ORIGINAL ARTICLE

PROTECTIVE EFFECT OF FLAXSEED OIL AGAINST EXEMESTANE INDUCED OSTEOPOROSIS IN ANIMAL MODEL

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Background: Exemestane, an aromatase inhibitor is used as an adjuvant to chemotherapy for early breast cancer. The objective of this study was to see the effect of dietary flaxseed oil on bone mass density in mice model of osteoporosis induced by exemestane. **Methods:** This study examined three animal groups over 25 days: a Control Group on normal diet, Group A receiving exemestane (2 mg/Kg), and Group B receiving both exemestane and 10% flaxseed oil. Pre- and post-experiment weights were compared using paired *t*-tests. Serum oestradiol levels and femur trabecular diameter between the control and exemestane groups were compared unpaired *t*-tests. Chi-square test was used to analyse osteoclast cell counts between the two groups. **Results:** Exemestane caused statistically significant effect on weight loss ($p<0.05$) in Group A and B. The flaxseed group (Group B) showed a smaller increase in trabecular diameter ($p<0.05$) and a lesser pronounced decrease in serum oestradiol level ($p<0.05$) as compare to Group A. There was statistically significant difference in the number of osteoclasts between groups and Group A had more osteoclasts as compared to Group B ($p<0.05$). **Conclusion:** Combined administration of flaxseed with exemestane in mice resulted in preservation or minimal reduction of bone mass density in animal model.

Keywords: Breast cancer, Exemestane, Oestradiol, Flaxseed oil, Osteoporosis

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INTRODUCTION

Breast cancer is the third most common cancer in women around the world. The risk factors for development of breast cancer includes increasing age, obesity, family history, early menarche, low socioeconomic status, physical inactivity and use of combined oestrogen-progesterone hormone pills in menopause. Research has shown that women in their postmenopausal period have almost 10–50 fold higher levels of oestradiol in endometrium as well as in the breast cancer tissues as compared to blood.[1] The worldwide reported cases of breast cancer are around 1.3 million as stated by American Cancer Society.[2] Its occurrence is higher in Asian population than the developed countries of Europe and America. In South Asia, Pakistan has 2.5 times higher incidence than India and Iran, and usually patients present with advanced stage of disease resulting in high mortality.[3] According to Karachi Cancer Registry, the contribution of breast cancer is 34.6% to all cancers.[4] Projected incidence in Pakistan has been estimated to be 60.7% in 2025 in women age 60–64 years.[5]

The treatment options of breast cancer are radiotherapy, chemotherapy, hormonal therapy and surgery.[6] Exemestane is an aromatase inhibitor, approved by FDA for breast cancer.[7] It is a third-generation steroidal aromatase inhibitor (SAI), given to postmenopausal women having metastatic breast cancer. The drug is well tolerated by most of the patients and has superior efficacy as compared with Tamoxifen.[8] Exemestane irreversibly inhibits aromatase enzyme. The oral administration of Exemestane at the dosage of 10 mg/day is associated with 85–95% reduction in plasma oestrogen level.[9] However, it results in deep suppression of circulating oestrogen concentrations in postmenopausal women, frequently below the detection limits of standard assays. This leads to decrease in bone mass in postmenopausal women, leading to increased risk of bone fractures and osteoporosis. Hormonal therapy is the treatment of choice for osteoporosis but it has some side-effects and can be shifted to non-hormonal therapy.[10]

Flaxseed is a commonly used natural product which has good nutritional and health effects and benefits. Flaxseed oil is rich in α-Linolenic acid (ALA) which is a plant derived Omega-3 poly unsaturated fatty acid and recommended as nutrient. Flaxseed oil also contains sodium, potassium, carbohydrate, fibre, sugars, proteins and vitamin-D. Flaxseed oil has protective role in normal functions of the body systems and also has immune boosting effects.[11]

The present study aimed to determine the preventive role of flaxseed oil against the adverse effects of exemestane therapy in animal model.

METHODOLOGY

This was a laboratory-experimental study conducted from Feb to Jul 2022 at Khyber Girls Medical College and Khyber Medical University, Peshawar. The Animal

Ethics Committee, Khyber Medical University Guidelines were followed for the experimental study. Ethical approval was obtained from the Advanced Studies and Research Board (Reference No. ASRB001374/HS/KGMC). Sample size was calculated using G-power with an effect size of 0.25, α=0.05, and 95% power of the study. The total sample size was 30. Female albino mice aged 6–7 weeks, weighing 20–40 grams were obtained from the National Institute of Health (NIH), Islamabad and subdivided in to 3 groups according to the study protocol (Table-1).

A simple random sampling technique was used. Healthy adult female albino mice were included while sick and/or pregnant mice were excluded from the study. Mice were provided standard laboratory pellet feed and water freely available at all times, and maintained in their respective cages for 25 days with normal light/dark cycle.

After completion of the experiment, animals were sacrificed and femur bones were collected, sectioned and stained for morphological study (measurement of osteoclast cells) and scores were given as per standard histopathologic criteria for bone study (Table-2). Blood was collected through cardiac puncture for estimation of serum oestradiol levels with ELISA method. The trabecular diameter of the femur was measured. Data was analysed on SPSS-25. Quantitative variables were presented as mean values and categorical variables as frequencies and percentages. For continuous variables like weight and oestradiol levels, paired-sample *t*-test was applied; for categorical variables like number of osteoclast cells, Chi-square test was used, and $p \leq 0.05$ was considered statistically significant.

Table-1: Distribution of mice in different groups, drugs, and food categories

Group	Animals	Food and treatment given
Control	6	Standard food and water only
A	12	Standard food and water+exemestane 1 mg/Kg/day
B	12	Standard food and water+exemestane 1 mg/Kg/day+1 mL of 10% flaxseed oil/day

Table-2: Histopathologic criteria for bone assessment

Score 0	Normal osteoblast cells (no osteoclasts)
Score 1	Few osteoclast cells (<5% of most damaged bone surfaces are lined)
Score 2	Some osteoclast cells (5–25% of the most damaged bone surfaces are lined)
Score 3	Many osteoclast cells (25-50% of most damaged bone surfaces are line
Score 4	Numerous osteoclast cells (> 50% of most damaged bone surfaces are lined)

RESULTS

Paired *t*-test was applied for comparison of mean weight of three groups of mice before and after the experiment. In Group A and Group B there was statistically significant effect on weight loss as compared with Control Group ($p<0.05$). (Table-3).

When trabecular diameter was compared between Group-A and Group-B, a significant difference was found between two groups in diameter of trabeculae ($p<0.05$) (Table-4).

The experimental Groups A and B were categorized according to the number of osteoclasts as having 2, 3, or 4 osteoclasts in subgroup. There were statistically significant differences in the number of osteoclasts between Group A and B ($p<0.05$). (Table- 5).

The mean serum levels of oestradiol were compared between Groups A and B with paired *t*-test. There were significant differences in mean serum level of oestradiol between two groups ($p<0.05$). (Table-6).

Histological sections were cut for the femur bones of mice in the three different groups. The mice in Group A showed signs of adverse effects of the drugs and the bones were spongier with larger trabecular diameter on eosin and haematoxylin staining (Figure-1 A).

The mice in Group B in comparison to Group A showed little or no signs of adverse effects of the drug and the bones were less spongy and no erosion with smaller trabecular diameter on eosin and haematoxylin staining (Figure-1 B).

The large multinucleated cells in the histological section were osteoclasts representing re-modelling activity of bone in Group A, after exemestane administration to albino mice. The multinucleated cells in the histological section in Group B (Figure-1 C) were less frequent (Figure-1 D).

Table-3: Comparison of mean weight of groups pre- and post-experiment (Mean±SD)

Group	Weight before experiment	Weight after experiment	*t*-statistics	*p*
Control	27.80±2.168	27.60±2.510	1.000	<0.05
Group A	26.67±3.822	12.17±28.23	8.521	
Group B	27.50±1.732	26.08±1.730	9.530	

Table-4: Comparison of trabecular diameter between Group A and Group B (mm, Mean±SD)

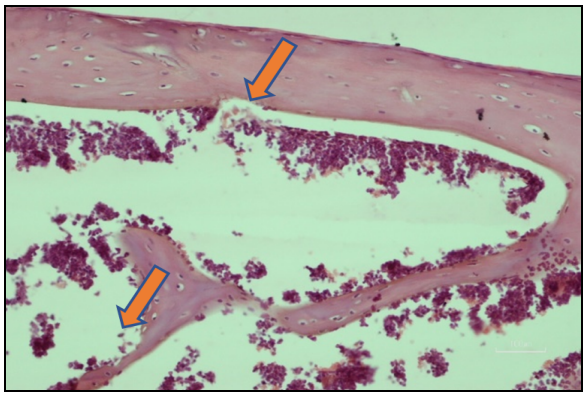
Groups	Trabecular diameter	95% CI	*t*-statistics	*p*
Group A	240.34±80.56	106.95–210.95	6.34	<0.05
Group B	81.39±32.46	105.34–212.56		

Table-5: Chi-Square test for relation between experimental groups and number of osteoclasts

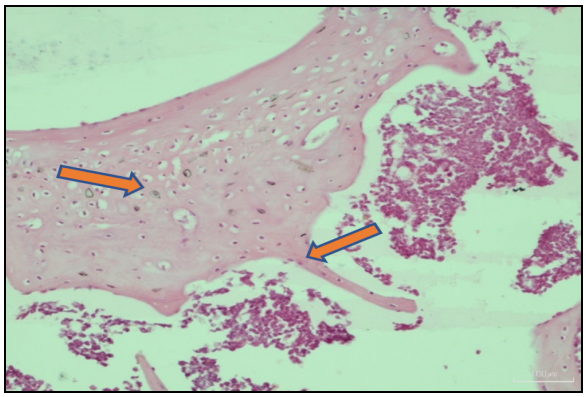
Experimental group	No. of osteoclasts in groups			Chi-square	*p*
	2	3	4		
Group A	5	7	0	11.07	<0.05
Group B	0	6	6		

Table-6: Comparison of mean oestradiol levels between group A and B

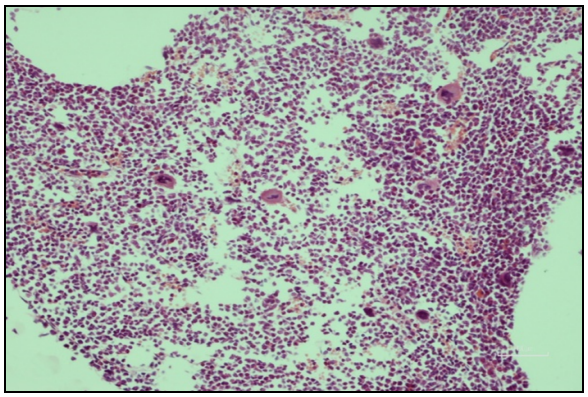
Groups	Oestradiol level (pg/ml)	95% CI	*t*-statistics	*p*
Group A	1.05±0.41	-2.14–1.03	5.94	<0.05
Group B	2.64±0.82	-2.15–1.02		



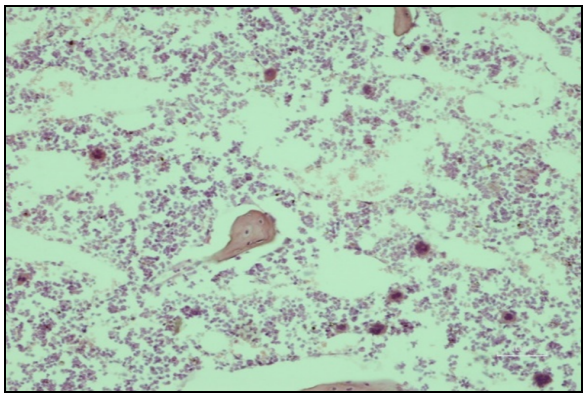
A: Histological section of femur showing large trabeculae in the exemestane only group. The arrow showing dilated trabeculae in the cortical bone with erosion of bone



B: Histological section of femur showing cortical bone in the exemestane and flaxseed treated group. The arrow showing normal trabecular bone with no signs of cortical bone erosion



C: Large multinucleated giant cells in Group-A (H & E stain)



D: Osteoclasts in Group B (H & E stain)

Figure-1: Histological assessment of bone and osteoclast cells

DISCUSSION

In the current study, there was significant weight loss in Group A and Group B when they received the drugs, compared to their weight before treatment. The weight loss was more in Group A from pre-treatment weight as compared to Group B. Weight loss in postmenopausal women with breast cancer on exemestane only was reported to be more than 2 Kg in 28% women as compared to 72% who either maintained weight or gained more than 2 Kg. These changes were reported after patients were followed for two years.[12] A study by G Francini reported significant loss of fat mass (not body weight) in obese patients after 12 months of exemestane therapy as compared to tamoxifen. Switching adjuvant therapy from tamoxifen to exemestane after 2 years may carry advantage in terms of weight loss for obese patients but this benefit may prove detrimental to patients with lower body mass index. The circulating oestrogen levels are reduced by exemestane and other aromatase inhibitors. Oestrogens have direct effect on cellular constituents of adipose tissue and adipocytes. This effect may explain the association of exemestane use with circulating oestrogen levels and that in turn explains its effects on body weight and fat mass. Another explaination for reduction in fat mass may be explained by reduced appetite with exemestane therapy, though this change was not statistically significant.[13] The Group B also demonstrated effect on weight loss. A trial conducted on flaxseeds using 15 gm/day suggested that flaxseed has a good impact on oestrogens which was increased in obese post menopausal women than normal weight women.[14] The effect of flaxseed on BMI and fat content of breast tissue is documented by several observational and experimental studies in women with early breast cancer and without breast cancer.[15]

The use of flaxseed along with exemestane demonstrated effects on trabecular size. There were statistically significant differences in the trabecular size at the lower end of femur with flaxseed oil use as compared to exemestane group alone. The trabecular bone has the ability to withstand greater strain as compared to cortical bone. The BMD decreases as trabecular size increases and its mineralization decreases.[16] The effect of flaxseed on BMD in Wistar rats in an experimental study demonstrated significant effects on BMD in maxilla and mandible. Flaxseed extract was more effective in increasing bone density than the group receiving Ca/Vitamin D.[17] The secoisolariciresinol diglycoside (SDG), the active

metabolite in flaxseed lignans has potential oestrogenic effect on BMD in early life in female rats when oestrogens are low. This study reported that the effect of flaxseed extract on BMD in female rats was more than male rats. This is partly explained by the positive effect of lignans in addition to other flaxseed compounds on immature rats. This indicates that female rats are more sensitive than male rats to lignans and other flaxseed extract compounds.[18]

Exposure to purified SDG during early life has a positive effect on developing bones. This effect was demonstrated by increased bone strength in femur of female rats when measured by dual energy X-ray absorptiometry and biomechanical strength by a 3-point bending test.[19] Flaxseed enhances the protective effect of low-dose oestrogen therapy on vertebral BMD, three-dimensional microarchitecture and bone strength in ovariectomized rats. Flaxseed exerts a stronger effect on bone density outcome when combined with low dose than when combined with ultra-low-dose oestrogen therapy.[20]

There were statistically significant differences in the mean levels of serum oestradiol between two groups, as estimated with ELISA. The mean serum oestradiol level was lower in exemestane only group as compared to exemestane plus flaxseed oil group. Exemestane lowers serum oestrogen levels by inhibiting peripheral conversion of oestrogen from androgen precursors.[21] Exemestane has demonstrated effectiveness in oestrogen receptor positive breast cancers. The lignan, a type of phytoestrogens, in flaxseed mimics the action of oestrogen in females. The level of oestrogen and progesterone in the ovariectomized rats treated with oestradiol and flaxseed oil was significantly increased as compared to rats ovariectomized only.[22] Phytoestrogens in flaxseed are steroid hormones that have an oestrogen-like structure, these hormones bind to oestrogen α and β receptors and produces a response without negative effects of synthetic oestrogens thus increasing oestrogen levels. Research on phytoestrogens effect on bone health in postmenopausal women have shown promising results.[23]

The number groups having 3 and 4 osteoclasts were more in Group B compared to Group A. None of the subgroups in Group A had 4 osteoclasts. The difference in the frequency of osteoclasts in groups was statistically significant. Some of the peptides isolated from flaxseed called cylolinipeptides A-I and their related derivatives have been described as osteoclast differentiation inhibitors. These peptides are very potent inhibitors of osteoclasts differentiation. In the initiation phase of bone metabolism the osteoblast recruits osteocytes that differentiate into osteoclasts and then the bone resorption begins.[24] The inhibition of osteoclasts by clolinopeprtides in the flaxseed is a possible explanation for the increased number of osteoclasts on histology in Group B.

The increase in BMD with flaxseed is in turn supported by the increased differentiation of precursor cells into osteoblasts. This increased differentiation is mediated by Alpha Linolenic Acid (ALA) in flaxseed that in turn increases bone mass.[25] Another mechanism by which ALA increases BMD is to enhance the absorption of calcium from the intestine that accelerates the deposition of calcium into the bone.[26] Apart from increased osteoblasts, and calcium deposition in the bones, the flaxseed increases organic matrix deposition in the bone. This organic matrix involves increased deposition of collagen type 1 that increases bone strength and resistance.[27]

The preventing the increase in the trabecular diameter, maintaining and enhancing the oestrogenic effects of its derivatives, decreasing the mitotic activity, inhibiting osteoclasts differentiation and increasing osteoblasts differentiation, increasing minerals and collagen deposition in the bones, flaxseed increases bone mass density and prevents osteoporosis.[28]

The protective effect of flaxseed oil against the side effects of exemestane in mice is supported by the literature. This may act as a promising remedy in reducing the exemestane induced side-effects in postmenopausal women with breast cancer.

The main limitation of our study was that we did not measure bone mass density. The radiological objective evidence about the bone mass density was not compared which may have shown better results.

CONCLUSION

Administration of flaxseed oil with exemestane resulted in minimum loss or maintenance of bone mass density in animal model. A large-scale study is required to further validate our results.

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SA: Concept and study design
SK: Statistical analysis
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US: Statistical analysis
SU: Lab work
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