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# BENEFICIAL EFFECTS OF SOYBEAN PROTEIN AND ISOFLAVONE EXTRACT SUPPLEMENTATION ON BONE DENSITY AND PLASMA LIPIDS IN FEMALES RATS

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The main purpose of this study was to evaluate the effect of supplementation of a soybean protein concentrate (20%) on bone mass and plasma lipid profile of adult female rats as well as to test the effectiveness of a soybean isoflavonoid extract (0.5%) in preventing bone loss due to ovariectomy. Results showed that isoflavones separated from the soybean extract contain a higher amount of isoflavonoid glycosides (daidzin and genestin) than the aglycones (diadzein and genistein). Data obtained revealed that both plasma total cholesterol and high density lipoprotein cholesterol(HDL-C) of rats were not significantly changed due to the presence of the soybean protein concentrate (20%) in the diet. On the other hand, there was a significant decrease in plasma low density lipoprotein cholesterol (LDL-C) in soy protein group. Lipid peroxidation was significantly reduced due to feeding soybean protein concentrate (20%) of the diet (approximately 30% reduction). Rats fed the soybean protein concentrate (20%) diet had significantly higher mean bone densities (10% increase) of right tibia as compared with control (protein-based diet, 20%). The ovariectomized rats fed a diet containing the isoflavonoid extract had significantly higher mean bone densities (15% increase) and bone calcium of right tibia than ovariectomized rats fed the control diet. In conclusion, the present study demonstrated that soybean protein and isoflavones could have the potential to reduce the risks of postmenopausal osteoporosis and cardiovascular diseases in such women.

## INTRODUCTION

Osteoporosis that is associated with ovarian hormone deficiency following menopause (postmenopausal osteoporosis) is by far the most common cause of age-related bone loss. A sharp decrease in ovarian estrogen production is the predominant cause of rapid horomone-related bone loss during the first decade after the menopause [Gruber *et al*., 1984]. Traditional therapies for postmenopausal osteoporosis have emphasized agents that inhibit bone resorption such as estrogen [Centrella & Canals, 1985], calcitonin [Canalis *et al*., 1988; Rudaman *et al*., 1981] and bisphosphonates [Bennet *et al*., 1984]. Although the most effective method to reduce the rate of postmenopausal bone loss is estrogen replacement therapy, it may be accompanied by side effects [Genant *et al*., 1989] and is thus recommended only for women who are at high risk of osteoporosis and who have no contraindications. Recent advances in bone biology have led researchers to suggest using a combination of anti-resorptive agents, such as estrogen, and formation-stimulating agents, such as growth hormone, toward a cure for osteoporosis [Turner, 1991]. However, the potential bone-forming agents available today either may have serious side effects (*e.g.* growth hormone, anabolic steroids), may not improve bone quality, or may not decrease susceptibility to fracture (*e.g.* sodium fluoride). Therefore, it would be most helpful to discover a naturally occurring substance that minimizes bone loss in postmenopausal women, thus decreasing the necessity for drug therapy.

Some reports have indicated that ipriflavone, a synthetic flavonoid derivative [Agnusdei *et al*., 1989], is effective in preserving bone mass in several models of experimental osteoporosis [Benvenuti *et al*., 1991; Yamazaki, 1986]. These promising reports regarding the beneficial effects of ipriflavone have led us to hypothesize that natural food sources with high concentrations of isoflavonoids might be equally effective in modulating bone mass due to ovarian hormone deficiency. Genistein is an isoflavone abundantly presents in soybean, and shows a structural similarity to estrogen, which suggests that it may act as a phytoestrogen. Recently, genisten has been shown to have a stimulatory effect on bone formation and an inhibitory effect on osteoclastic bone resorption. In addition, genistein has been reported to be as estrogen in maintaining bone mass in ovariectomized (OVX) animals [Morita *et al*., 1999]. The isoflavones found predominately in soybeans and soybean products are pharmacologically and structurally similar to the synthetic phytoestrogens that have been shown to be effective in preventing or reducing bone loss.

The main purpose of this study was to evaluate to what extent feeding a diet containing soybean protein concentrate may effect bone mass, plasma lipid profile and lipid peroxi-

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dation of adults female rats as well as to test the effectiveness of feeding a diet containing soybean isoflavonoids extract in preventing bone loss due to ovariectomy in rats.

## MATERIALS AND METHODS

**Extraction and determination of soybean isoflavone.** Two grams of soybean powder were refluxed for 1 h in 20 mL of 80/100 of methanol in a boiling water bath. The extract was centrifuged for 20 min at 1500 g at 5°C. The supernatant was obtained and evaporated to dryness using a rotary evaporator. After evaporation, the dried extract was dissolved in 5 mL of 80/100 of methanol. An aliquot was filtrated though a membrane filter (pore size of 0.4 mm) before analysis by HPLC [Kitada *et al*., 1986].

**Diet.** The composition of the basal diet used in the present study was described in Table 1.

**Animals.** The experiments were conducted on 30 female albino rats weighing 120±2.1 g (aged 2 months). The rats were housed in individual stainless steel screen bottom cages. The rats were fed a basal diet for one week for adaptation, water and diet were available *ad-libitum* in experiment I and 3 groups from experiment Π.

**Experiment I.** The rats were divided into 2 groups, each of 6 rats. The first group was fed a basal diet containing 20% protein as casein. The second group was fed a diet containing 20% soybean protein concentrate. After 8 weeks the animals were fasted overnight, blood samples were withdrawn by a fine capillary glass tube from the orbital plexus vein. Blood was collected in heparin-containing tube and centrifuged at 3000 rpm for 15 min; plasma was separated and stored at -20°C until analysis. Right tibia was excised.

**Experiment Π.** Rats were divided into 3 groups (each of 6 rats). The rats in groups 2 and 3 were ovariectomized (OVX). Groups 1 and 2 were fed a basal diet. Groups 3 was fed s basal diet containing a soybean isoflavonoid extract at a concentration of 0.5% per 100 g diet. The experiment was continued for 3 month. At the end of the experiment, the animals was killed and right tibia was excised.

Plasma total cholesterol, triglycerides, HDL-C and LDL-C were determined using the respective enzymatic methods: Allain *et al*. [1974], Fossati & Prencipe [1982], Arcol [1989], and Sharf *et al*. [1985]. Malondialdehyde (MDA) was determined with the colorimetric method [Ushyama & Mihara, 1978].

**Measurement of bone mineral density (BMD).** The bone mineral density of the tibial proximal metaphysic of rats from both exp. I and II was measured by dual-energy x-ray bone densitometer (DEXA) [Goda *et al*., 1995].

**Determination of tibial calcium.** Tibial bone of rats of both exp. I and II was cleaned and dried in an oven at 105°C and accurately weighed to a constant weight. After drying, tibias were dry ashed for 10 h at 55°C [Peterson *et al*., 1992]. Ash weight was recorded and dissolved in 2 mL of 0.3 mol/L HCl [Pallout *et al*., 1994] prior to analysis of Ca by atomic absorption (Perkin Elmer AAnazlyst model 300 atomic absorption spectrometry).

**Quality control**. A Standard Reference Material (SRM 1846) consisting of an infant formulae was obtained from the National Institute of Standards and Technology (Gaithersburg, Maryland, USA) and was treated in a similar manner to the unknown bone and food samples. An external standard procedure was adopted throughout the course of the study. The analysis of the SRM 1846 under the present condition gave recoveries of 95±2.92, % for Ca.

**Statistical analysis.** Results were subjected to Student's ttest and ANOVA analysis according to the method of Statgraphics Program Statistical Graphic System Version 2.6 [1987].

## RESULTS

### Isoflavonoid content of soybean

The isofalvonoid content of soybean extract is shown in Table 2. The results showed that soybean extract contained a higher amount of isofalvonoid glycosides (daidzin and genistin) than the aglycones (daidzein and genistein). The weight percentage of aglycone to total isoflavonoid in soybean was 7.573%.

TABLE 1. Composition of diets for rats (g/100 g diet).

| Diet ingredient | Experiment 1 | | Experiment 2 | | |
|---|---|---|---|---|---|
| | Basal diet (Group 1) | Soy protein concentrate (Group 2) | Basal diet (Group 1) | OVX (Group 2) | OVX +Soy isoflavone extract (Group 3) |
| Casein | 24* | 0.0 | 12 | 12 | 12 |
| Sucrose | 5 | 5 | 5 | 5 | 5 |
| Corn oil | 10 | 10 | 10 | 10 | 10 |
| Minerals | 4 | 4 | 4 | 4 | 4 |
| Cellulose | 4 | 4 | 4 | 4 | 4 |
| Vitamins | 1 | 1 | 1 | 1 | 1 |
| Soy protein | 0.0 | 50* | 0.0 | 0.0 | 0.0 |
| Isoflavone | 0.0 | 0.0 | 0.0 | 0.0 | 52** |
| Starch | 52 | 26 | 64 | 64 | 12 |

OVX : ovariectomized rats; * 50 g soy protein concentrate contains 20% protein; ** dried soy extract contains 900 mg of total isoflavonoid

TABLE 2. Isoflavonoid (mg/100 g) of soy bean (SBM) extracted with methanol.

| Isoflavonoid | Content (mg/100 g) |
|---|---|
| Daidzin | 94.67±1.55 |
| Genistin | 85.71±1.61 |
| Daidzein | 12.18±1.01 |
| Genistein | 2.60±0.13 |
| Total | 195.16±2.20 |

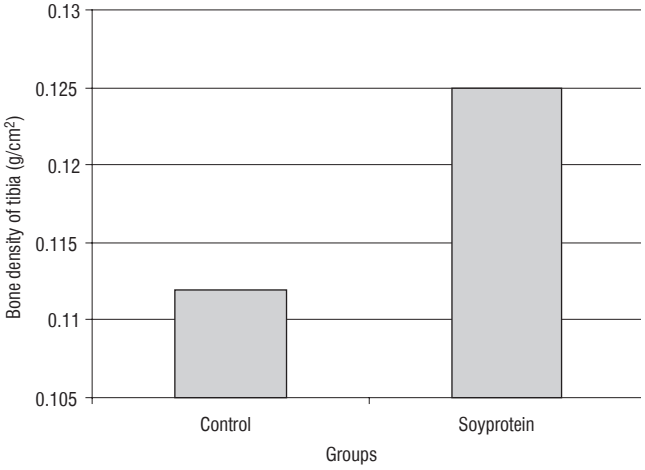


FIGURE 1. Effect soybean protein concentrate intake (20%) on bone mineral density of tibia.

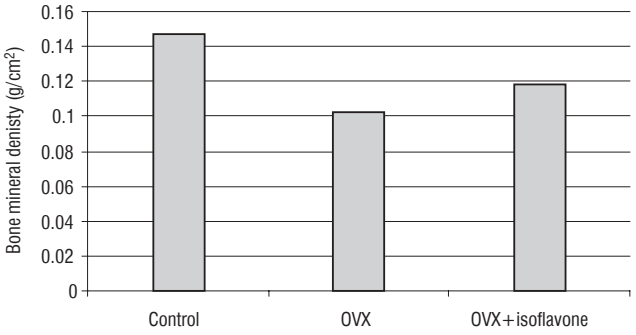


FIGURE 2. Effect of soybean isoflavonoid intake on bone mineral density of tibia.

## Lipid profile

Table 3 showed the results of lipid profile of plasma in control rats and these supplemented with soybean protein concentrate (20% of diet). Data obtained revealed that both plasma total cholesterol and high density lipoprotein cholesterol(HDL-C) did not significantly change due to the presence of soybean protein concentrate (20% of diet). On the other hand, there was a significant decrease in low density lipoprotein cholesterol (LDL-c) in soy protein group. Lipid peroxidation showed significant reduction due to soybean protein concentrate intake since MDA was decreased from 12.17 nmol/mL in the control group to 8.60 nmol/mL in the treated groups (approximately 30% reduction).

## Bone density in tibia

The results in Figure 1 showed that the rats fed the soybean protein concentrate diet had significantly ($p<0.01$) higher mean bone densities (10% increase) of right tibia compared with the control.

The results in Figure 2 showed that ovariectomized rats had significantly ($p<0.001$) lower densities of right tibia compared with the control. The ovariectomized rats (group 3) fed the soybean extracts isoflavonoid diet had significantly ($p<0.01$) higher mean bone densities (15% increase) of right tibia than the control ovariectomized rats (group 2).

## Calcium content in tibia

The results in Table 4 showed that the rats fed the soybean protein concentrate (20%) diet had significantly ($p<0.01$) higher mean bone calcium (60.6 mg) of right tibia as compared with the control (38.09%).

The Ca/Ash percentage revealed that Ca represented approximately 20% of ash in the group supplemented with soybean protein concentrate as the compared to control group (casein-based diet) (Table 4), thus indicating increased mineralization in rats fed the soybean protein concentrate.

The results in Table 5 showed a significant decrease in Ca content of the tibia (34.0±0.91 mg) due to ovariectomy (group 2) as compared with the control (66.61±0.711 mg). Supplementation of soybean isoflavonoid extracts could slow down the reduction of Ca content in ovariectomized

TABLE 3. Effect of soybean protein concentrate intake on lipid profile and lipid peroxidation in plasma of male albino rats.

| Groups | Total cholesterol (mg/dL) | HDL-C (mg/dL) | LDL-C (mg/dL) | MDA (nmol/mL) |
|---|---|---|---|---|
| Control | 109±4.51[a] | 70.72±1.72[a] | 38.43[a]±1.9 | 12.17[a]±0.383 |
| Soybean protein | 103.29±4.02[a] | 72.27±2.3.8[a] | 31.76[b]±1.64 | 8.60[b]±0.288 |

a,b, – the same superscripts in the same column indicate no significant differences at $p<0.05$

TABLE 4. Effect of soybean protein concentrate intake on bone composition of adult female rats.

| Groups | Total tibia (g) | | | | | |
|---|---|---|---|---|---|---|
| | Fresh (g) | Dry weight (g) | Ash (g) | Organic matter (g) | Ca (g) | Ca/ash (%) |
| Control | 0.456±0.04[a] | 0.3901±0.01[a] | 0.1405±0.01[a] | 0.1906±0.01a | 0.0381±0.01[a] | 27.23±1.83[a] |
| Soy protein concentrate | 0.495±0.03[a] | 0.2008±0.01[b] | 0.1308±0.02[b] | 0.2198±0.01[a] | 0.0606±0.02[b] | 46.31±1.44[b] |

a,b, – the same superscripts in the same column indicate no significant differences at $p<0.05$

TABLE 5. Effect of soybean isoflavones intake on bone composition of ovariectomized female rats.

| Groups | Total tibia (g) | | | | | |
|---|---|---|---|---|---|---|
| | Fresh (g) | Dry weight (g) | Ash (g) | Organic matter (g) | Ca (g) | Ca/ash (%) |
| Control | 0.422±0.01[a] | 0.3031±0.01[a] | 0.1408±0.01[a] | 0.1625±0.01a | 0.066±0.007[a] | 46.66[a]±2.41 |
| OVX | 0.371±0.01[a] | 0.2537±0.02[b] | 0.1303±0.01[b] | 0.1308±0.04[a] | 0.034±0.009[b] | 24.73±1.27[b] |
| OVX+ soybean isoflavones | 0.384±0.02[a] | 0.2561±0.02[b] | 0.1290±0.02[b] | 0.1428±0.03[a] | 0.0381±0.002[b] | 29.55±1.21[b] |

a,b, – the same superscripts in the same column indicate no significant differences at $p<0.05$

rats (38.11±0.27 mg). Bone dry weight of ovariectomized rats was significantly lower than that of the control animals (Table 5). Ash content of ovariectomized rats was lower than that of the controls but the decrease was not significant (Table 5). The Ca/Ash percentage revealed that Ca represented approximately 29.55% of ash in ovariectomized group 3 supplemented with soybean isoflavonoid extracts as compared to the ovariectomized group 2. This indicated the increased mineralization in rats fed the soybean isoflavonoid extracts.

## DISCUSSION

In the present work, isoflavones separated from the soybean extract contained a higher amount of isoflavonoid glycosides (Daidzin and genestin) than the aglycones (diadzein and genistein). Genistein is considered the major active isoflavone in soybean. Kishida *et al*. [2000] showed that the isoflavonoid content of soybean was approximately 207 mg/100 g. Farmakalidis & Murphy [1985] reported that variety and environment (location) affect the concentration of daidzin and genistin. Our results were consistent with those of Kishida *et al*. [2000] who showed that weight percentage of aglycone to total isoflavonoid in soybean was 7.573.

Soy protein is a major component of the diet of food and is increasingly important in the human diet. Friedman & Brandon [2001] and Uesugi *et al*. [2002] reported that soybean protein and isoflavone are of potentially beneficial effects on bone metabolism and on serum lipids. The data showed that LDL cholesterol and lipid peroxidation were significantly decreased in the soybean protein concentrate supplemented group. Anthory *et al*. [1995] reported that a soy bean protein diet is rich in phytoestrogens. Hwang *et al*. [2001] showed that phytoestrogens are potent low density lipoprotein antioxidant. Uesugi *et al*. [2002] showed that LDL cholesterol was decreased significantly in the postmenopausal Japanese supplemented with soybean isoflavone. The results in the present study showed that supplementation of soybean protein concentrate (20%) increased both bone density and calcium content in tibia as compared with the group fed the casein-based diet (20%). Horiuchi *et al*. [2001] studied the effect of soy protein intake on bone metabolism in postmenopausal Japanese woman. They suggested that a high soy protein intake is associated with a higher bone mineral density and a lower level of bone resorption. Arjmandi *et al*. [1996] reported that soybean protein (22%) intake prevents bone loss in an ovariectomized rats model of osteoporosis, compared with the casein diet (22%). Omi *et al*. [1994] reported a positive effect of a soybean milk-based diet on the bone density of six week-old female rats. They suggested that this positive effect of a soybean milk-based diet might be due to enhanced intestinal calcium absorption. Although we did not assess intestinal calcium absorption in this study, the enhanced intestinal absorption of calcium along with modulation of parathyroid hormone may be of beneficial effects of soybean protein on calcium absorption, as it has been suggested by Kalu *et al*. [1988]. Additionally, high-protein diets have been linked with increased urinary calcium excretion [Hegsted & Linkswiler, 1981], possibly due to the oxidation of sulfur-containing amino acids. Hypercaiuria is thought to be minimized by plant-based diets, because animal-based diets are higher in sulfur-containing amino acids. Also, isoflavonoids in soybean protein seem to differ from estrogen with respect to uterotrophic activity [Arjmandi *et al*., 1996].

The data in the present study concerning bone density and bone calcium support the observations of other investigators that bone loss due to ovarian hormone deficiency is prevented by intake of a soybean isoflavonoid extract [Arjmadi *et al*., 1996]. Additionally, estrogen suppressed the ovariectomy-induced rise in concentration of biochemical markers of bone turnover, alkaline phosphatase and tartrate-resistant acid phosphatase [Raisz, 1988].

In accordance with pervious evidence [Arjmadi *et al*., 1996], rats in the OVX group had lower densities of the right tibia, the loss of which was prevented in animals receiving the soybean isoflavonoid extract. A number of animal- and clinical studies have investigated the effects of protein or genistien intake on bone loss. In an ovariectomized rat model, the intake of genistein was found to exert modest effects on bone retention [Anderson *et al*., 1995]. Aral *et al*. [2000] estimated the interaction among dietary intakes of isoflavones, plasma concentrations and urinary excretions, bone mineral density and bone formation and resorting biomarkers. The subjects were 41 female volunteers (40–69-year-old; premenopause - 7; postmenopause – 34). They found a significant negative correlation between plasma genistein and urinary deoxypyridinoline. Urinary equol, a metabolite of daidzein, showed a significantly positive correlation with plasma osteocalcin. They suggested that genistein is useful in preventing bone loss, and equol may have a beneficial role on bone formation. Since the plasma and urinary isoflavones are as useful as bone biomarkers, it is hypothesized that a high intake (not excess) of isoflavones reduces the risk of osteoporosis. Parallel results were reported by Ebisawa & Koshihara [2000] who studied effects of dietary isoflavone-extract (IF) from soybean Hypocotyl on bone metabolism in ovariectomized young and old female rats. They suggested that dietary isoflavone has beneficial effects on bone metabolism in ovariectomized young rats, but not in the old rats. Morita *et al*.

[1999] investigated the effect of intake of genistein on the expression of type 1 collagen (COL 1), alkaline phosphatase (AP), osteopontin (OP) and osteocalcin (OC) genes that have been associated with bone formation in mouse osteoblastic cell (Mc3t3-El cells ) and OVX mice. Their results indicated that genistein exhibited estrogenic action in bone of OVX animals without estrogenic action in the uterus. Branca & Valtuena [1998] reported a 5% increase in total body bone mineral content after only 3 months of consuming soy flour (an average of 53 mg isoflavones/day) in early postmenopausal women.

## CONCLUSIONS

The present study demonstrated that soybean protein concentrate (20%) supplementation for 8 weeks had a beneficial effect on bone mass, improved plasma low density lipoprotein and decreased lipid peroxidation in adult female rats. Also, soybean isoflavones intake in adult ovariectomized rats reduced bone loss. These effects could have the potential to reduce the risks of postmenopausal osteoporosis and of cardiovascular diseases in such women.

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