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Does Flaxseed Uptake Reverse Induced-Bone Loss in Ovariectomized Rats?

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Abstract: To examine a potential role of flaxseed in postmenopausal bone loss, 30 wistar rats were divided randomly into 5 groups and given a basal diets and supplemented one with flaxseed for 12 weeks. The treatments groups were as followed: sham operated groups+basal diet, ovariectomized (OVX) + basal diet, OVX + 5% of flaxseed supplementation (FS), OVX+ 50% (FS), OVX+100% (FS). An increase ALP activity and urinary excretion of phosphorus and calcium were observed in OVX groups. The serum phosphorus and calcium were significantly lowered in OVX group than the sham group. The treatment with FS did not improve the later parameters at 50 and 100% of supplementations, while a decreased urinary excretion of phosphorus and calcium were noted. The administration of whole flaxseed at 5, 50 and 100% of supplementation, did not appear to have such as beneficial effect on the bone mineral density of femurs of treated animals. These findings provide evidence that dietary supplementation with flaxseed can prevent postmenopausal bone loss.

Key words: Bone loss, flaxseed supplementation, bone mineral density, biochemical markers, ovariectomized rats

INTRODUCTION

Postmenopausal osteoporosis is attributed to ovarian hormone deficiency and has resulted in a significant morbidity and mortality (Maclaughlin *et al.*, 2006). Indeed, the decline in levels of circulating estrogens produces an increased rate of bone loss rendering the skeleton more prone to bone fractures (Lerner, 2006). Several studies have shown that Estrogen Replacement Therapy (ERT) maintains skeletal mass and reduces fractures risk in postmenopausal women (Delmas, 2002). The protective effect of estrogens on bone tissue is believed to be due primarily to their antiresorptive action (Riggs *et al.*, 2002). However, long term ERT has been associated with increased risk factor of breast and uterine cancer (Barett-Connor and Stuenkel, 2001).

Recently, many researchers have reported that phytoestrogens intake plays a role in preventing the development of some chronic diseases such as age-related bone loss (Branca, 2003; Tsuang *et al.*, 2008), memory loss (Luine *et al.*, 2006) and cardiovascular disease (Cassidy and Hooper, 2006). Phytoestrogens are plant compound with estrogen-like biological activity. The three main classes of phytoestrogens are isoflavones, coumestans and lignans. They are abundant in many dietary sources

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such as soybeans, oilseed (e.g., flaxseed), clover and alfalfa sprouts. Currently, flaxseed have received considerable interest in the potential health benefits (Power and Thompson, 2007), especially in preventing bone loss associated with estrogen deficiency in postmenopausal women (Arjmandi *et al.*, 1998). Flaxseed is a rich source of lignans, which are reported to have both weak estrogenic and anti-estrogenic activities (Carreau *et al.*, 2008). In previous human studies on estrogen and bone metabolism, the diet of postmenopausal women was supplemented with ground flaxseed in the amounts of 5, 10 and 40 g (Hutchins *et al.*, 2001; Haggans *et al.*, 1999). Dodin *et al.* (2005) showed that a daily supplementation of flaxseed with the amount of 40 g for 12 months produced a favorable, but not clinically significant, effect on blood cholesterol and caused no significant change in Bone Mineral Density (BMD) or symptoms in healthy menopausal women. Whereas, other data showed that flaxseed can potentially exert positive effects on bone of postmenopausal women (Arjmandi *et al.*, 1998). Although the numbers of finding that have been conducted in menopausal women to evaluate the effects of phytoestrogens (especially of flaxseed) diet on bone metabolism, remain controversial (Arjmand, 2001). In our knowledge, few studies were evaluated the effect of whole flaxseed on bone loss in ovariectomized rats treated with whole flaxseed. Thus, the purpose of this study was to determine whether dietary supplementation of whole flaxseed at the following amounts 5, 50 and 100% for 12 weeks would prevent bone loss in ovariectomized rats.

MATERIALS AND METHODS

Animals and Diets

Three month old female virgin wistar rats (colony room of biology department, Faculty of Sciences, Kenitra Morocco) initially weighing 240.3 ± 1.6 g. The rats were sham operated or underwent bilateral ovariectomy after being anesthetized with chloral hydrate (0.5 ml /100g, Sigma-aldrich, laborchemikalien GmbH, Germany). In the ovariectomized rats (OVX) a ventral incision was made to expose the ovaries which were removed after ligation of the uterine horn. The following groups were formed after 2 weeks postsurgery: sham operated control rats (n = 6), OVX rats (n = 6), these two groups received a basal diet, the OVX rats (n = 24) received a basal diet containing 5, 50 and 100% of ground flaxseed (whole flaxseed). The basal diet was made in (Neuroendocrine physiology Unit, Kenitra; Morocco). For 100 g of food, dry weight, was composed of: Protein 14%, wheat flour 70%, cellulose 5%, corn oil 4%, sucrose 5% and salts 2%. To avoid any defaults in vitamins and minerals, these components were given to rats in solution form three times on weeks. All rats were maintained on a 12 h light/ 12 dark cycle in a temperature and humidity-controlled room. They were allowed free access to food and water *ad libitum* during the 12 weeks of experiment. Diets were stored at 4°C and fresh diets were provided to the rats every 2 days. Food consumption was measured for each feeding. Body weight was recorded weekly.

Blood Collection

After 12 weeks of feeding, rats were anesthetized with chloral hydrate (0.5 mL/100 g, Sigma-aldrich, laborchemikalien GmbH, Germany). Blood samples were collected and plasma was separated by centrifugation at $1500 \times g$ for 20 min at 4°C. Aliquots of plasma were frozen and kept at -20°C for later analyses.

Measurements of Serum Calcium, Phosphorus and Alkaline Phosphatase

The calcium (Ca) concentrations of serum samples was measured using standard colorimetric methods with commercial kits (Biolabo, Casablanca, Morocco). Serum alkaline phosphatase (ALP) activity and phosphorus (P) were determined using a BioSystems BTS 310 photcolorimeter and Standard BioSystems reagents.

Urine Collection

Twelve hours of urine collection was initiated 1 day before termination of the study. Urine was collected in acid-washed tubes and total volume was measured and acidified with 0.03 mL of 6 mol L⁻¹ HCl per 1 mL of urine. Samples were frozen at -20°C until analysis. Urine calcium and creatinin were determined using an automate analyser (Automate Vitros 250). The phosphorus was analysed by (BioSystems BTS 310 photocolormeter) using a Standard BioSystems reagents.

Bone Analysis

The femoral bones were carefully removed at necropsy. These bones were freed from soft tissue and weighed.

Bone Mineral Density

The femora were dried overnight at 100°C, weighted and then ashed at 550°C for 48 h. The ashed samples were extracted with HNO₃, 2N. The amounts of calcium and magnesium were analysed by complexometry. The phosphorus was determined colorimetrically.

Statistical Analysis

Data are reported as mean±SEM. The one way analysis of variance (ANOVA) tests were used for comparisons between every two groups. Parametric ANOVA was performed when data were sampled from populations with equal variance. If not, nonparametric methods were selected. Thus, a Kruskal-Wallis test was first performed. If it indicated a significant difference among groups (p<0.05), the Mann-Whitney U-test was used to determine specific differences. The level of significance was set at p<0.05 for all statistical tests.

RESULTS

Effects of Ovariectomy OVX, Enriched Diet of Flaxseed at 5, 50 and 100% on Body Weight, Feed Intake and Organ Weights in Rats

The average weekly food intake throughout the experiment did not differ between the studied groups. The treatment groups started with similar mean body weights. All rats gained weight during the study, but the weight gains of rats in the OVX groups were significantly higher (25.3%) than those of the sham animals (p<0.05). Ovariectomy caused atrophy of uterine tissue, indicating the success of the surgical procedure. However, there were no differences in the liver weights among any of the treatment groups (Table 1).

Effects of Ovariectomy OVX; Enriched Diet of Flaxseed at 5, 50 and 100% on Serum Activities of ALP and Serum and Urinary Excretion of Calcium and Phosphorus

OVX resulted in a significant increase in serum ALP and urinary excretion of phosphorus and calcium (Table 2 versus sham, p<0.05), suggesting that OVX increased the bone turnover rate.

Table 1: Effects of ovariectomy OVX; enriched diet of flaxseed at 5, 50 and 100% on body weight, feed intake and organ weights in rats

Measures	Sham	OVX	OVX 5%	OVX 50%	OVX 100%	
Feed intake (g day ⁻¹)	170.8±2.3	176.1±2.3	174.90±2.3	172.60±2.3	169.9±2.3	
Body weight (g)	Initial	240.6±3.8	240.8±4.1	240.50±4.8	240.80±5.1	240.6±4.6
	Final	245.2±4.9 ^a	266.1±6.2 ^b	246.40±5.2 ^a	252.40±7.3 ^a	249.8±8.7 ^a
	Gain (%)	4.60	25.30	5.90	11.60	9.20
Organ weights (g/100 g b.wt.)	Uterus	0.53±0.01 ^a	0.13±0.01 ^b	0.14±0.01 ^{b0}	13.00±0.01 ^b	0.12±0.01 ^b
	Liver	2.50±0.20	2.67±0.54	2.45±0.37	2.54±0.22	2.49±0.18

Values are presented as Mean±SEM, n = 6 in each group. Values that do not share the same letter(s) (a, b) are significantly (p<0.05) different from each other

Table 2: Effects of ovariectomy OVX, enriched diet of flaxseed at 5, 50 and 100% on serum activities of ALP and serum urinary excretion of calcium phosphorus

Measures	Sham	OVX	OVX 5%	OVX 50%	OVX 100%
s-CA (mg L ⁻¹)	97.20±01.9 ^a	85.80±02.3 ^b	100.60±01.4 ^a	87.80±01.53 ^b	84.60±05.0300 ^b
s-P (mg L ⁻¹)	64.10±03.2 ^a	28.70±01.8 ^b	58.10±09.20 ^a	52.20±6 ^a	59.30±05.1500 ^a
s-ALP (UI L ⁻¹)	199.80±31.8 ^a	385.40±17.8 ^{b,c}	277.00±86.07 ^{a,c}	326.80±34.8 ^{b,c}	528.40±82.600 ^b
Ca/Crea (mg mg ⁻¹)	0.01±00.001 ^a	0.09±00.03 ^b	0.01±00.001 ^a	0.01±00.001 ^a	0.01±00.005 ^a
P/Crea (mg mg ⁻¹)	1.40±00.25 ^a	14.01±00.3 ^{b,c}	1.50±00.96 ^a	1.10±00.27 ^{a,c}	1.10±00.240 ^{a,c}

Values are presented as Means±SEM, n = 6 in each group. Values that do not share the same letters (^{a, b}) are significantly (p<0.05) different from each other

Table 3: Mineral contents of left Femur from OVX rats¹

Contents	Sham	OVX	OVX 5%	OVX 50%	OVX 100%
Dry weight	0.31±0.02	0.27±0.02	0.32±0.02	0.25±0.02	0.27±0.02
Ash content	0.20±0.02	0.18±0.02	0.20±0.02	0.17±0.02	0.18±0.02
Calcium (mg L ⁻¹)	190.38±2.70	183.36±2.70	190.38±2.70	185.37±2.70 ^b	188.38±2.70
Phosphorus (mg L ⁻¹)	0.21±0.01	0.20±0.01	0.21±0.01	0.22±0.01	0.20±0.01
Magnesium (mg L ⁻¹)	73.43±0.51	66.38±0.51	73.43±0.51	67.42±0.51	70.54±0.51

¹Each value is expressed as the Mean±SD (n = 6)

Treatment of OVX rats with flaxseed diet at different concentrations 50 and 100% did not reduce the high levels of ALP activity which remain significantly higher compared to those in the sham group and OVX 5% group (Table 2, versus OVX and Sham, p<0.05).

The serum (P) and (Ca) were significantly lower in OVX group than the sham group and OVX 5% group (Table 2, OVX versus sham, p<0.05). The treatment with flaxseed at 50 and 100% did not alter the levels of serum (P) and (Ca).

The Mineral Contents of the Left Femur for the Rats

The Table 3 shows the mineral contents of the left femur for the rats. The values of dry weight and ash content of the left femur for the rats in sham group and OVX5% were higher than the OVX and treated groups although the difference was not significantly different.

DISCUSSION

In the present study, positive effects of dietary flaxseed on bone mineral density and biochemical markers of bone remodeling in ovariectomized rats was evaluated. The ovariectomized rat model of osteoporosis was taken as an experimental model for conducting this study (Kalu, 1991). Following ovariectomy, there is an increase in cancellous bone turnover rates, with increase in bone resorption which results in a net loss of cancellous bone at some skeletal sites, particularly metaphyseal regions of the long bones. Cancellous bone osteopenia is well-established 1-2 months after ovariectomy with persistent long-term changes in cancellous bone structure (Wronski *et al.*, 1988).

Following ovariectomy at the end of the experiment, the body weight of ovariectomized rats increased significantly (25.3%) and the uterine weight decreased greatly (75%) compared with those of sham animals (Table 1) indicating that the animals had become estrogen deficient. The administration of flaxseed (which is a phytoestrogen) at different concentrations has inhibited the increase of body weight and did not affect the uterine weight. Some reports have attributed the mechanism of action of some phytoestrogens to their high binding affinities to the intracellular estrogen receptors. They support that these compounds may act in target tissues as agonist or antagonists in the absence of endogenous estradiol (Migliaccio and Anderson, 2003). This is in agreement with the present work indicating that the weight of the uteri of the treated animals did not differ significantly from that of non- treated-groups.

The ovariectomized rats have shown significant increase in biochemical parameters. They were characterized by an increase in ALP activity and urinary excretion of phosphorus and calcium. This indicate an increase in the osteoblastic and osteoclastic activity respectively (Hadjidakis and Androulakis, 2006). The concentrations of serum (Ca) and (P) were decreased after ovariectomy in this study. Similar to these results, Kalu (1984) has shown that the levels of serum (Ca) were decreased in the ovariectomy. Contrary to this finding, Ohta *et al.* (1992) reported that both concentrations of (Ca) and (P) were on the increase in ovariectomized groups. Those results suggest that ovarian hormone deficiency following ovariectomy is marked by reduced intestinal calcium absorption and may contribute to the accompanying bone loss (Kalu, 1984).

Treatment for 3 months with whole flaxseed at different concentrations (5, 50 and 100% successively) revealed a significant increase in ALP activity (as a marker of bone formation) and significant decrease in urinary excretion of (Ca) and (P) (as a marker of bone resorption), thus showing an enhancement of osteoblastic activity and a reduction of osteoblastic activity.

Flaxseed is a rich source of lignans with potential weak estrogen and antiestrogenic activity similar to that of the isoflavones found in soy (Adlercreutz *et al.*, 1987; Kuiper *et al.*, 1998). The estrogen receptors in osteoblasts and osteoclasts were found in rats and human (Wiren *et al.*, 2002; Maran *et al.*, 2003; Denger *et al.*, 2008). Lignans (Lee *et al.*, 2007) as well as other phytoestrogens including daidzein and genistein (Masayoshi, 2006) were proven to have an anabolic effect on bone metabolism and prevented bone loss. Thus the mechanism of action of flaxseed on bone is possibly that the lignans affect the bone through estrogen receptors.

Flaxseed is also a very rich source of α -linolenic acid (ALA) (Cunnane *et al.*, 1993), which is known to decrease bone turnover and increase bone mineral density in the femur and lumbar bones (Teegarden *et al.*, 1999). Previous studies in our laboratory reported that feeding ovariectomized rats with flaxseed oil (10%) may help in preventing osteoporosis associated with estrogen deficiency (Boulbaroud *et al.*, 2008).

In the present study, the administration of whole flaxseed at different concentrations (5, 50 and 100%) did not appear to have such as beneficial effect on the bone mineral density of femurs of treated ones. These results agree with those reported in human studies which found that flaxseed had no significant changes in bone mineral density or symptoms in healthy menopausal women (Dodin *et al.*, 2005).

In summary, the consumption of flaxseed may offer a potential alternative therapy for the treatment of osteoporosis in ovarian deficient-women. Thus in the present experiment, the biological effects observed cannot attributed to particular constituents, as many compounds are present in the flaxseed. Whereas additional studies are needed to demonstrate their efficacy in humans and to elucidate their mechanism of action in animals.

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