Potent Role of Dietary Phytoestrogen Plants Cultivated in Egypt Against Osteoporosis in Ovariectomized Rats

Nahed M Hassan, Rasmia A Hassan, Lobna M Abou Setta, Mehrevan M Abd El-moniem, Hanaa H Ahmed and Faiza M Hammouda

Chemistry of Medicinal Plant Department, National Research Center, Giza, Egypt. Medical Biochemistry Department, NRC, Giza, Egypt. Hormones Department, NRC, Giza, Egypt.

Abstract: The current study included seven groups of female Sprague Dawley rats which were classified as gonad intact control group and six ovariectomized groups: the first ovariectomized group was fed on the standard diet devoid of phytoestrogen, the second and third ovariectomized groups were fed on standard diet contained defatted soybean or crushed soybean (200g/kg diet) respectively instead of casein. The fourth ovariectomized group was fed on standard diet contained soybean extract (200g/kg diet). The last two ovariectomized groups were fed on standard diet contained freshly ground flaxseed (100g/kg diet), yellow or brown respectively. The treatment was started after 3 months of ovariectomy and continued for other 3 months. Serum calcium (Ca), phosphorus (P), osteocalcin (OC) and interleukin-6 (IL-6) levels were determined. Urine deoxypyridinoline (DPD) level was also estimated. Bone mineral density (BMD) and bone mineral content (BMC) of right femur bone of each rat were measured using DEXA technique. The obtained data revealed that ovariectomy decreased serum Ca, P and OC levels whereas; it increased serum IL-6 and urine DPD levels. DEXA results revealed that ovariectomy decreased BMD and BMC of the proximal, distal and mid areas of rat femur bone. The selected phytoestrogens could improve all the studied bone biochemical markers significantly. DEXA results also showed that treatment with these phytoestrogens could increase both BMD and BMC of rat femur bone in almost all areas.

Key words: Osteoporosis, soybean, flaxseed, bone biomarkers, bone density.

INTRODUCTION

Osteoporosis is an aged-related disease characterized by loss of bone mass and deterioration of the architecture of bone tissue, resulting in increased bone fragility (Cauley et al., 2002). Postmenopausal women are actually at risk for osteoporosis, and the bone loss is associated with reduction in estrogen levels as a result of the cessation of menses (Marcus, 1996). Hormone replacement therapy (HRT) has been used for many years to slow the development of osteoporosis and help alleviate other menopausal symptoms. However, there is mounting evidence that HRT may be linked with greater risks for some diseases including cancer, coronary vascular diseases (Rossouw et al., 2002) and thromboembolism (Huliey et al., 2002). Therefore, there is a need for alternative treatments to HRT that provide benefits to bone without adverse side effects.

Isoflavones are a subclass of flavonoides with a chemical structure similar to 17β-estradiol, the most potent, naturally occurring estrogen (Knight and Eden, 1996 and Anderson and Gamer, 1998). Isoflavones bind to estrogen receptors, affecting estrogen-regulated processes (Kuiper et al., 1997and Messina and Messina, 2000). Cells vary in their distribution of the putative estrogen receptor alpha (ERα) and the newly discovered ERβ, depending on the tissue. For instance, bone tissue has a great amount of ERβ (Potter et al., 1998).

Isoflavones are referred to as phytoestrogens (plant estrogens) (Kuiper et al., 1997 and Messina and Messina, 2000). The plant food sources high in phytoestrogens are numerous and includes soybeans, flaxseeds and other certain fruits and vegetables high in polyphenolic compounds.

The main isoflavones in soybeans are genistein and daidzein (Potter et al., 1998). Glycitein is also present in much smaller amount (Kuiper et al., 1997). Genistein binds with a much greater affinity to ERβ than to ERα (Kuiper et al., 1997, Potter et al., 1998,and Anderson et al., 1999). The different tissue distribution of α- and
β-receptors points to the possibility of tissue-selective effects of the isoflavones (Messina, 1999), as they appear to have different effects in different tissues (Kuiper et al., 1998). Isoflavones, therefore, could influence several biological processes.

Studies have shown that some phytoestrogens including genistein are ER-β selective and can bind with a greater affinity than estradiol, indicating that their action is separate and distinct from that of mammalian estrogen. Genistein binding to ER is nearly identical to selected ER modulators (SERM) (Setchell, 2001). Therefore, the consumption of soy may have beneficial effects on skeletal tissues. Dietary soy was found to slow the rate of bone loss after ovariectomy in rats (Arjmandi et al., 1996 and Harrison et al., 1998). The increased bone strength due to dietary soy was attributed to an increased efficiency of the intestine to absorb calcium (Omi et al., 1994). Furthermore, the conservation of calcium with dietary soy compared with other protein sources may be due to a reduction in the urinary excretion of calcium (Messina and Messina, 2000).

Among the edible plant foods, flaxseeds are by far the richest source of lignans, which are reported to have both weak estrogenic and anti-estrogenic activities (Ayers and Loike, 1990). Lignans are structurally similar to tamoxifen, which has beneficial effects on bone (Turner et al., 1988). Lignans present in flaxseeds may also possess antioxidant properties. In vivo and in vitro findings indicated that free radicals generated in the bone environment enhance osteoclast formation and bone resorption. Hence, flaxseeds may reduce the rapid rate of bone loss experienced by postmenopausal women, in part, by enhancing antioxidant status (Bahram and Arjmandi, 2001). Also, flaxseeds are considered as a rich source of polyunsaturated fatty acids (PUFA's), especially α-linolenic acid (18:3 n-3). Alpha-linolenic acid (omega-3) has a beneficial effect on decreasing the rate of bone resorption by inhibiting the biosynthesis of prostaglandins (Tashjian et al., 1972). Also, it has been reported that high n-3 fatty acid intake enhances calcium absorption, decreases calcium loss and increases bone calcium (Claassen et al., 1995; Kruegel and Horrobin, 1997 and Das, 2000). In addition, inhibition of cytokine production has been implicated as a potential mechanism of the favorable effects of fatty acid on bone; with higher intake of n-3 fatty acids inhibites the synthesis of pro-inflammatory cytokines such as interleukin 6 (IL-6), interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-α) (Endres et al., 1989 and Meydani et al., 1991). These cytokines (IL-6, IL-1, TNF-α) have been found to be active in the pathophysiology of osteoporosis, increasing osteoclast formation, activity and life-span (Manolagas and Jilka, 1995). Therefore, there are a plethora of biologically plausible pathways whereby PUFA's may regulate the factors involved in bone metabolism, such as prostaglandins, cytokines, insulin-like growth factor-1 and calcium. One or a combination of these factors may have an effect on bone (Kruegel and Horrobin, 1997; Das, 2000; Kettler, 2001 and Watkins et al., 2001).

The principal goal of the current study was to explore the potential role of Egyptian dietary phytoestrogens namely; soybeans and flaxseeds in management of postmenopausal osteoporosis in experimental ovariectomized rat model.

**MATERIALS AND METHODS**

**Plant Materials:**

- Soybeans cultivated in Egypt were obtained from the Egyptian Company for Seeds, Oils and Chemicals (El-Korma), Cairo, Egypt.
- Flaxseeds cultivated in Egypt, with either brown or yellow colour, were purchased from the Egyptian Herbal Market, Cairo, Egypt.

Soybeans and flaxseeds with brown or yellow colours were precisely identified and differentiated in the Research Institute for Oily Crops, Cairo, Egypt.

Preparation of plant materials:

1. 3kg of soybeans were crushed and stored at -20°C until use.
2. 3kg of soybeans were defatted with hexane (3x6L) using ultrasonic wave bath at 65°C for 1 hour each time, Hexane extract was evaporated, under reduced pressure until dryness to give dark yellow oily yield (547g). Defatted soybeans were dried (2.2kg) and stored at -20°C until use.
3. Crushed soybeans (5 kg) were defatted as previously stated, the defatted soybeans was dried and extracted with 80% MeOH (3x10L) using ultrasonic wave bath at 80°C for 1 hour and the extract was concentrated under reduced pressure until complete removal of MeOH and then lyophilized to give brown crude powdered extract (650 g).
4. 2kg of each of brown or yellow flaxseed were freshly ground immediately before use to avoid oxidation.
Bioassay:

70 female rats (5 months old) weighting 150-160g were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt. The animals were kept in wire bottomed cages at room temperature (25±2°C) under a 12 h dark-light cycle. Twenty rats were untreated and considered as control groups; ten of them underwent surgical ovariectomy. The control groups were fed on the standard diet devoid of phytoestrogens which consisted of casein 10%, salt mixture 4%, vitamin mixture 1%, corn oil 10%, and cellulose 5% and completed to 100g with corn starch (A.O.A.C., 1995). The other fifty rats underwent surgical ovariectomy and were classified into 5 groups (8 rats for each). The first two groups were fed on standard diet contained defatted soybean or crushed soybean (200 g/kg diet) instead of casein. The third ovariectomized group was fed on standard diet contained soybean extract (200 g/kg diet) (Bahr et al., 2005). The last two ovariectomized groups were fed on standard diet contained freshly ground flaxseeds (100 g/kg diet) (Ward et al., 2001), yellow or brown, respectively. The treatment period was continued for three months following three months of surgical ovariectomy.

At the end of the experimental period all animals were fasted overnight and the urine of each animal was collected for measurement of deoxypyridinoline (DPD) during the 24-hour period before slaughtering. Then, the blood samples were collected from the retro-orbital plexus (Schermer, 1967) under diethylether anaesthesia. The blood samples were left to clot and the sera were separated by cooling centrifugation (4°C) at 3000 rpm for 10 min. Serum samples were stored at -80°C until analysis. The right femur bone of each animal was carefully dissected, cleaned and stored in 10% formalin buffer for measuring bone mineral density (BMD) and bone mineral content (BMC) by dual energy X-ray absorptiometry (DEXA). Quantitative estimation of serum Ca level was carried out using Nitrous oxide flame atomic absorption spectrometer Varian (Spectr AA220) equipped with cross flow mebulizer for serum Ca measurements. The most optimum wave length (422.7 nm), has been used for Ca determination, Ca lamp current was 10 mA. Spectrophotometric method was used for quantitative determination of serum phosphorus level according to the method described by Cottenie et al., 1982. Briefly, this method depends on measuring phosphate concentration in the presence of V" and Mo" where orthophosphate forms a yellow coloured phosphovandomolybdate complex that shows an optimal absorption at 430 nm. Serum osteocalcin level was measured by enzyme linked immunosorbent assay (ELISA) procedure according to Coleman et al.1988 using kit purchased from Biosource Europe S. A. Co. Quantitative determination of serum interleukin-6 (IL-6) was carried out using a solid phase sandwich enzyme linked immunosorbent assay technique (ELISA) according to the method described by Dosquet et al.1994 using kit purchased from R&D Systems Co. USA. Metro DPD is an assay for quantitative measurement of deoxypyridinoline (DPD) in urine according to the method described by Conti et al.1998 using kit purchased from Quidel Corporation Specialty Products Co. USA. Creatinine in urine was measured colorimetrically according to the method of Bartles et al.1972 using kit purchased from Biodiagnostic Co. Egypt.

Statistical Analysis:

The results were expressed as mean ± S.E. Statistical differences between means were carried out using student "t"(Snedecor and Cochran, 1967). A probability value P<0.05 and P<0.01 were considered to be statistically significant while that corresponding to P > 0.05 was considered to be non-significant.

RESULTS AND DISCUSSION

Phytoestrogens have been known to have a role in modulating hormone related diseases based on their structural similarity to the estrogens (17β-estradiol and diethylstilbestrol). The presence and position of hydroxyl groups (OH) in the phytoestrogen compounds are considered one of the prerequisites for estrogenic activity (Martucci and Fishman, 1993). Also, the antioxidant potencies of isoflavones (phytoestrogenic compounds) are structurally related and closely associated with the presence of OH groups at positions 4` and 5` and the position of the aromatic ring. The estrogen receptor is capable of binding several structurally diversified compounds that include natural estrogens and isoflavones. Two of the major isoflavones are genistein and daidzein, which are the common diphenolic compounds of soybeans and have a structure resembling the structure of the potent synthetic estrogens diethylstilbestrol and hexestrol (Kettler, 2001). Flaxseeds are the richest source of lignans, which have been reported to have both weak estrogenic and antiestrogenic activities. Lignans are structurally similar to tamoxifen, which has beneficial effects on bone. Flaxseeds are also rich in polyunsaturated fatty acids (PUFA) especially α-linolenic acid (18:3 n-3) (Ayers and Loike, 1990 and Zava and Duwe, 1997).
Calcium and Phosphorus levels in serum: The present data showed significant decrease in serum calcium (Ca) and phosphorus (P) levels (P<0.01) in ovariectomized (OVX) rats when compared with the gonad intact control (Table 1).

The results in Table (1) revealed that ovariectomized rats fed on crushed soy showed significant increase (P<0.01) in each of serum Ca and P levels as compared to the ovariectomized control rats. Ovariectomized rats fed on defatted soy revealed non-significant increase (P>0.05) in serum Ca level with concomitant significant increase (P<0.05) in serum P level when compared with the ovariectomized control rats. Ovariectomized rats fed on soy extract showed significant increase (P<0.01) in serum Ca level accompanied with non-significant increase (P>0.05) in serum P level when compared with the ovariectomized control rats. Feeding ovariectomized rats with yellow flaxseeds produced non-significant increase (P>0.05) in serum Ca level with concomitant significant increase (P<0.05) in serum P level as compared to the ovariectomized control rats. Ovariectomized rats fed on brown flaxseeds showed significant increase (P<0.01) in serum Ca level accompanied with non-significant increase (P>0.05) in serum P level when compared with the ovariectomized control rats (Table 1).

Ovariectomy was suggested to induce loss of extraskeletal effects of estrogen on intestinal and renal Ca handling which in turn led to an increase in whole body losses of Ca (Vincent et al., 2003 and Zhang et al., 2007). Also, ovariectomy has been shown to alter phosphate homeostasis, with a significant reduction in serum phosphorous level (Hietala, 1993). Moreover, Reddy et al. 2003 reported that ovariectomy and concurrent calcium deficiency resulted in an increase in urinary excretion of Ca and P with concomitant decrease in femoral weight and density.

The work of Bahram and Arjmandi, 2001 raises the possibility that the protective effect of soy on bone may be due to its positive effect on intestinal calcium absorption. Arjmandi et al. 1998 reported that there was a significant increase in insulin-like growth factor-1 (IGF-1) mRNA in the isoflavone-treated rats compared to the control. It has been suggested that IGF-1 could stimulate renal 25-hydroxyvitamin D-1-alpha hydroxylase activity, thereby enhancing Ca and P absorption in the intestine and increasing the maximal renal tubular reabsorption of P (Saggese et al., 1995). Moreover, Cross et al. 2004 suggested that nutritional soy or genistein can optimize extrarenal, 25 dihydroxyvitamin D3 synthesis to participate in promoting the translocation of Ca against the concentration gradient which exists across the intestinal cell membrane for controlling extracellular fluid Ca++. This ensures an adequate concentration of Ca and P for deposition as hydroxyapatite crystals onto the collagen fibrils in bone (Daryl and Granner, 2000).

Flaxseed lignans are active estrogens only after metabolism by intestinal flora into mammalian lignans: enterolactone and enterodiol (Setchell and Adlercreutz, 1988). Lignans are structurally similar to estrogen and share with estradiol several features such as the diphenolic ring that is essential for binding to estrogen receptors (Meksicek, 1995). Therefore, flaxseed lignans via estrogen receptors could increase serum Ca level through their effects on intestinal and renal Ca handling as the major mechanisms for their actions on modulating Ca homeostasis (Vincent et al., 2003). Furthermore, Dick et al. 2005 reported that estrogen, and in turn compounds that mimic estrogen could reduce renal Ca excretion via increasing renal Calbindin D28k mRNA level (Criddle et al., 1997). Similarly, these active ingredients, lignans, in flaxseeds with their estrogen-like action could stimulate renal biosynthesis of 1, 25 dihydroxyvitamin D3 which is one of the major regulators of Ca and P metabolism, stimulating intestinal Ca and P absorption (Erben, 2001).

2. Osteocalcin and interleukin-6 (IL-6) levels in serum and DPD level in urine: The current results revealed that ovariectomy resulted in significant decrease (P<0.01) in serum osteocalcin level with concomitant significant increase (P<0.01) in serum interleukin-6 (IL-6) and urine deoxypyridinoline (DPD) levels as compared to gonad intact control (Table 2).

The data in Table (2) revealed that ovariectomized rats fed on crushed soy, defatted soy, soy extract, yellow flaxseeds or brown flaxseeds showed significant increase (P<0.01) in serum osteocalcin level with concomitant significant decrease (P<0.01) in each of serum IL-6 and urine DPD levels when compared to the ovariectomized control rats.

The reduction in serum osteocalcin level due to ovariectomy is in agreement with Horcajada-Molteni et al., 2000. Osteocalcin is a non-collagenous protein in bone and its synthesis is stimulated by 1, 25 dihydroxyvitamin D3 (1, 25 (OH)2 D3) (Matsunaga et al., 1999). Ovariectomy and estrogen deficiency could decrease 1,25 (OH)2 D3 production as well as plasma 1,25 (OH)2 D3 level through a reduction of 1,25 (OH)2 D3 receptors expression and bioreponse (Liel et al., 1999). In consequence, serum osteocalcin level was reduced due to estrogen deficiency through ovariectomy in the present study. Regarding the increased serum IL-6 level due to ovariectomy in the current study, Kalaitzidis and Gilmore, 2005 stated that the loss of estrogen resulted in an increase in pro-inflammatory cytokines, IL-1, IL-6 and tumor necrosis factor- alpha.
of the proximal 225-bp sequence of the promoter (Pottratz et al., 1994 and Ray et al., 1994). Thus, loss of estrogen as a result of ovariectomy induced marked increase in serum IL-6 in the present study. With respect to the elevated urinary DPD level in ovariectomized rats in the present study, it is well known that bone turnover is increased by ovariectomy and the activity of osteoclasts is higher than that of osteoblasts, hence osteoporosis develops (Manna et al., 2004) Urinary DPD is one of the most important bone turnover markers and its elevated level in urine indicated the increased bone turnover due to ovariectomy (Rona Brynin, 2002). Soy containing daidzein could increase the viability of osteoblasts and increase alkaline phosphatase activity (ALP) and osteocalcin synthesis of osteoblasts. ALP and osteocalcin are phenotypic markers for early stage differentiated osteoblasts and terminally differentiated osteoblasts respectively (Chiechi et al., 2002 and Jia et al., 2003). The effect of genistein on osteoblastic cells appears to be the same as that of diadzein. Choi et al.2001 reported that soybean extract promotes anabolic functions of osteoblast-like cells and that agreed with Yamaguchi et al.2002 who reported that genistein and daidzein stimulated protein synthesis in osteoblast-like cells in vitro. Fernandes et al.2003 reported that soy protein has an anti-inflammatory property that can down-regulate pro-inflammatory cytokines including IL-6 and may also protect against bone loss by decreasing osteoclast activation and bone resorption. Genistein in soy has been found to inhibit osteoclastic activity directly by a mechanism independent of cellular attachment and at doses similar to those inhibiting tyrosine kinase autophosphorylation. Also, genistein could decrease acid secretion by osteoclasts, thus decreasing bone dissolution (Williams et al., 1998). This explains the antiresorptive effect of feeding ovariectomized rats on soy in its different formulations in the present study as indicated by a decreased serum IL-6 level and urine DPD level as bone resorption markers. Moreover, it was reported (Criddele et al.1997) that isoflavones could reduce bone turnover induced by ovariectomy as indicated by measuring urine DPD level. Flaxseeds containing lignans with potent estrogen–like action could induce the synthesis and production of 1, 25 (OH)_{2}D, which is the most important factor for osteocalcin expression in osteoblasts. It has been found that 1, 25 (OH)_{2}D, stabilized osteocalcin mRNA on both the transcriptional and post-transcriptional levels (Mosavin and Mellon, 1996) This explains the elevated serum osteocalcin level, a marker of bone formation (Christenson, 1997) in the ovariectomized rats fed on flaxseeds. This result indicated the anabolic action of flaxseeds on bone. On the other hand, the antiresorptive effect of flaxseeds has been reported by Yin et al.. 2004 who attributed the potent inhibitory activity of flaxseeds on bone resorption induced by hyperparathyroidism to the reactive flaxseed lignans. Lignans could bind to estrogen receptors and act in the same manner as estrogen so that these compounds have an inhibitory effect on IL-6 production mediated by the inhibition of IL-6 gene transcription through estrogen receptor activation (Pottratz et al., 1994 and Ray et al., 1994). Moreover, flaxseed oil is enriched in the long chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Bahram and Arjomandi, 2001and Kettler, 2001), therefore, it is capable to modulate the ratio of omega 6: omega 3 fatty acids in the membranes and to decrease the production of the inflammatory cytokines IL-1, IL-6 and tumor necrosis factor-α (TNF-α) (Caughey et al., 1996). Also, the antiresorptive action of flaxseeds is well documented in the present study by the decreased urine DPD level in the ovariectomized rats fed on flaxseeds. This finding is greatly supported by Bahram and Arjomandi, 2001who demonstrated that flaxseeds reduce the rate of bone loss in postmenopausal women, in part, by enhancing antioxidant status which in turn impairs osteoclast activity and decreases bone resorption as well as bone resorptive markers. Also, it was demonstrated (Schlemer et al., 1999) that lignans induce significant decrease in bone turnover as indicated by lowering urine DPD level.

1. Bone mineral density and bone mineral content:-The results of the current work showed significant decrease (P<0.01) in bone mineral density (BMD) (Table 3) in different areas of the right femur bone of ovariectomized rats when compared to the gonad intact control. The results in Table (3) revealed that ovariectomized rats fed on crushed soy exerted significant increase (P<0.01) in BMD of mid area of right femur bone while non-significant change was detected in BMD of each of the proximal, distal and total area of right femur bone as compared to the ovariectomized control rats. Also, no change in BMD of proximal, mid, distal and total areas of right femur bone of ovariectomized rats fed on defatted soy was demonstrated. Also, feeding ovariectomized rats on soy extract resulted in significant increase (P<0.01) in BMD of proximal, mid, and total areas of right femur bone while no change in BMD of the distal area was detected as compared to the ovariectomized control rats. Ovariectomized rats fed on yellow flaxseeds showed significant increase in BMD of proximal (P<0.05), mid, distal and total areas (P<0.01) of right femur bone as compared to the ovariectomized control rats (Table 3). However, feeding ovariectomized rats on brown flaxseeds led to significant increase in BMD of the mid and total areas (P<0.01) while no change in BMD of
each of proximal and distal areas of the right femur bone was observed as compared to the ovariectomized control rats.

The results of the current work showed significant decrease (P<0.01) in bone mineral contents (BMC) (Table 4) of each of proximal, mid and total areas while significant decrease (P<0.05) in BMC in distal area of right femur bone of ovariectomized rats when compared to the gonad intact control.

Regarding the BMC of the right femur bone of ovariectomized rats fed on different formulae of phytoestrogenic plants, the data in Table (4) revealed that feeding on crushed soy resulted in significant increase (P<0.01) in BMC of the four areas of right femur bone (proximal, mid, distal and total area) as compared to the ovariectomized control rats. Feeding ovariectomized rats on defatted soy led to significant increase (P=0.01) in BMC of the mid area while no change in BMC of each of proximal, distal and total areas of right femur bone was detected when compared with the ovariectomized control rats (Table 4). Ovariectomized rats fed on soy extract expressed significant increase (P<0.01) in BMC of proximal, mid and total areas of right femur bone and non-significant increase (P=0.05) in the distal area as compared to the ovariectomized control rats (Table 4). Feeding ovariectomized rats on yellow flaxseeds resulted in significant increase (P<0.01) in BMC of the four areas (proximal, mid, distal and total area) of the right femur bone when compared to the ovariectomized control rats (Table 4). Ovariectomized rats fed on brown flaxseeds expressed significant increase (P<0.01) in BMC of each of mid, distal and total area of right femur bone while no change in BMC of proximal area was detected when compared with the ovariectomized control rats.

Westerlind et al. 1997 observed a reduction of bone structure parameters of more than 50% within few weeks after ovariectomy, thus, it has been reported that ovariectomy greatly reduced BMD resulting from increased bone turnover (Picherit et al., 2000) and decreased calcium absorption efficiency as well as calcium homeostasis which contribute to the reduction in BMD and BMC in ovariectomized rats (Gaumet et al., 1997). The observed improvement in BMD and BMC of different areas of right femur bone of ovariectomized rats fed on crushed soy or soy extract may be attributed to:

1. The efficacy of soy protein to enhance intestinal Ca absorption (Bahram and Arjmandi, 2001).
2. The dual effect of active soy compounds namely daidzein and genistein. Daidzein could increase the viability of osteoblasts (Chiechi et al., 2002 and Jia et al., 2003). Also, daidzein may have a stimulatory effect on the proliferation and differentiation of osteoblastic cells (Sugimoto and Yamaguchi, 2000). Moreover, daidzein has been found to increase IGF-1 mRNA in osteoblasts (Arjmandi et al., 1998) and it could increase the production of bone morphogenetic proteins by osteoblasts and thus it has a direct stimulatory effect on bone formation (Jia et al., 2003). Therefore, daidzein has an important role in stimulation as well as acceleration of bone formation. From another point of view, genistein has been found to decrease osteoclastic activity via inhibition of tyrosine kinase (Blair et al., 1996). Ishimi et al. 1999 reported that genistein is able to reduce ovariectomy – induced increase of bone marrow hemopoiesis which stimulates bone resorption. Thereby genistein has a key role in inhibiting bone resorption. Soy protein which has anti-inflammatory properties and can down-regulate the pro-inflammatory cytokines participates with genistein in protection against bone loss by decreasing osteoclast activation and bone resorption (Fernandes et al., 2003). Therefore, through these synergistic multipotent pathways, soy protein and isoflavones could preserve bone mineral density as well as bone mineral content in ovariectomized rats.

Regarding the significant positive effects of flaxseeds on BMD and BMC of different areas of right femur bone of ovariectomized rats, we could discuss these findings as follows: 1) flaxseed oil is enriched in omega-3 fatty acids which have multifunctions with respect to bone tissue; this type of long chain fatty acids could increase intestinal Ca absorption, protect bone Ca content and preserve bone density (Kettler, 2001), 2) flaxseeds containing omega-3 fatty acids have anti-inflammatory properties so that they could decrease the production of pro-inflammatory cytokines and thus inhibit osteoclastic activity and reduce bone resorption process (Caughey et al., 1996).

3. Yin et al., 2004 attributed the powerful inhibitory activity of flaxseeds on bone resorption induced by hyperparathyroidism to their reactive constituent lignans. Lignans have estrogen-like action and via their affinity to estrogen receptors, they could inhibit bone resorbing cells 'osteoclasts' and thus reduce bone resorption.

4. Flaxseeds could also reduce bone loss by stimulating the antioxidant defense system and enhancing the antioxidant status (Bahram and Arjmandi, 2001), thereby inhibiting bone resorption pathway and playing a role in the maintenance of skeletal health (Lucas et al., 2002). Thus, flaxseeds via all these mechanisms could conserve bone mineral density and bone mineral content in ovariectomized rats.
Table 1: Effect of different treatments with soy and flaxseed on serum Ca and P levels of ovariectomized rats.

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Ca mg/dl</th>
<th>P mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad intact control</td>
<td>9.06± 0.16</td>
<td>8.1± 0.2</td>
</tr>
<tr>
<td>Ovariectomized (OVX) control</td>
<td>6.90± 0.10**</td>
<td>6.70± 0.3**</td>
</tr>
<tr>
<td>OVX+ Crushed soy</td>
<td>8.20± ±0.20**</td>
<td>7.90±0.1**</td>
</tr>
<tr>
<td>OVX+ Defatted soy</td>
<td>7.40± ±0.45***</td>
<td>7.50±0.15**</td>
</tr>
<tr>
<td>OVX+ Soy extract</td>
<td>9.00± ±0.29***</td>
<td>7.06±0.13***</td>
</tr>
<tr>
<td>OVX+ yellow Flaxseeds</td>
<td>7.20 ± 0.5***</td>
<td>7.60±0.11***</td>
</tr>
<tr>
<td>OVX+ brown Flaxseeds</td>
<td>7.90 ± 0.26**</td>
<td>7.10±0.13***</td>
</tr>
</tbody>
</table>

Values are means ± SE for 8 rats/group, a: Differences in relation to control group, b: Differences in relation to ovariectomized control rats, **: Significant change at P<0.05, ***: Significant change at P<0.01, NS: Non-significant change at P>0.05.

Table 2: Effect of different treatments with soy and flaxseeds on serum osteocalcin, IL-6 and urine DPD levels of ovariectomized rats.

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Osteocalcin ng/ml</th>
<th>IL-6 pg/ml</th>
<th>nmol DPD/mmol creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad intact control</td>
<td>8.10±0.12</td>
<td>20±0.5</td>
<td>3.40±0.1</td>
</tr>
<tr>
<td>Ovariectomized (OVX) control</td>
<td>5.60±0.19**</td>
<td>21.50±0.3**</td>
<td>6.40±0.12**</td>
</tr>
<tr>
<td>OVX+ Crushed soy</td>
<td>7.90±0.06**</td>
<td>21.30±0.3**</td>
<td>5.50±0.28**</td>
</tr>
<tr>
<td>OVX+ Defatted soy</td>
<td>7.90±0.16**</td>
<td>22.03±0.5**</td>
<td>4.20±0.29**</td>
</tr>
<tr>
<td>OVX+ Soy extract</td>
<td>7.80±0.09**</td>
<td>21.30±0.25**</td>
<td>4.40±0.21**</td>
</tr>
<tr>
<td>OVX+ yellow Flaxseeds</td>
<td>7.80±0.1**</td>
<td>21.60±0.3**</td>
<td>2.80±0.12**</td>
</tr>
</tbody>
</table>

Values are means ± SE for 8 rats/group, a: Differences in relation to control group, b: Differences in relation to ovariectomized control rats, **: Significant change at P<0.01.

Table 3: Effect of different treatments with soy and flaxseeds on bone mineral density (BMD) of right femur bone of ovariectomized rats.

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>BMD mg/cm²</th>
<th>Proximal</th>
<th>mid</th>
<th>distal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad intact control</td>
<td>120.6±0.65</td>
<td>119.5±1.2</td>
<td>127.5±0.8</td>
<td>120.2±1.2</td>
<td></td>
</tr>
<tr>
<td>Ovariectomized (OVX) control</td>
<td>110.3±0.7**</td>
<td>110.1±0.75**</td>
<td>113.1±0.7**</td>
<td>117±0.4**</td>
<td></td>
</tr>
<tr>
<td>OVX+ Crushed soy</td>
<td>112.5±0.9**</td>
<td>113.8±0.5**</td>
<td>113.8±1.2**</td>
<td>118.4±0.6**</td>
<td></td>
</tr>
<tr>
<td>OVX+ Defatted soy</td>
<td>110.5±0.0**</td>
<td>110.7±0.4**</td>
<td>113.7±0.1**</td>
<td>117.1±0.1**</td>
<td></td>
</tr>
<tr>
<td>OVX+ Soy extract</td>
<td>115.5±1.4**</td>
<td>114.6±0.7**</td>
<td>113.4±0.4**</td>
<td>119.6±0.5**</td>
<td></td>
</tr>
<tr>
<td>OVX+ yellow Flaxseeds</td>
<td>121.3±0.7**</td>
<td>115.6±0.5**</td>
<td>121.2±0.4**</td>
<td>121.5±0.6**</td>
<td></td>
</tr>
<tr>
<td>OVX+ brown Flaxseeds</td>
<td>110.6±0.4**</td>
<td>115.6±0.5**</td>
<td>113.25±0.1**</td>
<td>119.6±0.1**</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE for 8 rats/group, a: Differences in relation to control group, b: Differences in relation to ovariectomized control rats, **: Significant change at P<0.05, ***: Significant change at P<0.01, NS: Non-significant change at P>0.05.

Table 4: Effect of different treatments with soy and flaxseeds on bone mineral content (BMC) of right femur bone of ovariectomized rats.

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>BMC mg</th>
<th>Proximal</th>
<th>mid</th>
<th>distal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad intact control</td>
<td>61.7±0.5</td>
<td>200.7±0.7</td>
<td>66±1.1</td>
<td>274.8±1.1</td>
<td></td>
</tr>
<tr>
<td>Ovariectomized (OVX) control</td>
<td>44.4±0.6**</td>
<td>131.2±0.9**</td>
<td>46.9±0.6**</td>
<td>231.9±0.9**</td>
<td></td>
</tr>
<tr>
<td>OVX+ Crushed soy</td>
<td>59.6±0.9**</td>
<td>174.2±1.2**</td>
<td>55.8±1.2**</td>
<td>259.9±0.7**</td>
<td></td>
</tr>
<tr>
<td>OVX+ Defatted soy</td>
<td>45.02±0.7**</td>
<td>153.3±0.8**</td>
<td>47.1±0.8**</td>
<td>232±0.4**</td>
<td></td>
</tr>
<tr>
<td>OVX+ Soy extract</td>
<td>69.2±0.3**</td>
<td>197±0.6**</td>
<td>50.0±2.1**</td>
<td>274.7±1.2**</td>
<td></td>
</tr>
<tr>
<td>OVX+ yellow Flaxseeds</td>
<td>60.0±0.7**</td>
<td>186±0.18**</td>
<td>61±1.1**</td>
<td>294.6±0.7**</td>
<td></td>
</tr>
<tr>
<td>OVX+ brown Flaxseeds</td>
<td>45.02±1.0**</td>
<td>180±1.4**</td>
<td>53.1±0.4**</td>
<td>276.9±0.2**</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE for 8 rats/group, a: Differences in relation to control group, b: Differences in relation to ovariectomized control rats, **: Significant change at P<0.05, ***: Significant change at P<0.01, NS: Non-significant change at P>0.05.

Conclusion:

Diets supplemented with either one of the phytoestrogens: crushed soy, soy extract or ground flaxseeds could markedly modulate the majority of bone biomarkers as well as BMD and BMC in ovariectomized rats as an animal model for postmenopausal osteoporosis. The biological effects of phytoestrogens are mostly mediated by the interaction with estrogen receptors. In addition, these phytochemicals, particularly soy, have the ability to stimulate bone formation and inhibit bone resorption simultaneously beside its anti-inflammatory properties which help in more and more inhibition of bone resorption. Flaxseeds also, have the capability to inhibit bone resorption via their anti-inflammatory activity, potent estrogen-like action and antioxidant property. Actually, phytoestrogens can prevent postmenopausal bone loss and thus represent a potential alternative natural therapy for a range of hormone-dependent postmenopausal symptoms. Meanwhile, these encouraging results provide new concepts for the development of effective therapeutic modalities for preserving bone mineral density in postmenopausal women.
ACKNOWLEDGMENTS

Financial support by the Academy of Research and Technology is gratefully acknowledged. Thanks are due to the Research Institute for Oily Crops, Cairo, Egypt for the identification of the Soybean and flaxseeds.

REFERENCES


