Manipulation of flaxseed inhibits tumor necrosis factor-alpha and interleukin-6 production in ovarian-induced osteoporosis

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ABSTRACT

الأهداف: تقييم تأثير الحبوب الكاملة من بذور الكتان، وزيت بذور الكتان على السيتوكينات المولدة للالتهاب وذلك في الفئران التي أجريت لها عملية استئصال المبيض، وكذلك تأثير مثل هذه الأغذية على مرض هشاشة العظام.

الطريقة: أجريت هذه الدراسة في قسم الغذاء والتغذية بكلية الزراعة، جامعة الملك سعود، الرياض، المملكة العربية السعودية وذلك خلال الفترة من أكتوبر إلى ديسمبر 2009م. شملت الدراسة 48 جرذياً من النوع دوللي سبراغو والتي تبلغ أعمارها 3 أشهر، وقد تم تقسيمها عشوائياً إلى المجموعات التالية: المجموعة 1 وهي التي أطعمت طعاماً خادعاً مع تزويدها بحمية محددة، والمجموعة 2 وأجريت لها عملية استئصال المبايض مع حمية أساسية، والمجموعة 3 وأجريت لها نفس العملية مع إعطاؤها 20% من بذور الكتان، والمجموعة 4 وأجريت لها العملية مع 40% من بذور الكتان، والمجموعة 5 أجريت لها نفس العملية ولكن مع 5% من زيت بذور الكتان، والمجموعة 6 وخضعت لنفس العملية مع 10% من زيت بذور الكتان. لقد تم استئصال كلي المبيضين في الجرذان التي خضعت لعملية استئصال المبايض. استمرت هذه التجربة لمدة شهرين، وخلالها تم قياس وتحليل معدلات كلاً من: ألكالين فوسفاتيز، وانترليوكين– 6، وعامل تنخر الأورام ألفا، والكالسيوم، والفسفور، والمغنيسيوم.

النتائج: أشارت نتائج الدراسة إلى زيادة معدلات انترليوكين– 6، وعامل تنخر الأورام ألفا في الجرذان التي خضعت لعملية استئصال المبايض مقارنة بالمجموعة 1، في حين لم يكن هناك اختلافاً ظاهراً في معدلات كلاً من: ألكالين فوسفاتيز، والكالسيوم، والفسفور، والمغنيسيوم بين كافة المجموعات المشاركة في التجربة. وكان هناك نقصاً واضحاً في معدلات انترليوكين– 6، وعامل تنخر الأورام ألفا وذلك في المجموعات التي احتوت حميتها على بذور الكتان (المجموعة 3 و4) وكذلك المجموعات التي تناولت زيت بذور الكتان (المجموعة 5 و6).

خاتمة: اقترحت الدراسة إمكانية تأثير بذور وزيت الكتان على الوقاية من مرض هشاشة العظام الناتج عن نقص هرمون الاستروجين وذلك عن طريق خفض عمليات هدم العظام. ونحن بحاجة إلى المزيد من الدراسات من أجل تقييم عمليات الأيض لمثل هذه الأغذية وكيفية تأثيرها على عمليات هدم وبناء العظام.

Objectives: To evaluate the potential effects of whole flaxseed (FS), and/or flax oil (FO) incorporation into the diet on the level of pro-inflammatory cytokines in ovariectomized (OVX) rats model of osteoporosis.

Methods: This study was performed in the Food Science &Agriculture Collage, King Saud University, Kingdom of Saudi Arabia from October to December 2009. Forty-eight, 3-month-old female Sprague-Dawley rats were randomly divided into 6 groups: Group 1 - sham + control diet; Group 2 - OVX rats + basal diet; Group 3 - OVX + 20% whole FS; Group 4 - OVX rats + 40% FS; Group 5 - OVX rats + 5% FO; Group 6 - OVX rats + 10% FO. All OVX rats underwent bilateral ovariectomy. The experiment was continued for 2 months. Serum bone alkaline phosphatase (B-ALP), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), calcium (Ca), phosphorous (P), and magnesium (Mg) were measured.

Results: A significant increase of serum IL-6 and TNF-α concentrations were observed between OVX rats when compared with Group 1, while there was no significant difference in the activity of B-ALP, serum Ca, P, and Mg among all groups. A remarkable significant decrease of serum levels of IL-6 and TNF-α was observed in the group of rats that were fed with FS (Groups 3 and 4) and FO (Groups 5 and 6).

Conclusion: This study suggests that FS and FO might be useful in the prevention of estrogen-deficiency induced osteoporosis via decreasing osteoclastogenesis. Further studies are needed to demonstrate their efficacy in humans by using bioactive components of FS, and to clarify their mechanism of action.

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Ovarian hormone deficiency is a major risk factor for osteoporosis in postmenopausal women.[1,2] Estrogen replacement therapy (ERT) has long been used to alleviate postmenopausal symptoms and lowers the risk of osteoporosis. However, estrogen treatment is associated with a higher risk of certain types of cancer or contraindications.[3,4] As estrogen inhibits bone resorption, its deficiency will increase bone loss.[5] Estrogen down-regulates bone-resorbing cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and prostaglandin (PG) E2.[6] Phytoestrogens, are compounds found in plants and plant products that possess some estrogenic or antiestrogenic activity. Recent evidence suggests that phytoestrogens are non-steroidal plant compounds found in many fruits, vegetables, and grains. These phytoestrogens may act as estrogen antagonists in mammary gland, and in contrast, they act as estrogen agonists in bone.[7] Lignans, one type of phytoestrogen, are diphenolic compounds similar in structure to endogenous non-steroid hormones, and are hypothesized to act in vivo to alter hormone metabolism and subsequent bone metabolism.[8] Flaxseed (FS), one of the edible plant foods is the highest rich source of lignans, which are reported to have both weak estrogenic and anti-estrogenic activities.[9] Flaxseed is also a rich source of polyunsaturated fatty acid (PUFA), especially linolenic acid (18:3 [n-3]).[10] Alpha linolenic acid (ALA) may decrease the rate of bone resorption by inhibiting the biosynthesis of cytokines, such as PG, ILs and TNF.[11] Lignans present in FS may also possess antioxidant properties. Oxygen-derived free radicals, resulting from excessive production of reactive oxygen species perturb the normal redox balance of osteogenesis including bone formation and resorption. These findings indicate that free radicals have marked capacity to degenerate the bone metabolism, and enhance osteoclast formation and bone resorption. A previous study[12] demonstrated that oxidative stress is involved in the pathogenesis of bone loss in female rats due to chronic inflammatory diseases, aging, and osteoporosis. Therefore, FS may reduce the rapid rate of bone loss experienced by postmenopausal women in part, by enhancing antioxidant status and exerting a positive effect on the bone.[13] In a follow up study,[13] FS can potentially exert positive effects on bone of postmenopausal women. They assigned 60 postmenopausal women not on hormone replacement therapy receiving either 40 g FS, or 40 g wheat as control supplement for 3 months, and showed no amelioration of serum and urinary biomarkers of bone metabolism in the early evaluation, or whether longer-term study using bioactive components of FS, such as lignans or its oil can elucidate a positive influence on BMD. The n-3 PUFAs have anti-inflammatory properties that was mediated by the production of anti-inflammatory eicosanoids, which in turn offset the production of pro-inflammatory eicosanoids through competitive inhibition within their common metabolic pathways.[14] Limiting the production of pro-inflammatory eicosanoids, such as PGE2 may be an important factor in minimizing the production of IL-6, IL-1, and TNF-α, which may mediate inflammation-associated bone abnormalities.[15] The FS derived ALA has been shown to decrease in vivo PGE2 concentrations in rat bones.[16] In addition, flaxseed oil (FO) has been shown to decrease TNF-α and IL-1 in human peripheral blood mononuclear cells. Modulation of the dietary n-6 to n-3 ratio has been shown to be beneficial in various clinical inflammatory diseases,[17,18] and animal models of bone metabolism.[19-21] Little published data indicated a response of bone metabolism during intestinal inflammation to a diet rich in ALAs derived from FO. This information initiated our interest to examine the effect of dietary intake of FS and/or FO as therapeutic functional foods on pro-inflammation cytokines induced in ovariectomized (OVX) rats model osteoporosis.

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Methods. ***Animals.*** This study was performed in the Food Science & Agriculture Collage, King Saud University, Kingdom of Saudi Arabia from October to December 2009. Forty-eight female Sprague-Dawley rats aged 3 months, and weighing between 180-250 g were obtained from the university animal facility. Throughout the experiment, rats were housed in stainless steel cages with available water ad libitum. All animals were kept under normal healthy conditions and fed basal diet.

Experiment procedure. Rats were randomly divided into 6 groups (n=8 each). After one week of habituation to the facilities, the animals were used for the study. All OVX rats underwent bilateral ovariectomy via a dorsal approach with a small midline dorsal skin incision, the sham-operation rats (Group 1) were subjected to sham surgery exposure without removing the ovaries. After 2 weeks of recovery from the operation, Group 1 and OVX control group (Group 2) received basal diet without any addition of FS or FO. The other 4 groups were: Group 3 - OVX + 20% FS diet; Group 4 - OVX + 40% FS diet; Group 5 - OVX + 5% FO diet; and Group 6 - OVX + FO 10% (**Table 1**). There were 8 rats in each group. Dietary treatment was started 2 weeks post-ovariectomy, and continued for 2 months. After 2 months, the animals were sacrificed by cervical

dislocation under anesthesia; blood samples were obtained, and left to clot. Serum was separated after centrifugation at 3500 rpm for 15 minutes, and frozen at -70 °C until analysis.

Analytic methods. Serum B-ALP (a marker of bone formation), IL-6 and TNF-α (marker of bone resorption) were measured using enzyme linked immunosorbent assay (ELISA) method kits, B-ALP [AviBion, Orgenium Laboratories Division, Vantaa, Finland], TNF-α & IL-6 ELISA [Ani Biotech Oy, Orgenium Laboratories Division, Vantaa, Finland). The reading was carried out using ELISA microplate reader (VERSA Max, Molecular Devices Corporation, MN, USA). Serum calcium (Ca), phosphorus (P), and magnesium (Mg) were measured by enzymatic method as cited in the United Diagnostics Industry.[22,23] This study was approved by the IBR at the King Saud University of Kingdom Saudi Arabia.

Statistical analyses were carried out using 2-way analysis of variance (ANOVA), and paired t-test for normally-distributed samples. Data were analyzed using the Statistical Package for Social Sciences version 17.1 (SPSS Inc, Chicago, IL, USA). $P<0.05$ was considered significant.

Results. ***Effects of ovariectomy, FS and FO on food intake, body weight, and relative organ weight.*** Data on food intake, body weights and organ weights are shown in **Table 2**. The average food intake in Group 2 was significantly higher from Group 1 and other treated groups. The weight gains of rats in Group 2 were significantly higher (38%) than those of Group 1. The OVX rats showed atrophy of uterine tissues, however, there were no differences in the liver weight among the study groups (**Table 2**).

Effects of ovariectomy, FS, and FO on biochemical markers. The OVX, a known stimulus of bone resorption, induced a significant increase in each markers of pro-inflammation cytokines (IL-6 & TNF-α), above the

Table 1 - Composition of the control diet based on American Institute of Nutrition (AIN 93)[22] standard diet.

Components	Control	Flax	Flax	Flax oil	Flax oil
		powder	powder		
Casein	200	200	200	200	200
Cornstarch	367.50	167.5	0	367.50	367.48
Dyetrose (Dextrinized cornstarch (90-94% tetrasaccharides)	132	132	132	132	132
Sucrose	100	100	100	100	100
Cellulose	50	50	15	50	50
Corn oil	100	100	100	50	0
Flaxseed powder	0	200	400	0	0
Flaxseed oil	0	0	0	50	100
Butylhydroquinone	0	0.02	0.02	0.02	0.02
l-Cystine	3.0	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
Mineral mix	35	35	35	35	35
Vitamin mix	10	10	10	10	10

Both diets were modified AIN93G standard rodent diet and contained either 20, 40% FS or 5, 10% FS

Table 2 - Effects on ovariectomized rats whole flaxseed and flax oil on food intake, body weight, and relative organ weight.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
			Flaxseed		Flax oil	
Food intake, g/day/rat	10.84 ± 0.41	15.38 ± 0.40	13.16 ± 0.21	11.07 ± 0.49	12.05 ± 0.63	11.04 ± 0.82
P-value		0.001	0.008	0.003	0.01	0.004
Body weight, g						
Initial	155.4 ± 5.03	154.6 ± 9.04	154.0 ± 8.14	154.2 ± 6.73	154 ± 8.97	154.2 ± 8.54
Final	210 ± 6.98	242.6 ± 10.93	245.4 ± 8.33	265.2 ± 9.69	235.6 ± 7.82	234.4 ± 10.39
P-value		0.0001	0.0001	0.0001	0.0001	0.0001
*Organ weight**						
Uterus	0.32 ± 0.03	0.21 ± 0.05	0.19 ± 0.03	0.18 ± 0.04	0.20 ± 0.04	0.19 ± 0.06
Liver	5.22 ± 0.356	4.90 ± 0.293	5.40 ± 0.174	5.86 ± 0.122	4.74 ± 0.29	4.60 ± 0.354

Values are expressed as mean ± standard error, *g/100 g body weight

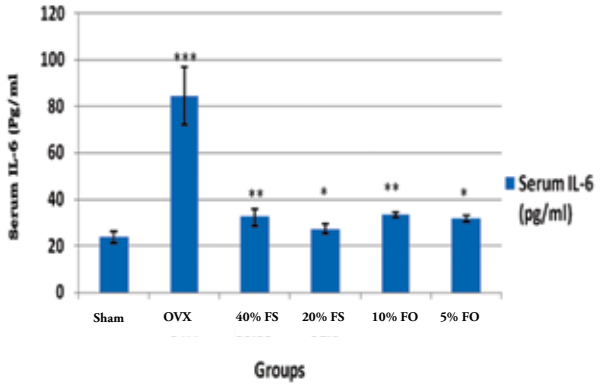

Figure 1 - Effect of dietary whole flaxseed (FS) and flax oil (FO) on serum interleukin (IL)-6. The IL-6 was determined by enzyme-linked immunosorbent assay method, ovariectomized rats (OVX) group compared with the Sham group. After treatment, FS groups (20% and 40%) compared with OVX group and FO groups (5% and 10%) compared with OVX group for 2 months. The plotted data represent the mean ± standard error for each dietary treatment group. For statistical differences between treatments **p*=0.03, ***p*=0.02, ****p*= 0.001

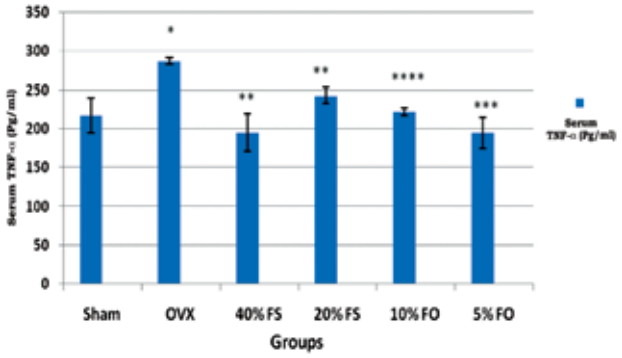

Figure 2 - Effect of dietary whole flaxseed (FS) and flax oil (FO) on serum tumor necrosis factor-alpha (TNF-α). The TNF-α was determined by enzyme-linked immunosorbent assay method. The ovariectomized rats (OVX) group was compared with the Sham group. After treatment, the FS groups (20% and 40%) was compared with the OVX groups, and FO groups (5% and 10%) were compared with the OVX group for 2 months. The plotted data represent the mean ± standard error for each dietary treatment group. For statistical differences between treatments. **p*=0.04, ***p*=0.03, ****p* =0.02, *****p*=0.0001

levels measured in Group 1. Thus, the mean value of serum IL-6 was 84.40 ±12.18 pg/ml in Group 2 versus 23.78 ± 2.62 pg/ml in Group 1 (*p*=0.01) (**Figure 1**), and that of TNF-α was 287.62 ±3.80 pg/ml in Group 2 versus 217.48 ± 22.19 pg/ml in Group 1 (*p*=0.04) (**Figure 2**). No significant difference was observed in the activity of serum B-ALP of Group 2 when compared with Group 1 (**Table 3**). For each marker, whole FS at Group 3, and Group 4 reversed the effect of OVX (**Figures 1 & 2**). Thus, the mean values of serum IL-6 was 32.33 ± 3.70 pg/ml (*p*=0.01) for Group 3, and 27.23 ± 2.03 pg/ml (*p*=0.02) for Group 4, and that of serum TNF-α was 195.50 ± 24.37 pg/ml (*p*=0.03) for Group 3, and 242.86 ± 0.78 pg/ml (*p*=0.03) for Group 4. The treatment of OVX rats significantly decreased the level of serum IL-6 in Group 5 (33.33 ± 1.19 pg/ml; *p*=0.03), and in Group 6 (31.60 ± 1.30 pg/ml; *p*=0.02), and serum TNF-α in Group 5 was 221.90 ± 4.70 pg/ml (*p*=0.03), and 194.96 ± 20.43 pg/ml (*p*=0.02) in Group 6 (**Figures 1 & 2**). There was no significant difference in the activity of serum B-ALP, Ca, P, and Mg among the different groups (**Table 3**).

Discussion. The present study showed positive effects of dietary FS on biochemical markers of bone remodeling in OVX rats model of osteoporosis.[24] It showed that the body weight significantly increased (63.1%), while the uterine weight decreased greatly (34.4 %) of OVX rats when compared with Group 1 (**Table 2**). This indicates that the animals had became estrogen deficient. The administration of FS rich in phytoestrogen at different concentrations has inhibited the increase of body weight, and did not affect the uterine weight.[24] The present data are consistent with some reports,[26,27] that has attributed the mechanism of action of some phytoestrogen to their high binding affinities to the intercellular estrogen receptors. They supported that these compounds may act in target tissues as agonist or antagonists in the absence of endogenous estradiol. This is in agreement with our results indicating that the weight of the uterus of the treated group did not differ significantly from that of the untreated groups.

Table 3 - The levels of biochemical parameters in ovariectomized rats, fed with whole flaxseed and flax oil after 2 months treatment.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
			Flax seed		Flax oil	
Serum Ca (mg/dl)	11.00 ± 0.21	11.15 ± 0.28	10.54 ± 0.33	11.23 ± 0.21	11.08 ± 0.20	11.15 ± 0.34
Serum P (mg/dl)	4.61 ± 0.16	5.13 ± 0.26	45.17 ± 0.14	4.73 ± 0.17	4.94 ± 0.16	4.75 ± 0.12
Serum Mg (mg/dl)	2.65 ± 0.04	2.61 ± 0.02	2.62 ± 0.07	2.65 ± 0.14	2.65 ± 0.04	2.61 ± 0.04
Serum B-ALP(pg/ml)	21.14 ± 1.56	20.30 ± 0.51	21.78 ± 1.84	22.48 ± 1.30	18.94 ± 0.85	19.08 ± 1.31

Values are expressed as mean ± standard error, Ca - calcium, P - phosphorus, Mg - magnesium, B-ALP - bone alkaline phosphatase

The obtained data showed that the OVX rats have significantly increased in biochemical parameters as observed with the increase of pro-inflammatory activity.[23] These results could be supported by the fact that pro-inflammatory cytokines are responsible for osteoclastogenesis and increased trabecular bone resorption after loss of sex steroids due to menopause.[28,29] The concentrations of serum Ca, P, and Mg showed no significant difference after ovariectomy, and these results are inconsistent with previous study[30] that serum Ca decreased in the OVX rats. In this study, 2 months treatment of OVX with whole FS and FO revealed significant decrease of serum IL-6 and TNF-α (markers of bone resorption) and no significant difference of serum B-ALP activity (a marker of bone formation), therefore a reduction of osteoclastic activity and establishment of osteoblastic activity, indicate down regulation of the bone turnover rate in OVX rats.

Flaxseeds is a rich source of lignans with diphenolic ring structures resembling those of endogenous estrogens that have potential weak estrogenic and antiestrogenic activity similar to that of the isoflavones found in soy.[31,32] Therefore, lignans as well as other phytoestrogens including daidzein and genistein,[33] were proven to have an anabolic effect on bone metabolism and prevented bone loss. The TNF-α stimulates osteoblasts to secrete other cytokines (IL-1B, IL-6) and PGE2, as well as osteoclasts to cause bone resorption.[34,33] Estrogen inhibits the IL-1, and TNF-α stimulated biosynthesis of IL-6 in stromal and osteoblastic cells.[36,37] It is hypothesized that suppression of the stimulatory effect of estrogen deficiency on bone metabolism is through the blocking any one of these cytokines.[38] This could help us to suggest that mechanism of action is possibly that the lignans in FS affect the bone through estrogen receptors.[39]

Several studies also suggested that FS lignans and soy isoflavones interfere with the normal physiologic activity and metabolism of estrogens, together with the ability to modulate estrogen metabolism that affect tissue exposure to biologically active estrogens (estradiol and 16-OHE1), which may inhibit pro-inflammation (IL-6 and TNF-α) and osteoclastogenesis.[40,41] There was an association between receptor activator of nuclear factor kappa-B ligand (RANKL) and the direct or indirect effect of TNF-α on osteoclastogenesis. The RANKL stimulate TNF-α expression in osteoclast precursor cells and homogenous populations of Raw264.7 cells.[42] The antioxidant and free radical scavenging properties of polyphenolic compounds in several plants has recently been reported.[43,44] Redox oxygen scavenges (ROS) are known to induce TNF-α expression, suggesting that ROS acts by increasing intracellular signals that induce TNF-α expression, rather than by augmenting signals that stimulate osteoclast formation and function.[45,46] Thus, in the present results, it can be suggested that TNF-α production in response to RANKL was reduced by FS lignan as polyphenolic compounds, and hence, FS were involved in affecting the viability and proliferation of osteoclasts, which may be associated with regulation of bone remodeling.

Flaxseed is also a very rich source of ALA,[47] which is known to decrease bone turnover and increase bone mineral density in the femur and lumber bones.[48] The FO contains approximately 56% ALA,[49] a precursor of eicosapentaenoic acid (EPA), has generated interest as a potential anti-inflammatory agent due to the ability of the ALA to be converted to EPA in human and animals.[30] The EPA-enriched diet is potent in preventing estrogen deficiency bone loss in OVX rats.[31] Thus, the present study suggested that FO might contribute to the prevention of osteoporosis by the beneficial effect on cytokines and prostaglandins production, and in consequent bone resorption markers. The limitation of the study: cannot be applied on human, difficult in volunteers and consents from the target people.

In conclusion, this study is encouraging and may be promising for the consumption of FS lignans as a potential alternative therapy to prevent osteoporosis associated ovarian deficient-women. However, the biological effects observed can attribute to particular contents, as many compounds are present in FS. Further studies are needed to demonstrate their efficacy in humans by using bioactive components of FS, and to clarify their mechanism of action.

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