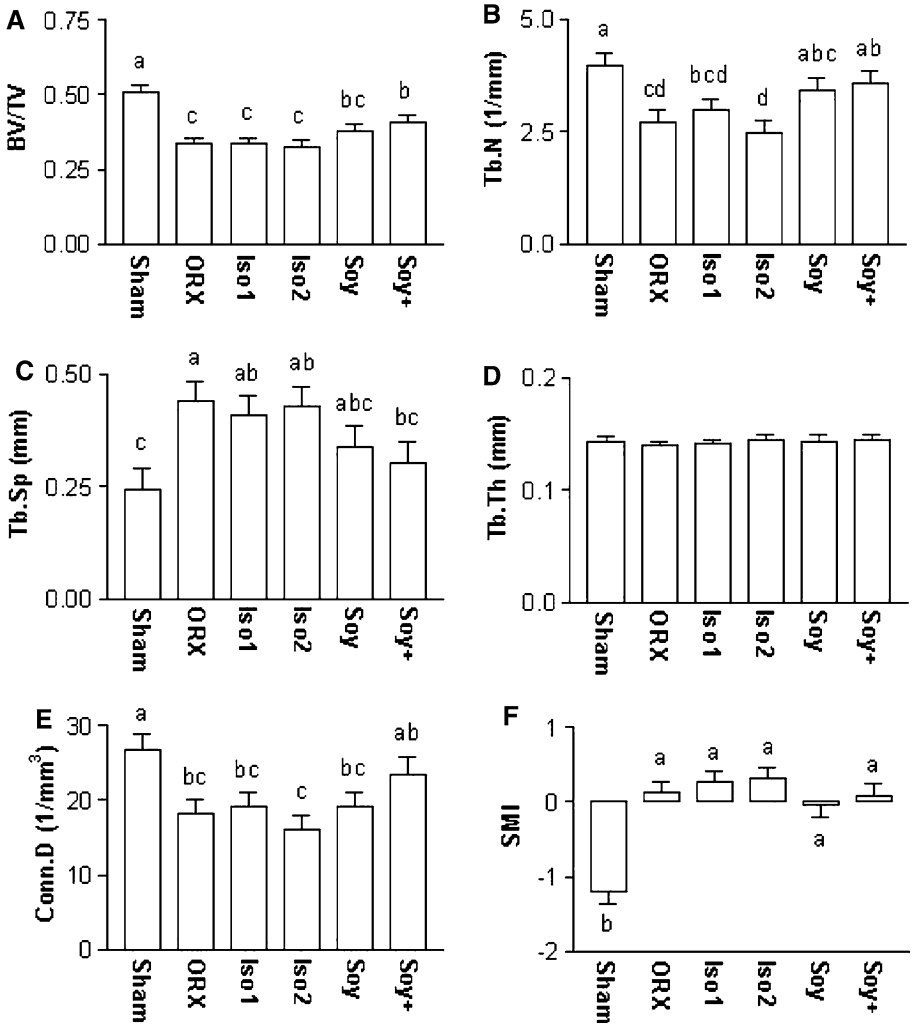

Fig. 1. Effects of orchidectomy (ORX) and treatment on femoral neck trabeculae in aged male rats. Microarchitectural parameters were (A) bone volume as a ratio of total volume (BV/TV), (B) trabecular number (Tb.N), (C) trabecular separation (Tb.Sp), (D) trabecular thickness (Tb.Th), (E) connectivity density (Conn.D), and (F) structural model index (SMI). Bars represent mean ± SE; $n = 8$ rats per group. Bars that share the same letter are not significantly different ($P > 0.05$).

Results

Bone Area, BMC, and BMD

The effects of orchidectomy and isoflavone treatment on femoral area, BMC, and BMD are presented in Table 1. Orchidectomy significantly ($P < 0.05$) reduced bone area, BMC, and BMD by 2.7%, 14.4%, 12.1%, respectively, in comparison to the Sham group. None of the dietary treatments prevented these losses with the exception of the modest effect of Iso2 on bone area.

Trabecula and Cortical Microarchitectures

Femoral neck microarchitectural parameters of trabecular bone are shown in Fig. 1. Orchidectomy caused significant reduction in BV/TV (34.5%), Tb.N (31.3%), and connectivity density (Conn.D; 32.4%) and an increase in Tb.Sp (78.9%) compared to Sham. The deterioration of femoral neck trabeculae due to orchidectomy was lessened by the soy protein diet with the high-dose of isoflavones (Soy+) as evidenced by greater BV/TV (22.5%) and Tb.N (31.6%) and lower

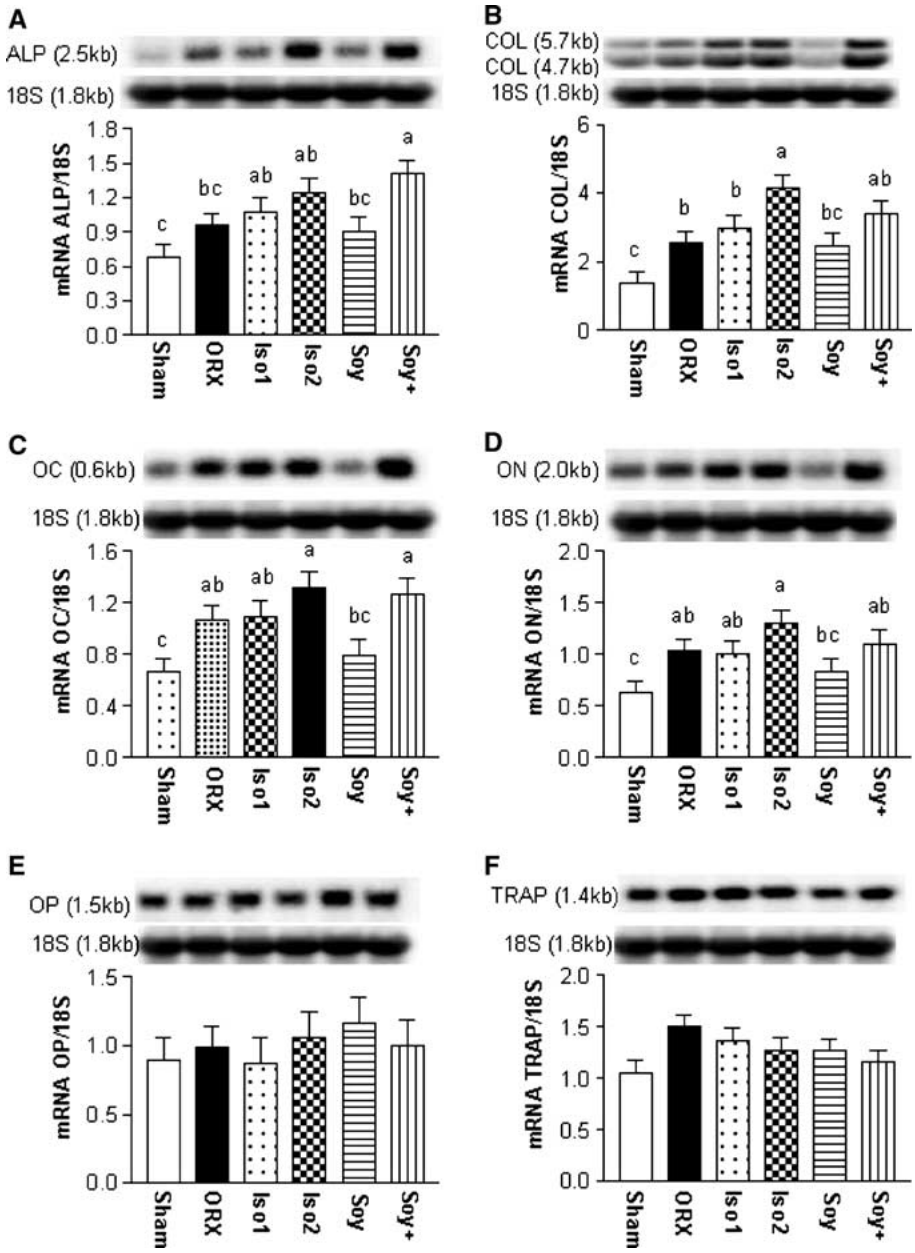

Fig. 2. Effects of orchidectomy (ORX) and treatment on femoral mRNA levels of (A) ALP, (B) COL, (C) OC, (D) ON, (E) OP, and (F) TRAP in aged male rats. A representative blot from $n = 4$ is presented and the same membrane was stripped and rehybridized with 18S RNA as an internal control to normalize gene expression. Bars that share the same letter are not significantly different ($P > 0.05$).

Tb.Sp (31.4%) compared to the ORX control group. Rats fed the soy protein diet containing 600 mg isoflavones (Soy) had a slightly higher BV/TV and Tb.N and lower Tb.Sp compared to ORX control rats. Casein based diets, irrespective of the dose of isoflavones had no such effects on these properties. As was the case for the proximal and distal femur sites, Tb.Th was neither changed by ORX nor any of the treatments at the femoral neck. The SMI values of neck of femur were −1.190 and 0.120 in Sham and ORX, respectively. Treated ORX rats had SMI values similar to those of ORX control.

Cortical thickness and porosity of the femoral neck were not affected by either orchidectomy or diet (data not shown).

Messenger RNA Levels of Select Bone Matrix Proteins and TRAP Using Northern Blot

To explore the role of soy isoflavone on bone at the molecular level, mRNA levels of select bone matrix proteins and TRAP were assessed. ORX increased the gene expressions of ALP, COL, OC, and ON by 41.2%, 86.0%, 61.3%, and 64.1%, respectively (Fig. 2A–2D) compared to Sham group. This ORX-induced elevated gene expressions were further upregulated by isoflavone treatments, albeit with varying degrees. ALP gene expressions were increased by Iso1, Iso2, and Soy+ by 12.7%, 30.3%, and 46.9%, respectively in comparison with ORX animals (Fig. 2A). There were 61.3% and 33.0% increases in COL mRNA levels compared to

ORX in groups that received Iso2 and Soy+ (Fig. 2B), respectively. Similarly, mRNA levels of OC were upregulated by Iso2 (23.0%) and Soy+ (18.4%) (Fig. 2C). Nonetheless, mRNA levels of ON were elevated above that of ORX by Iso2 (25.8%) only (Fig. 2D). However, OP and TRAP gene expression were not significantly altered by either ORX or treatments (Fig. 2E and 2F).

Discussion

As more men and women are living longer, the prevalence of osteoporosis and its related fractures is also increasing [1, 14]. In adult men, hypogonadism is considered a risk factor accounting for 5% to 33% of osteoporosis [15]. This percentage is believed to be higher in the elderly men because available gonadal hormone levels are decreased with aging [5, 6]. In fact, compromised bone status has been observed with gonadal hormone deficiency in elderly men [5, 6] as well as aging male animal models [9, 16].

One of the possible dietary interventions to minimize the effects of gonadal hormone deficiency on bone is the inclusion of soy protein and its isoflavones in the diet. We have previously shown that soy protein and its isoflavones are able to prevent bone loss as well as rebuild bone mass after its loss due to gonadal hormone deficiency in female rats [17, 18]. Additionally, we have recently reported that soy protein and its isoflavones exert protective effects on whole body BMD in aged, orchidectomized male rats [9]. However, in the present study, soy protein and its isoflavones were unable to protect against orchidectomy-induced femoral bone loss [9]. Although, in our experience, the doses of isoflavones as well as the treatment duration in the present study are not below adequate, our results are in contrast with those observed in ovariectomized rats [17, 18] and aged intact male rats [19]. This lack of agreement between our previous studies and the present study may be attributed to the differences in age and gender between the studies (older male rats versus younger female rat models) as well as hormonal status (orchidectomy in addition to aging versus intact aged rats).

In addition to BMD, bone microarchitecture plays an important role in bone quality and resistance to bone fracture. Bone microarchitecture has been reported to deteriorate with age in both men [20] and male animal models of osteoporosis [21]. In the present study, soy protein with a high dose of isoflavones showed significantly favorable effects on the trabecular region of femoral neck by preventing orchidectomy-induced reduction in BV/TV and Tb.N along with an increase in Tb.Sp. These parameters were obtained using 3-dimensional (D) μCT and agree with our previous findings of similar parameters obtained by 2-D histomorphometry [9]. Regarding cortical microstructure in the present study, neither cortical thickness nor porosity were altered by any of the surgical or dietary manipulations. This lack of response of cortical microstructure compared to trabecular microstructure may be due to the slow remodeling processes of cortical bone [22] and low levels of Haversian remodeling system in rats [23].

In this study, the protective effect of soy protein with a high dose of isoflavones on trabecular microarchitecture may be a consequence of effects on mRNA levels of bone matrix proteins such as ALP, COL, and OC. Isoflavones have been reported to increase the protein synthesis of COL and OC by inducing osteoblast differentiation [24, 25]. Another bone matrix protein OP, however, was not affected by either ORX or the dietary treatments. Osteopontin has been shown to inhibit hydroxyapatite growth and interrupt mineral apposition [26]. Osteopontin has also been reported to suppress the proliferation of osteoblasts and the synthesis of OC [26]. Additionally, OP may play an important role in osteoclast lineage by increasing number of TRAP positive-multinucleated cells which lead to elevation of membrane attachment and resorption area [27]. Therefore, the lack of the effects of isoflavones on this protein should be considered a positive event.

Since the synthesis of bone matrix proteins, e.g., COL and OC is viewed to be mediated by Runt-related transcription factor (Runx)2 [7], soy isoflavones might stimulate the production of Runx2. This is supported by studies that have demonstrated the ability of soy isoflavones to increase the production of insulin-like growth factor-I (IGF-I), transforming growth factor beta (TGFβ), and bone morphogenic protein (BMP) [18, 25, 28–30]. These osteogenic growth factors have been shown to activate Runx2 in the upstream of bone matrix formation pathway [7, 31]. We have reported that soy protein with normal isoflavone content induces the expression of IGF-I as indicated by higher femoral mRNA levels in ovariectomized rats [18]. The findings of human studies [29, 30] also suggest that soy isoflavones increase IGF-I. For instance, soy protein supplementation containing 80 mg isoflavones has been shown to significantly increase serum IGF-I levels in both postmenopausal women [29] and men [30]. Jia et al. [25] have reported the upregulation of BMP in osteoblast cells isolated from calvarias of newborn Wistar rats in the presence of the soy isoflavone, daidzein. Genistein has been shown to cause overexpression of TGFβ1 as shown in bone marrow cells aspirated from iliac crest of healthy perimenopausal women [28]. In brief, the mechanism(s) of action of isoflavones on production of bone matrix components may be mediated through the upregulation of Runx2 under the control of IGF-I, TGFβ, and/or BMP. However, there is a need for further evaluation of the bone forming role of isoflavones in relation to osteogenic growth factors such as IGF-I, BMP, TGFβ and their influence on transcription factor Runx2.

Another factor to consider in skeletal health is bone resorption. In this study, we found that ORX increased mRNA levels of TRAP, a marker of osteoclast, by 42.8 % compared with Sham. This increase of mRNA TRAP by ORX was reduced in Iso1, Iso2, Soy, and Soy+ by 29.3%, 50.7%, 52.9%, and 76.2%, respectively. Although these data tended to increase ($P = 0.0834$), they did not reach statistical significance.

Our earlier report [9] and those of other investigators [32, 33] demonstrate that urinary deoxypyridinoline (Dpd), a biomarker of bone resorption is significantly increased in both animal models and men with low testosterone and/or estrogen levels. Since Dpd is the final product of bone matrix degradation by osteoclasts, its elevated levels may indicate increased activity and/or number of osteoclasts. This notion is given credence from the findings of Huber and colleagues [34] that gonadal hormone deficiency increases the number of hematopoietic precursors capable of forming osteoclasts *in vitro*. In terms of dietary treatments soy isoflavones, genistein and daidzein, have been shown to effectively lower the number of mature osteoclasts by inducing osteoclast apoptosis [35, 36] and suppressing osteoclastogenesis [28, 37]. The anti-bone resorptive properties of total or individual isoflavones have also been reported in both animal [38] and human studies [29].

In conclusion, soy protein and its isoflavones appear to exert pronounced positive effects on bone microstructure of femoral neck in an aged rat model of osteoporosis despite a lack of apparent effect on the mineral density of that bone. These favorable effects could be through the modulation of the rate of bone formation- and resorption-related gene expressions. Soy is, at present, known for its benefits to postmenopausal women; the results of this study warrant examining the bone protective effects of soy protein and its isoflavones in men at risk for osteoporosis.

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