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Hormonal and Metabolic Benefits of FlaxSeed Oil in Ovariectomized Rats

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Abstract

Menopause is associated with a decline in circulating estrogen levels, with a negative impact on bone, metabolism homeostasis and health. Flaxseed is the seed of the flax plant, and its supplementation shown to decrease the menopausal symptoms and improves the quality of life in postmenopausal women. This study investigated the effects of flaxseed oil supplementation on hormonal, lipid, hepatic, and bone homeostasis in ovariectomized rats. Thirty adult females were divided into three groups: I: Sham operated control group, II: Bilateral ovariectomized group (OVX) and III: Bilateral ovariectomized flaxseed oil-supplemented group (FLAX). All rats were subjected to measurement of plasma levels of Estradiol, FSH, MDA, TAC, TG, TC, HDL, BALP and IL-6. Histological examination of H&E-stained bone and liver tissues samples was performed. Ovariectomy results in a decrease in plasma estrogen level, increased plasma FSH level, increased oxidative stress, decreased total antioxidant capacity, increase in TG, TC, LDL, atherogenic index, decreased HDL, increase in BALP and IL-6. In addition to histopathological bone and liver changes in the form of bone demineralization, increased osteoclasts, bone erosions and liver congestions, apoptosis and inflammatory infiltrates. Flaxseed oil supplementation to ovariectomized rats had a marked beneficial effect and results in improvements in all the mentioned post-ovariectomy changes, in addition to improvements in the histopathological bone and liver findings. It is concluded that supplementation with flaxseed oil is of great value in decreasing or inhibiting the hormonal, lipid, bone and liver impairments and imbalances induced by ovariectomy.

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INTRODUCTION

Menopause is associated with a decline in circulating estrogen levels, with subsequent negative crucial impact on bone, metabolic homeostasis and health **[1,2]**. Further, the menopause-related lower circulating estrogen levels result in a decrease in the antioxidant capacity, and subsequently postmenopausal women have high levels of oxidative stress **[3]**, which could be restored to normal by oestrogen replacement therapy **[4]**.

An effective experimental model of menopause is the ovariectomized (OVX) rat. In this rat model, metabolic and osteoporotic postmenopausal manifestations could be studied, besides providing the advantages of studying application of different strategies to improve menopause-related dyshomeostasis **[5,6]**.

Menopausal estrogen replacement therapy has been considered as an effective treatment of menopausal related hormonal, metabolic and bone changes. However, studies related the estrogen replacement with the increased risk of cancer breast and uterine endometrium **[7,8]**.

Alternative strategies for health improvement of menopausal effects have been developed to reduce the risk caused by estrogen replacement therapies. These strategies include non-pharmacological approaches such as exercise training, and include the use of herbal medicines **[9,10]**.

Flaxseed is the seed of the flax plant (Linum usitatissimum L.), and it is commonly named linseed, was discovered to be a rich source of lignans, and so it exerts a strong antioxidant effect **[11]** and an anti-inflammatory effect **[12,13]**. Moreover, flaxseed supplementation shown to decrease the menopausal symptoms and improves the quality of life in postmenopausal women **[14]**.

This study aim to investigate the effects of flaxseed oil supplementation on ovarian, lipid, hepatic and bone homeostasis in ovariectomized rats. Also, to investigate the possible underlying mechanism(s) of flaxseed oil.

Materials and Methods:

Experimental Protocol:

Site of Research:

Physiology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

Animals:

Thirty adult female Wistar rats, initially weighing 140-180 grams, were purchased from the Animal Farm (Helwan), Egypt, and were housed in Animal House of Ain Shams Medical Research Centre (5 rats / cage) with suitable ventilation, temperature of 22-25^{o}C, 12 hours light dark cycle and free access to food (standard rat chow) and water *ad libitum.*

After one week of acclimatization, rats were randomly and equally divided into the following three groups:

GROUP I: Sham operated control Group (control): rats of this group were subjected to all surgical procedures of ovariectomy except for removal of the ovaries. This group received distilled water daily by gavage for four successive weeks in equivalent dose to flaxseed oil given to the supplemented group.

GROUP II: Bilateral ovariectomized Group (OVX): rats of this group were subjected to removal of both ovaries by ovariectomy operation. This group received distilled water daily by gavage for four successive weeks in equivalent dose to flaxseed oil given to the supplemented group.

GROUP III: Bilateral ovariectomized flaxseed oil-supplemented Group (FLAX): rats of this group were subjected to removal of both ovaries by ovariectomy operation. Then, received 4 ml/kg flaxseed oil daily by gavage for four successive weeks. **[15]**

All rats care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared according to guidelines of animal use and according to National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No 8023, revised 1978).

Ovariectomy operation:

After one week accommodation in the animal house of Ain Shams Medical Research Centre, rats were subjected to surgical intervention by either sham-operation or bilateral ovariectomy.

Ovariectomized rats were subjected to bilateral ovariectomy, as described by **Ingie and Griffith [16]**. ***In the sham-operated groups***, rats were subjected to the same surgical manipulation except for removal of the ovaries.

After one week recovery, each group was assigned to the respective daily supplementation.

Experimental Procedures:

At the end of the 4 weeks experimental period, all rats were subjected to the following measurements:

On the day of sacrifice, overnight fasted rats were weighed and anaesthetized with i.p. injection of Pentobarbitone (40 mg/kg B.W.). Then, an abdominal midline incision made and blood samples collected from abdominal aorta into a heparinized tube. Blood of heparinized tubes was centrifuged, and the separated plasma was used for subsequent determination of:

Ovarian function tests: plasma Estradiol and FSH were measured by using Enzyme linked immunosorbent assay (ELISA) kits (Cusabio, China)

Oxidative stress markers: plasma malondialdehyde (MDA) and total antioxidant capacity (TAC) were measured by Colorimetric Method, using kits supplied by Bio-Diagnostic, Egypt.

Lipid profile: plasma triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL) & low-density lipoprotein cholesterol (LDL) were calculated. Atherogenic index was calculated according to **Grundy et al. [17]** as follows: Atherogenic index = Total cholesterol / HDL-C.

Bone specific alkaline phosphatase (BALP): BALP was measured by using enzyme-linked immune sorbent assay (ELISA) kits (Cusabio, China)

Inflammatory markers: interlukin-6 (IL6) was measured by using Rat Interleukin-6 ELISA Kit (MyBioSource, USA)

Histological Examination of bone and liver Tissues: Samples of median lobe of liver, and samples of left femoral bone in all studied groups were fixed in 10% formalin. Paraffin embedded sections of 5-μm thickness were stained with Hematoxylin and Eosin (H&E) examined by light microscopy for histopathological studies **[18].**

Statistical Analysis

All results in this study were expressed as mean ± SEM. Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA) program, version 20.0 was used to compare significance between each two groups. One Way ANOVA (Analysis Of Variance) for difference between means of different groups was performed on the results

obtained in the present study. Differences were considered significant when P is ≤ 0.05.

Results of the study are demonstrated in the following tables and figures:

Results:

Table-1: Mean± SEM of plasma estradiol, FSH, malondialdehyde (MDA), total antioxidant capacity (TAC), bone-specific alkaline phosphatase (BLAP) and inteleukin-6 (IL-6) in the different studied groups.

	Control	OVX	FLAX
ESTRADIOL (ng/ml)			
Mean			9.03
± SEM	12.8	5.12	± 0.37
P	± 0.98	± 0.55	< 0.001
P*		< 0.001	< 0.001
FSH (ng/ml)			
Mean	14.5	53.7	26.8
± SEM	± 0.64	± 2.07	± 3.08
P		< 0.001	< 0.001
P*			< 0.001
MDA (nmol/ml)			
Mean			64.2
± SEM	31.2	125.4	± 5.43
P	± 2.23	± 11.2	< 0.001
P*		< 0.001	< 0.001
TAC (nmol/ml)			
Mean			52.9
± SEM	68.9	24.7	± 2.10
P	± 3.80	± 3.62	< 0.01
P*		< 0.001	< 0.001
BALP (u/l)			
Mean	43.2	103.4	64.3
± SEM	± 38.0	± 86.6	± 49.8
P		< 0.001	< 0.05
P*			< 0.001
IL-6 (pg/ml)			
Mean	30.7	103.3	68.6
± SEM	± 1.54	± 5.68	± 2.79
P		< 0.001	< 0.001
P*			< 0.001

Values are mean ± SEM of 10 rats in each group.
P: Significance from the control group by LSD, at least P≤0.05
P*: Significance from the ovariectomized (OVX) group by LSD, at least P≤0.05
FSH: follicle stimulating hormone
MDA: malondialdehyde
TAC: total antioxidant capacity
BALP: bone-specific alkaline phosphatase
IL-6: inteleukin-6

Table (1) shows a significant decrease in plasma estadiol levels, associated with a significant increase in plasma FSH level in the OVX group and in the FLAX group when each was compared to the control group. However, the FLAX group estadiol level was significantly increased, and the FSH level was significantly decreased when compared to the OVX group.

In addition, table (1) shows a significant decrease in plasma TAC levels, associated with a significant increase in plasma MDA level in the OVX group and in the FLAX group compared to

the control group. With flaxseed oil supplementation, the FLAX group TAC level was significantly increased, and the MDA level was significantly decreased when compared to the OVX group.

Moreover, table (1) shows a significant increase in each of plasma BALP and IL-6 levels in the OVX group and in the FLAX group compared to the control group. With flaxseed oil supplementation, the FLAX group BALP and IL-6 levels were significantly decreased compared to the OVX group.

Table-2: Mean± SEM of lipid profile in the different studied groups.

	Control	OVX	FLAX
TG (mg/dl)			
Mean	86.9	123.7	98.2
± SEM	± 3.23	± 4.15	± 2.47
P		< 0.001	< 0.05
P*			< 0.001
TC (mg/dl)			
Mean	132.70	281.9	180.2
± SEM	± 2.47	± 10.5	± 5.2
P		< 0.001	< 0.001
P*			< 0.001
HDL (mg/dl)			
Mean	59.3	31.9	46.1
± SEM	± 1.76	± 4.15	± 1.59
P		< 0.001	< 0.01
P*			< 0.01
LDL (mg/dl)			
Mean	73.4	250.0	134.1
± SEM	± 2.52	± 11.2	± 5.05
P		< 0.001	< 0.001
P*			< 0.001
Atherogenic index			
Mean	2.24		3.93
± SEM	± .070	9.48	± .171
P		± 1.08	NS
P*		< 0.001	< 0.001

Values are mean ± SEM of 10 rats in each group.
P: Significance from the control group by LSD, at least P≤0.05
P*: Significance from the ovariectomized (OVX) group by LSD, at least P≤0.05
TG: triglycerides
TC: total cholesterol
HDL: high density-lipoprotein cholesterol
LDL: low density-lipoprotein cholesterol

Table (2) shows a significant increase in all of plasma TG, TC and LDL associated with a significant decrease in HDL level in the OVX group and in the FLAX group compared to the control group. However, upon flaxseed oil supplementation, the FLAX group TG, TC and LDL levels were significantly decreased, and the HDL level was significantly increased when compared to the OVX group.

The calculated atherogenic index was significantly increased in the OVX group compared to the control group, and was significantly decreased in the FLAX group compared to the OVX group.

Histopathological results:

Bone:

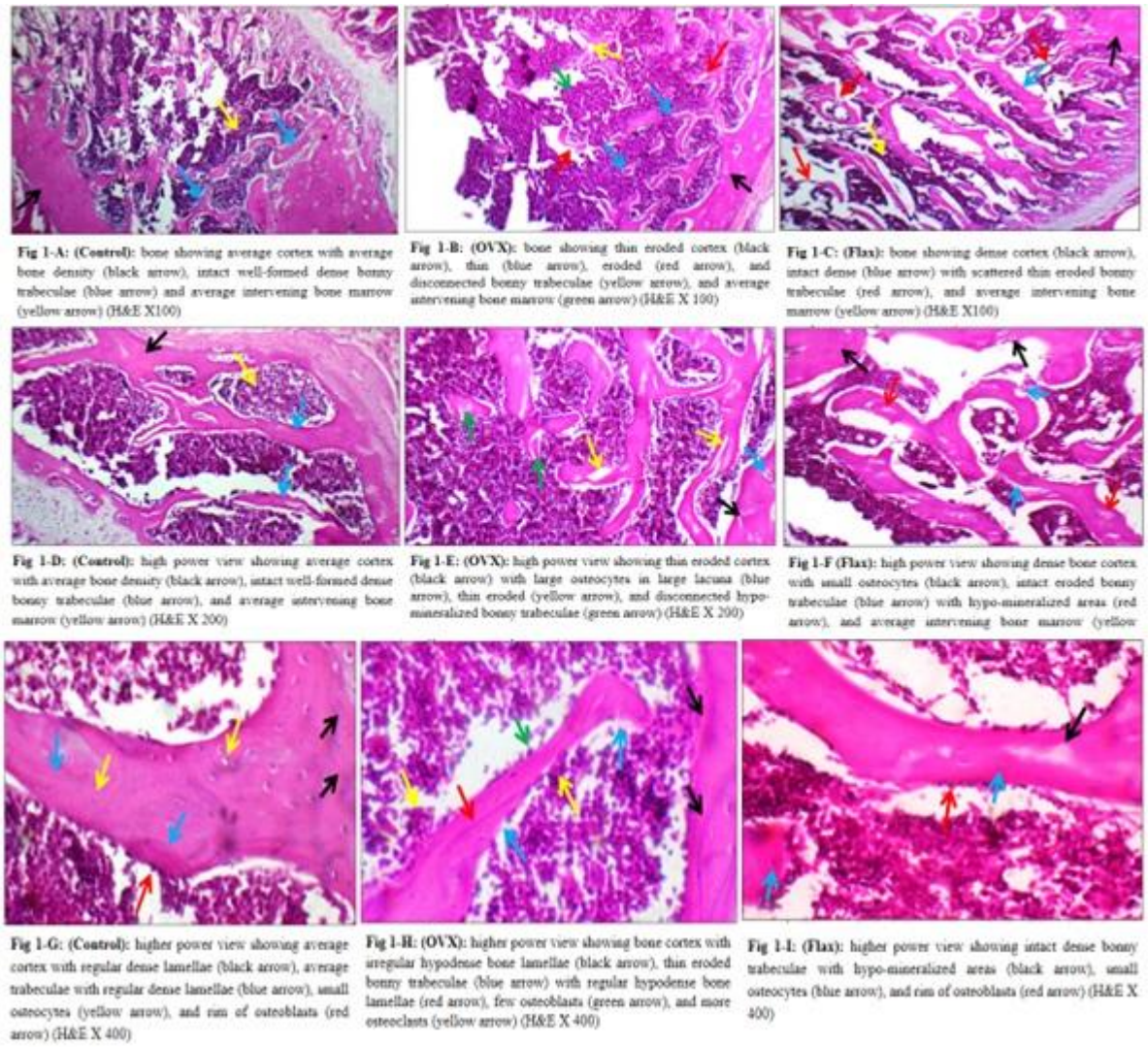

Figure (1): Bone histopathological changes in the different studied groups

- **Group 1; Control:** bone showed average cortex with average bone density, regular dense bone lamellae an small osteocytes, intact well-formed dense bonny trabeculae with regular dense bone lamellae, small osteocytes, rim of osteoblasts and few osteoclasts, and average intervening bone marrow **(Fig 1-A, D & G)**
- **Group 2; OVX:** bone showed thin eroded cortex with regular hypodense bone lamellae and large osteocytes, thin, eroded and disconnected hypo-mineralized bonny trabeculae with irregular hypodense bone lamellae, large osteocytes, few osteoblasts with marked osteoclastic activity, and average intervening bone marrow **(Fig 1-B, E & H)**
- **Group 3; Flax:** bone showed dense cortex with regular dense bone lamellae and small osteocytes, intact dense with scattered thin eroded bonny trabeculae with regular dense bone lamella, few large osteocytes, hypo-mineralized areas, rim of osteoblasts with scattered osteoclasts, and average intervening bone marrow **(Fig 1-C, F & I).**

Liver:

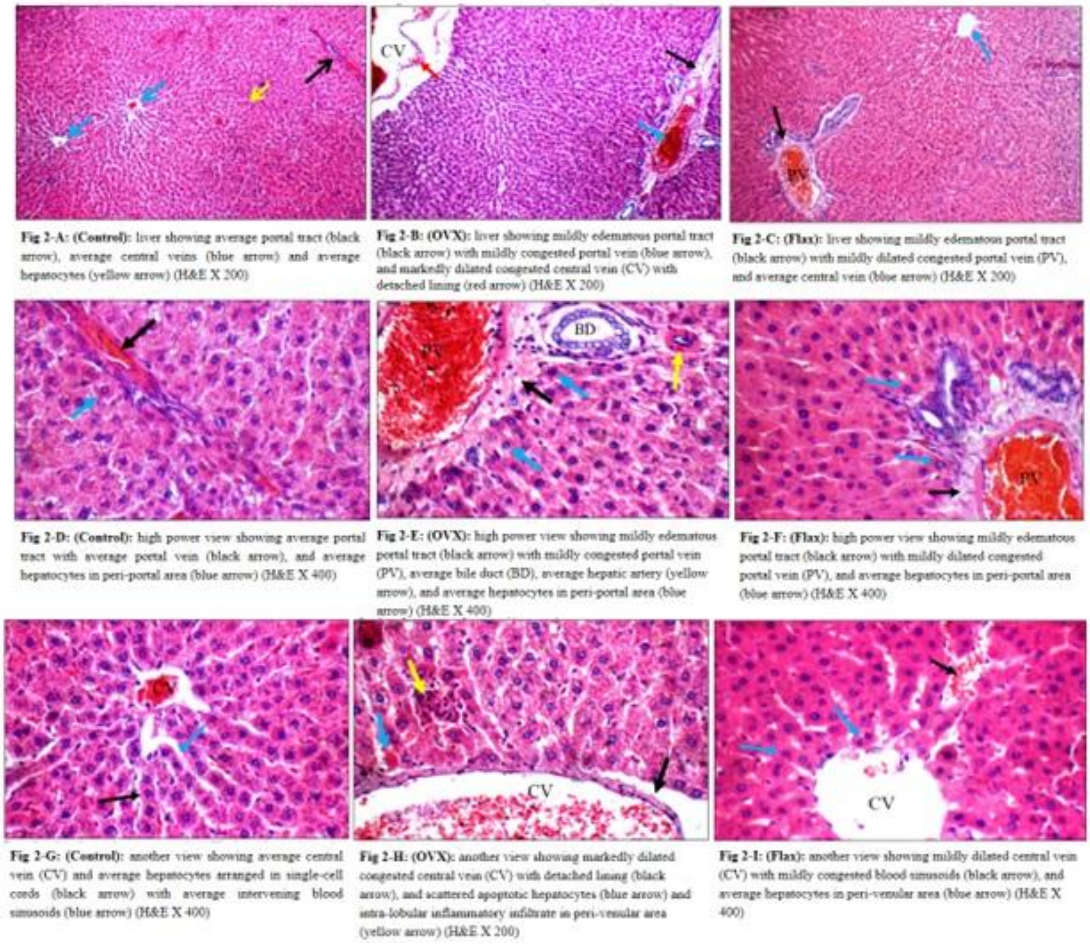

Fig 2-A: (Control): liver showing average portal tract (black arrow), average central veins (blue arrow) and average hepatocytes (yellow arrow) (H&E X 200)

Fig 2-B: (OVX): liver showing mildly edematous portal tract (black arrow) with mildly congested portal vein (blue arrow), and markedly dilated congested central vein (CV) with detached lining (red arrow) (H&E X 200)

Fig 2-C: (Flax): liver showing mildly edematous portal tract (black arrow) with mildly dilated congested portal vein (PV), and average central vein (blue arrow) (H&E X 200)

Fig 2-D: (Control): high power view showing average portal tract with average portal vein (black arrow), and average hepatocytes in peri-portal area (blue arrow) (H&E X 400)

Fig 2-E: (OVX): high power view showing mildly edematous portal tract (black arrow) with mildly congested portal vein (PV), average bile duct (BD), average hepatic artery (yellow arrow), and average hepatocytes in peri-portal area (blue arrow) (H&E X 400)

Fig 2-F: (Flax): high power view showing mildly edematous portal tract (black arrow) with mildly dilated congested portal vein (PV), and average hepatocytes in peri-portal area (blue arrow) (H&E X 400)

Fig 2-G: (Control): another view showing average central vein (CV) and average hepatocytes arranged in single-cell cords (black arrow) with average intervening blood sinusoids (blue arrow) (H&E X 400)

Fig 2-H: (OVX): another view showing markedly dilated congested central vein (CV) with detached lining (black arrow), and scattered apoptotic hepatocytes (blue arrow) and intra-lobular inflammatory infiltrate in peri-venular area (yellow arrow) (H&E X 200)

Fig 2-I: (Flax): another view showing mildly dilated central vein (CV) with mildly congested blood sinusoids (black arrow), and average hepatocytes in peri-venular area (blue arrow) (H&E X 400)

Figure (2): Liver histopathological changes in the different studied groups

- **Group 1; Control :** liver showed average portal tracts with average portal veins and average hepatocytes in peri-portal area, average central veins with average hepatocytes arranged in single-cell cords with average intervening blood sinusoids **(Fig 2-A, D & G)**
- **Group 2; OVX:** liver showed mildly edematous portal tracts with mildly congested portal veins and average hepatocytes in peri-portal area, and markedly dilated congested central veins with detached lining, scattered apoptotic hepatocytes with mild intra-lobular inflammatory infiltrate in peri-venular area **(Fig 2-B, E & H)**
- **Group 3; Flax:** liver showed mildly edematous portal tracts with mildly congested portal veins and average hepatocytes in peri-portal area, and mildly dilated central veins with mildly dilated congested blood sinusoids, and average hepatocytes in peri-venular area **(Fig 2-C, F & I)**

Discussion:

The present study investigated the effects of the flaxseed oil supplementation on hormonal, metabolic, liver and bone changes in ovariectomized rats. Ovariectomy results in a decrease in plasma estrogen level, increased plasma FSH level, increased oxidative stress, decreased total antioxidant capacity, increase in TG, TC, LDL, atherogenic index, decreased HDL, increase in BALP and IL-6. In addition to histopathological bone and liver changes in the

form of bone demineralization, increased osteoclasts, bone erosions and liver congestions, apoptosis and inflammatory infiltrates. Flaxseed oil supplementation to ovariectomized rats had a marked beneficial effect and results in improvements in all the mentioned post-ovariectomy changes, in addition to improvements in the histopathological bone and liver findings.

Ovariectomy is an experimental model similar to postmenopausal period, which is associated with increased risks of bone and metabolic diseases. In addition, the lack of estrogen and the increased oxidative and inflammatory stress seem to be major underlying causes of increased risk of diseases, morbidity and mortality during this period [**19]**.

As the impairment of estrogen secretion can contribute to several postmenopausal dyshomeostatic pathways, we first evaluated the effects of ovariectomy on plasma levels of estrogen and FSH. In the present study, a lowered estradiol and elevated FSH levels were detected in ovariectomized rats. Similar findings were detected regarding this effect of ovariectomy on hormonal balance of pituitary-ovarian axis [**20]**, where a lowered estrogen and significantly elevated FSH levels were found in serum of ovariectomized rat group compared with sham group.

Ovariectomy-induced decreased estrogen level, results in changes in the plasma lipid levels and increased tendency for body fat deposition. The increased TG, TC, LDL, atherogenic index, and the decreased HDL in OVX group of the present study is in support to these findings. These detected changes in plasma lipids are in agreement with previous studies [**19,21,22]**, that all detected similar changes in lipid profile in ovariectomized rats.

The ovarian hormone deficiency also increases the generation of reactive oxygen species (ROS), which could result in cell damage or death. MDA level as a marker of lipid peroxidation was increased in the OVX group of this study, and at the same time, the plasma TAC was decreased, reflecting the increased oxidative stress and the improper antioxidant status in absence of estrogen in ovariectomized rats. The estrogen decrease is a major cause of defective production of natural antioxidant enzymes in the body, which results in increased Reactive Oxygen Species (ROS) and Oxidative Stress [**23].** ROS produce lipid peroxidation, which increases the level of Malondialdehyde (MDA) in plasma, reflecting the low antioxidant status [**24]**.

In the present study, OVX showed an increase in BALP and IL-6. Similar findings were detected of increased serum alkaline phosphatase level [**25**] and a higher expression of the proinflammatory cytokines IL-6 in ovariectomized rats than in the Non-ovariectomized rats [**26**]. These findings, together with the histopathological morphology of bone tissue of OVX rats of the present study, which showed eroded cortex with hypodense bone lamellae, thin, eroded and disconnected hypo-mineralized bonny trabeculae, few osteoblasts with marked osteoclastic activity, indicate the osteoporotic bone changes of OVX rats. BALP is an enzyme that can be used as an index for the rate of bone turnover, giving an idea about the relation between bone mineralization, bone resorption and bone formation. Its level is increased in osteoporosis due to a high bone turnover rate **[27**]. IL-6 is a bone-resorbing proinflammatory cytokine, that activates

osteoclastogenesis through enhancing osteocyte-mediated osteoclast differentiation [**28**]. The detected bone osteoporotic changes induced by ovariectomy is in agreement with many literatures that ovariectomy attenuated bone microstructure and bone mass [**29**], increases expression of the proinflammatory cytokine IL-6 [**26**], and reduce femoral bone mineral density [**30**]

OVX group of the present study also showed hepatic structural changes, demonstrated histopathologically as mildly edematous portal tracts with mildly congested portal veins and average hepatocytes in peri-portal area, and markedly dilated congested central veins with detached lining, scattered apoptotic hepatocytes with mild intra-lobular inflammatory infiltrate in peri-venular area. These OVX-induced hepatic changes are believed to be due to oxidative stress, which increases lipid peroxidation, with the production of reactive compounds that have the ability to induce membrane lesions and disruption of membrane permeability [**21**], and also capable of cross-linking DNA proteins, with the resultant of cell apoptosis [**31**].

Flaxseed oil supplementation to ovariectomized rats in the present study produced marked improvements in the hormonal, metabolic, bone and liver changes detected in the OVX unsupplemented group. The mechanisms by which flaxseed exerts these improvements are not clear. The high efficacy of flaxseed against postmenopausal changes may be due to high content of polyunsaturated omega-3 fatty acids (e.g. α-linolenic acid), phytoestrogens oestrogen-like compounds), soluble fiber [**32],** beside its antioxidant **[33]** and anti-inflammatory [**34]** capacity.

Previous studies have investigated the effects of flaxseed oil or its bioactive in postmenopausal women and on the quality of life. The quality of life improved and the postmenopausal symptoms decreased with flaxseed supplemented diet in women [**14]**. An earlier study reported similar beneficial effects of flaxseed in posmenopausal women **[35]**. However, some studies concluded no significant effects of flaxseed on postmenopausal quality of life [**36].**

Flax group showed an increase in plasma estrogen and a decrease in plasma FSH compared to the OVX group. Similar findings were detected where the level of oestrogen in ovariectomized rats treated with flaxseed oil was significantly increased in comparison with the OVX group [**37]**.Thus, phytoestrogens of flaxseed oil exert positive effects in situations of hypoestrogenism, and it was suggested that they bind to estrogen receptors, specifically β receptor, which is predominant in the bone and brain [**38]**,

Moreover, the dyslipidemia detected in the OVX group of the present study was reversed by flaxseed oil supplementation to ovariectomized rats. The role omega-3 fatty acid supplementation in correction of dyslipidemia and improving lipid profile, by lowering the TG, TC, LDL, and by increasing the HDL levels is also shown in other studies **[39,40,41**].

FLAX group in the present study showed an enhanced plasma TAC together with reduction in the lipid peroxidation product MDA, reflecting the superior role played by flaxseed oil as an antioxidant agent that restore the oxidant/antioxidant imbalance induced bet ovariectomy. These data are in agreement with previous reports that flaxseed oil restored the

disturbed enzymatic antioxidant barrier in many disease states [**42,43].**

Meanwhile, the BALP and the IL-6 plasma levels were decreased in the FLAX group of rats of the current study compared to OVX rats, this was associated with improvements in the histopathological bone studies in the form of regaining of the dense cortex with regular dense bone lamellae. This effect is believed to be due to the antioxidant and the anti-inflammatory properties of flaxseed oil components. Flaxseed oil supplementation was able to significantly decrease the elevated level serum of the proinflammatory cytokine IL-6 [**44**], to lower bone resorption marker in postmenopausal women [**45**]. It was observed that OVX rats fed flaxseed oil and sesame oil showed improved bone histopathological morphology, less destruction and elongated trabeculae, and aid in the prevention of osteoporosis associated with estrogen deficiency [**46].** The bone-protective effect of flaxseed is also supposed to be due its high content of PUFA. PUFA are well known anti-inflammatory agents that inhibit the production of proinflammatory cytokines [**34]**, increase the production of insulin-like growth factor-1, thus improving the mechanical properties of cortical bone [**47]** and decrease bone loss in ovariectomized animals by downregulation of osteoclastogenic factors [**48**], and reduce bone loss in ovariectomized mice**.**

Hepatic histopatholigical studies in FLAX group of the present study revealed improvements in the hepatic morphology than that detected in OVX rats, suggesting a hepatoprotective role of flaxseed oil in ovariectomy-induced hepatic changes. In accoirdance with these findings, previous experimental work concluded that flaxseed oil supplementation maintained the normal level of hepatic enzymes, prevented carbon tetrachloride (CCl4)-induced hepatotoxicity and reduced the level of hepatic lipid peroxide [**49**]. Moreover, it was suggested that observed that the antioxidant property of flaxseed oil phenols, flavonoids and other phytochemical components are able of reducing oxidative stress and inflammation, thereby are protective against organ toxicity and damage [**50]**.

From the results obtained in this study, it could be concluded that supplementation with flaxseed oil is of great value in decreasing or inhibiting the hormonal, lipid, bone and liver impairments and imbalances induced by ovariectomy. However, further studies are needed to elucidate the underlying detailed mechanisms of action and the effect of different dosage forms of flaxseed oil that make it possible to use flaxseed oil as an alternative or complement supplementation to increase life quality and to reduce symptoms of menopause.

Limitation of the study: The authors recommend more studies for investigating the comprehensive effects of flaxseed oil administration in postmenopausal states on the effects of menopause on other organ, specially the cardiovascular system. Also, for investigating the different doses and durations of flaxseed oil supplementation on liver and bone postmenopausal changes.

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Competing interests: None

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