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THERAPEUTIC EFFECT OF FLAXSEEDS ON OSTEOPOROSIS IN ALVEOLAR BONE OF RATS

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ABSTRACT

BACKGROUND: Osteoporosis is a bone disorder, occurs due to disruption of bone remodeling. Primary osteoporosis is observed in post-menopausal women and aging population. Glucocorticoids are implicated as a main cause for secondary osteoporosis. Pharmacological treatments for osteoporosis cause adverse effects thus using natural herbs is preferable. Flaxseeds may has a key role in enhancing bone mass.

OBJECTIVES: To assess the therapeutic efficacy of flaxseeds against glucocorticoids induced osteoporosis in alveolar bone of rats.

MATERIALS AND METHODS: A total of 45 adult albino female rats were divided into three equal groups, Control group I, Glucocorticoid group II and Flaxseed treated group III. All rats will be fed on basal diet (BD). Rats in group II and III received Methylprednisolone 5 mg/kg 5 days per week by subcutaneous injection for 4 weeks. After induction of osteoporosis, rats in group III were fed with BD supplemented with 7% flaxseeds daily for 8 weeks. After 12 weeks, rats were euthanized. The mandible was dissected and prepared for scanning electron microscope (SEM) and energy dispersive x-ray microanalysis (EDX).

RESULTS: It was found that the alveolar bone in the control group had normal ultrastructural traits. There were alterations to the ultrastructure of the cortical bone surface in the glucocorticoid group. The trabecular bone also had wide marrow spaces and poorly developed trabeculae. Compared to group II, group III had a more regular cortical bone surface with well architecture trabeculae and narrow marrow spaces.

CONCLUSION: Flaxseeds is suggested as a therapeutic approach for alveolar bone osteoporosis.

KEYWORDS: Flaxseeds, glucocorticoids, osteoporosis, alveolar bone, Scanning electron microscope (SEM).

RUNNING TITLE: Flaxseeds effects on Rat's osteoporosis.

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INTRODUCTION

Bone is a highly adaptable tissue that undergoes constant remodeling in order to meet the physiological needs of the body. Deregulation in this dynamic process, however, results in a variety of bone disorders, such as osteoporosis (1).

Osteoporosis is a medical disorder which is marked by a diminution in bone mass, loss of bone tissue, and deterioration of bone microarchitecture. The weakening of bone density and the subsequent increase in susceptibility to fractures have been observed (2).

There are two distinct subtypes of osteoporosis, namely primary and secondary osteoporosis. Primary osteoporosis is generally classified into two main types: Type I and Type II. Type I osteoporosis is a frequently encountered skeletal disorder that is prominent among women in the postmenopausal stage, mostly attributed to a deficiency in estrogen levels. Type II osteoporosis is predominantly linked to the process of aging in both males and females (3). Certain medical conditions or medications that impair normal bone development can lead to secondary osteoporosis. Diabetes mellitus, gastrointestinal disorders, hyperthyroidism, hepatic disease, hematologic disorders, and chronic glucocorticoids are all risk factors (4).

Glucocorticoids (GCs) are frequently used to inhibit immunological activity or inflammation. They are commonly utilized in clinical settings and are recommended as treatment option for a variety of inflammatory disorders, autoimmune diseases, lung diseases, tumors, and organ transplantation (5). Even in the first two months of therapy, high-dose oral GC treatment significantly reduced bone mineral density (BMD) (6). Two consequences of GCs on bone homeostasis are diminished calcium absorption from the gastrointestinal tract and decreased reabsorption of

calcium in the kidney. GCs may also suppress gonad activity and reduce the anabolic effects of sex hormones on osseous tissue resulting in bone loss (7). Glucocorticoid-induced osteoporosis is recognized by a diminution in bone synthesis associated with an initial, though temporary, elevation in bone resorption. The presence of considerable bone loss can be linked to a combination of accelerated bone turnover and an unfavorable remodeling equilibrium. This occurs when there is an initial increase in the rate of remodeling, which is subsequently paired with a decrease in bone formation at the level of the individual bone multicellular unit (BMU) (7).

The Wnt/β-catenin signaling pathway is known to have a substantial impact on the direct effects of glucocorticoids on bone formation. The improved synthesis of sclerostin results in a decline in the differentiation process of osteoblast precursors into fully mature osteoblasts, as well as an elevation in the occurrence of apoptosis in both osteoblasts and osteocytes. This effect is attributed to the binding of sclerostin to the co-receptors for frizzled, Lrp4 and Lrp5 (8).

Furthermore, the influence of glucocorticoids on bone resorption is mediated through the modulation of osteoblastic cells and osteocytes, leading to a decrease in the expression of osteoprotegerin (OPG) and a boost in the expression of receptor activator of nuclear kappa B ligand (RANKL) and macrophage colony stimulating factor (M-CSF). This alteration in gene expression ultimately results in an enhancement of osteoclast quantity and function (9).

The financial implications, time commitment, and potential adverse reactions associated with pharmacological interventions for osteoporosis are frequently high. In addition, the extended utilization of hormone replacement therapy (HRT) is associated with an increased susceptibility to cerebral infarction, stroke, and malignancies such as breast cancer (10).

The development of nutraceutical therapy using natural herbs for osteoporosis prevention and treatment with fewer side effects is one promising approach (11).

Three groups of phytoestrogens, including isoflavones, lignans, and coumestans are present in many fruits, seeds, and whole grains. They have a molecular structure similar to natural estrogen and can stimulate estrogen's activities. The binding of these substances to estrogen receptors results in the activation of estrogen-related processes and stimulation of calcium absorption (12).

Linum usitatissimum, or flaxseeds, has gained attention as a potential source of bioactive substances that can be incorporated into daily diet. It contains abundant amounts of lignans, which have estrogenic properties and are found in mammalian phytoestrogens (13). Moreover, it has been observed that estrogen possesses the ability to reduce the synthesis of pro-inflammatory cytokines such as interleukins (IL-1, IL-6, and IL-7) and tumor necrosis factor alpha (TNF-α), which are known to play a crucial role in the progression of osteoporosis (14).

Lemay et al. (15) have stated that the consumption of flaxseeds at a daily dosage of 40 g leads to a reduction in menopausal symptoms, comparable to the effects obtained with HRT. Within this particular context, it is hypothesized that flaxseeds could potentially yield positive impacts relative to bone health and metabolic alterations that arise as a consequence of menopause.

On the other hand, Alpha-linolenic acid (ALA), which is abundant in flaxseed flour, is crucial for maintaining or building bone mass (16). Researchers investigated that administration of flaxseed powder and extract would improve the morphological changes in aged menopausal female rats (17).

The alveolar bone comprises the maxillary and mandibular bones, serving as a structural support for the teeth. It undergoes continuous and extensive remodeling due to the emergence of teeth and the functional demands of mastication. The capacity of the alveolar bone to remodel is crucial for the teeth's ability to adapt to their position (18).

The utilization of SEM to reveal the surface topography of bone presents a special approach for observing the previous metabolic activity of bone cells across wide bone surface areas. Scalloped pits are observed on bone surfaces during resorption, whereas smooth surfaces are typical for quiescent bone surfaces (19).

Few investigations have been directed on the phytoestrogens in flaxseeds and their role in human well-being. Moreover, the absence of effective nutraceutical intervention for osteoporosis in persons undergoing GC-treatment necessitates a particular emphasis on therapeutic approaches. Hence, the reason behind conducting this investigation is elucidated. The null hypothesis presented in this study postulates that there will be no apparent variations in the alveolar bone structure of rats across different groups. Any contrary findings would serve to disprove this hypothesis.

MATERIALS AND METHODS

The study was carried out subsequent to obtaining approval from the research ethical committee (No.0465-6/2022) at the Faculty of Dentistry, Alexandria University. The population size of these animals was determined based on sample size calculations conducted in the Department of Biomedical Informatics and Medical Statistics at the Medical Research Institute of Alexandria University.

Materials

Experimental animals:

Forty-five adults female Sprague Dawley rats weighing 180 ± 10 grams (approximately 8 weeks old) were utilized in this study. Rats were obtained from the animal house of Medical Research Institute, Alexandria University. The animals were placed in specialized wire mesh cages and kept under optimal circumstances of adequate ventilation throughout the whole duration of the experiment (12 weeks).

Basal diet preparation:

All rats were fed on basal diet (BD) throughout the whole experimental period. The diets were administered to the rats using specialized feeding cups designed to prevent food loss and contamination. Furthermore, ad libitum of tap water was available and monitored on a daily basis (20).

Glucocorticoids administration:

Methylprednisolone was obtained from Pfizer Inc. It was provided in both solvent and solute forms, which were combined to ensure that all rats were administered a dosage of 5 mg/kg. Rats in experimental groups II and III were administrated with Methylprednisolone at a dosage of 5 mg/kg, five days per week, through subcutaneous injection for a duration of four weeks to induce osteoporosis (21).

Flaxseeds preparation

Flaxseeds were prepared at Faculty of Agriculture, Alexandria University. After induction of osteoporosis, rats in group III were fed with basal diet supplemented with 7% flaxseeds daily for 8 weeks (22).

Method

Experimental design

Rats were randomly allocated into three distinct groups (using computer generated random numbers):

Group I: (Negative control group): 15 rats received basal diet and ultimately injected with vehicle [1 ml phosphate buffer solution (PBS)] in order to regulate the impact of injection-induced stress on rats.

Group II: (GC group): 15 rats received basal diet and were given Methylprednisolone 5 mg/kg (5 days per week) through subcutaneous injection for four weeks (21).

Group III: (Flaxseed group): 15 rats were given Methylprednisolone 5 mg/kg (5 days per week) through subcutaneous injection for four weeks and then received basal diet supplemented with 7% flaxseed powder daily for 8 weeks (22).

Euthanasia of experimental animals:

All animals across all groups were euthanized with overdose of diethyl ether after the experimental period which lasted for 12 weeks. Mandibles were carefully dissected, and the right molar segment was prepared for examination using a scanning electron microscope (SEM) and energy dispersive x-ray microanalysis (EDX).

Tissue preparation for Scanning Electron Microscopy

The mandible was separated along the midline, particularly between the central incisors. The portion of the mandibular molar teeth, along with the adjacent alveolar bone, was exclusively processed for SEM. The specimens were immersed in a solution with 2.5% glutaraldehyde in phosphate buffer (pH=7.3) for a duration of 48 hours. Following this fixation period, the specimens were rinsed two times using the same phosphate buffer. Subsequently, the specimens went through dehydration using a graded series of aqueous ethanol solutions, including concentrations of 50%, 70%, 90%, and 100%, with each specimen being subjected to this process for a duration of one hour. Afterwards, the samples underwent air-drying and were mounted onto aluminum SEM stubs using silver paint and a thin layer of gold was applied onto the samples by a process known as a sputter coater (23).

The specimens were observed with SEM (Joel JSM-5300, Tokyo, Japan) at the Electron Microscope Unit located inside the Faculty of Science at Alexandria University. This was done in order to investigate the surface properties of the alveolar bone across various groups.

Energy Dispersive X-ray (EDX): (24)

Energy Dispersive X-ray is an analytical approach used to characterize a sample's chemical or elemental composition. This kind of X-ray was utilized to compare the amounts of calcium and phosphorus in the alveolar bone of all groups.

Statistical Analysis

To evaluate the overall differences between the three groups, the data obtained from EDX was collected and analyzed using IBM SPSS software package version 20.0. Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. **(**Armonk, NY: IBM Corp**).** The Shapiro-Wilktest was used to verify the normality of distribution of variables. **ANOVA** was used for comparing the three studied groups and followed by **Post Hoc test (Tukey)** for pairwise comparison. Significance of the obtained results was judged at the 5% level.

RESULTS

Scanning Electron microscopic results:

Control group (Group I)

The buccal cortical plate surfaces of the alveolar bone exhibited a widespread pattern characterized by smooth and typical surface topography, displaying regular and clearly delineated nutritive channels (Fig. 1.A). The trabecular bone surface exhibited prominent bone trabeculae and sufficient marrow spaces (Fig. 1.B).

Glucocorticoids group (Group II)

The buccal cortical plates of the alveolar bone exhibited a widespread presence of roughness and porosity, along with an irregular surface topography. The presence of nutritive canals with irregular outlines was observed. The observation revealed the presence of numerous resorptive pits characterized by irregular resorptive craters and deep erosions, which were found to be distributed across the buccal cortical plate (Fig. 2.A).

Besides, the trabecular bone has wide bone marrow spaces and delicate bone trabeculae (Fig. 2.B).

Flaxseed group (Group III)

The alveolar bone's buccal cortical plate displayed a more comprehensive enhancement compared to group II, resulting in the restoration of the normal structure. An enhanced level of smoothness and uniformity in the topography of the alveolar bone was noted. Furthermore, it was observed that the nutrient canals exhibited a narrow and consistent opening (Fig. 3.A).

In comparison to group II, it was observed that the trabecular bone presented reduced marrow spaces and increased thickness of bone trabeculae (Fig. 3.B).

Energy dispersive x-ray analysis (EDX):

By analyzing the means and standard deviation of the different groups, calcium and phosphorus levels were calculated. Table 1 compares the calcium and phosphorus levels among the three groups. In comparison to the control group I, calcium and phosphorus levels were significantly lower in groups II and III (P1 <0.05). However, group III had significantly more calcium and phosphorus levels than group II (P2 < 0.05). There were statistically significant differences between the three groups. (P1, P2&P3 <0.05).

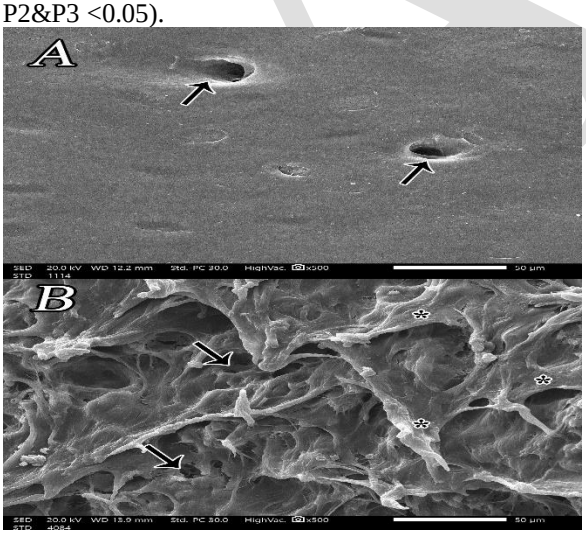

Figure (1): Scanning electron micrograph (SEM), group I control. A: showing the buccal cortical plate of alveolar bone with smooth surface areas and regularly bordered nutritive canals (arrows). **B**: revealing exposed trabecular alveolar bone with normal well-developed bone trabeculae (asterisks) and narrow marrow spaces (arrows). (X500)

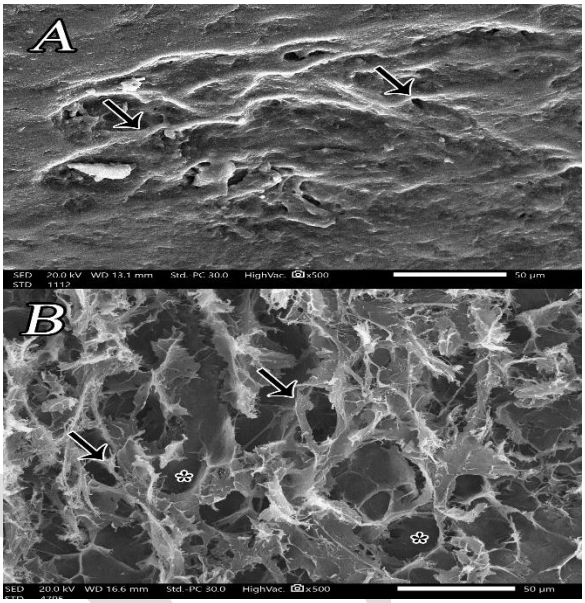

Figure (2): SEM, group II, Glucocorticoid group, A: showing the buccal cortical plate of alveolar bone with generalized resorption and porosity. Note the irregular outline with massive abrasion and discontinuity of the cortical plate (arrows). **B:** showing appearance of poorly developed bone trabeculae (arrows) with wide marrow spaces (asterisks). (X500)

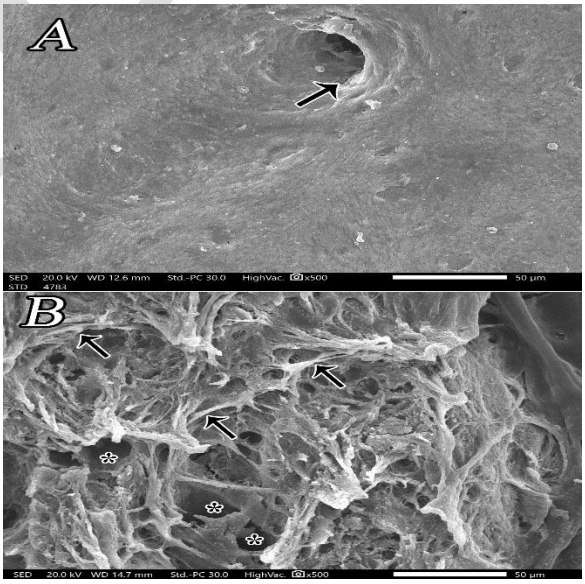

Figure (3): SEM, group III, Flaxseeds group, A: revealing nearly smooth and uniform buccal cortical plate of alveolar bone with relatively regular outline of the nutrient canals (arrow). **B:** showing well-formed architecture of alveolar bone trabeculae (arrows)

having narrow bone marrow spaces in between (asterisks). (X500)

Table (1): Comparison between the three studied groups according to calcium and phosphors.

	Group I	Group II	Group III	F	p
Calcium	40.20 ± 8.05	11.73[a] ± 2.72	25.04[ab] ± 2.65	23.058*	0.002*
Sig. bet. grps.		p_1=0.001*, p_2=0.026*, p_3=0.044*			
Phosphors	15.86 ± 0.33	9.03[a] ± 0.90	13.07[ab] ± 1.16	47.222*	<0.001*
Sig. bet. grps.		p_1<0.001*, p_2=0.018*, p_3=0.003*			

Data was expressed using Mean ± SD.

F: **F for One way ANOVA test**, Pairwise comparison between each 2 groups was done using **Post Hoc Test (Tukey)**

p: p value for comparing between the studied groups.

p_1: p value for comparing between **Group I** and **Group II**

p_2: p value for comparing between **Group I** and **Group III**

p_3: p value for comparing between **Group II** and **Group III**

*: Statistically significant at $p \leq 0.05$

a: significant with **Group I**

b: significant with **Group II**

DISCUSSION

The purpose of this research was to examine the therapeutic impact of flaxseed on glucocorticoid-induced osteoporosis in the alveolar bones of rats. The influence of GCs on bone physiology may be attributed to the disruption of the equilibrium between bone resorption and bone formation, vascular occlusion, and the initiation of apoptotic signaling pathways (25).

An immediate and substantial suppression of osteocalcin, a marker of decreased osteoblastic function, that is correlated with bone loss over time, is a sign of the skeletal consequences caused by GCs. Additionally, GCs may increase osteoclast survival by decreasing the ratio of OPG to RANKL in osteoblasts (21).

The study's ultrastructural assessment demonstrated that the control group revealed a uniform and normal alveolar bone structure, along with a clearly defined nutritive canal.

On the other hand, the ultrastructural investigations of alveolar bone revealed that GC treated group exhibited prevalent roughness and porosity with irregular defined nutritive canals. This finding aligns with the results of a prior investigation conducted by Kasem et al. (26); who reported that prednisolone injection dramatically reduced the cortical thickness in the osteoporotic group when compared to the healthy control group. Moreover, Ton et al. (27) suggested that prednisolone significantly inhibits bone remodeling, decreases bone formation at even small doses, and may have other undesirable effects on bone strength.

The limited availability of efficacious treatments for reducing the risk of osteoporosis in patients undergoing GC therapy has prompted a particular emphasis on preventive strategies, including the utilization of dietary interventions that protect bone health.

The findings of this study indicate that the group treated with flaxseed exhibited an improvement in bone architecture, as observed by scanning electron microscopy. This coincides with the results of Ragheb et al. (22), who proposed that combining flaxseeds in dietary supplements is advisable for preventing the risk of bone loss caused by glucocorticoid usage.

Additionally, the results of the current study are consistent with the research conducted by Arjmandi et al. (28), which showed that the addition of Flaxseeds to the diet has a beneficial impact on bone metabolism among postmenopausal women.

Maíra et al. (29) reported that the inclusion of flaxseed flour in an individual's diet shown notable characteristics contributing to the enhancement of bone mineral density and femur resistance over a period of 180 days.

Flaxseed flour is characterized by its elevated levels of alpha-linolenic acid (ALA), as reported in previous studies (30). Additionally, many researchers examined the potential of ALA to enhance the functionality of primary osteoblasts by upregulating the expression of β-catenin, RUNX2, and osterix proteins and genes. Further, they concluded that ALA significantly improved the expression of the β-catenin gene and protein in osteoblasts. This finding implies that ALA has the potential to facilitate osteogenic differentiation by activating the Wnt pathway (31).

An animal study demonstrated that the provision of ALA through the consumption of flaxseed flour had a positive impact on the bone integrity in female rats throughout the post-partum period (32). In addition to providing ALA, flaxseed flour is marked by a significant calcium content of 236 mg per 100 g of seed. It has been observed that ALA promotes retention of calcium in the gastrointestinal tract, hence facilitating the deposition of minerals in bones (30, 33)

Our findings are consistent with the study conducted by El-Saeed et al. (34), which examined the implications of flaxseed oil on bone homeostasis and its influence on the levels of bone formation markers. The researchers discovered that the consumption of flaxseed oil yields positive effects in the prevention of

osteoporosis. According to Longo et al. (35), it was proposed that the administration of Flaxseed oil, potentially due to its abundant ALA content, offered protection against alveolar bone loss induced by ovariectomy in rats.

The SEM observations in the current study were supported by the EDX results. In comparison to the glucocorticoid and flaxseeds groups, the calcium level in the control group showed the highest level. These findings are in agreement with those made by Fei et al., who performed an elemental analysis on an osteoporotic model and found that calcium ratios are decreased in cases of osteoporosis compared to control groups (36).

Regarding the EDX results of flaxseed group, calcium concentration showed a marked increase in in relation to glucocorticoid group. These results were supported by Hala et al, who found that diets supplemented by flaxseed increase femur bone density and calcium content in bone (37).

CONCLUSION

Based on the findings of this research, it is suggested that the administration of flaxseeds as a dietary supplement could serve as a viable therapeutic intervention for the prevention of alveolar bone loss induced by glucocorticoids. So, it's recommended to incorporate flaxseeds in routine diet to improve skeletal properties against glucocorticoid induced osteoporosis.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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