EFFECT OF RALOXIFENE IN THE PREVENTION OF OSTEOPOROSIS OF ALVEOLAR BONE INDUCED BY HIGH FAT DIET IN RATS.

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ABSTRACT

INTRODUCTION: Obesity is positively associated with many chronic disorders such as osteoporosis, as it decreases the osteoblastic cell formation, increases the osteoclastic activity and decreases the vascularity of the bone marrow as well. Raloxifene belongs to a class of drugs called selective estrogen receptor modulators (SERMs). It reduces the expression of bone turnover markers and increases bone mineral density.
OBJECTIVES: To investigate the effect of Raloxifene in the prevention of osteoporosis of alveolar bone induced by high fat diet in the rats.\
MATERIALS AND METHODS Thirty female albino rats were divided randomly into 3 equal groups, (10 rats each) as follows: Group I: control group, Group II: High fat diet group (59.28% of fats in their chow), Group III: High fat diet group (59.28%) with intake of Raloxifene 1mg/ kg once daily for 12 weeks. After 12 weeks all rats were sacrificed, molar segments of the mandibles were dissected and prepared for histological and histomorphometric analysis.
RESULTS: In high fat diet group, there was a significant deterioration in alveolar bone architecture and decrease in its surface area. Bone surface became irregular with multiple osteoclasts. It consisted of thin trabeculae enclosing wide fatty infiltrated bone marrow. In the high fat diet group treated with Raloxifene the alveolar bone surface and architecture relatively returned back to the normal. The histomorphometric results, revealed a significant decrease in the total bone surface area in high fat diet group and a significant restoration of the bone surface area in high fat diet group treated with Raloxifene in relation to control group.
CONCLUSIONS: High fat diet can induce alveolar bone osteoporosis and Raloxifene is an effective drug in restoring alveolar bone density and architecture.
KEYWORDS: Osteoporosis, obesity, high fat diet, alveolar bone, Raloxifene.

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INTRODUCTION

Osteoporosis is a disorder characterized by enhanced skeletal fragility due to reduction in both bone quantity and quality. The syndrome includes the triad of back pain, fractures without significant trauma (commonly at the spine, hip, distal radius, and proximal humerus) and low bone mineral density (BMD) (1).

Riggs and Melton established a classification of osteoporosis into primary and secondary forms (2). Primary osteoporosis was further divided into Type I, which occurred in patients between 50 and 70 years old and Type II for patients over 70 (senile osteoporosis). Type I includes mostly women with postmenopausal osteoporosis. It is clearly related to the loss of estrogen with menopause. Secondary osteoporosis occurs due to a particular medical condition or due to certain medications such as corticosteroids (3).

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health (4). Obesity is most commonly caused by a combination of excessive food intake, lack of physical activity, and genetic susceptibility. Few cases are caused primarily by genes, endocrine disorders, medications, or mental illness (5) .Obesity increases the likelihood of various diseases, particularly heart disease, type 2 diabetes, obstructive sleep apnea, certain types of cancer, and osteoarthritis (4)

Osteoporosis and obesity, two disorders of body composition, are growing in prevalence. Interestingly, these diseases share several features including a genetic predisposition and a common progenitor cell (6). Both adipocytes and osteoblasts are derived from a common multipotential mesenchymal stem cell. Obesity increases adipocyte differentiation and fat accumulation while decreases osteoblast differentiation and bone formation (7).

The increased circulating and tissue proinflammatory cytokines in obesity may promote osteoclast activity and bone resorption through modifying the receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and (RANK/RANKL) ligand/osteoprotegerin pathway (Interleukin-1, IL-6, tumor necrotizing Factor-α) thus increasing bone resorption which may lead to osteoporosis. (8,9)

Other studies have shown a selective increase in the production of reactive oxygen species (ROS) in the adipose tissues of obese rat (10). The ROS and oxidative stress inhibit osteoblastogenesis (11). Moreover, excessive secretion of leptin and decreased production of adiponectin by adipocytes in obese rats may either directly affect bone formation or indirectly affect bone resorption through up-regulated proinflammatory cytokine production. Adiponectin acts as an anti-inflammatory cytokine which suppresses TNF-α-. While leptin decreases the secretion of serotonin from the brain stem as serotonin acts as a neurotransmitter that increases bone formation (12).

High-fat intake may interfere with intestinal calcium absorption and therefore decrease calcium availability for bone formation. Free fatty acids can form unabsorbable

insoluble calcium soaps and therefore contributing to low calcium absorption. Moreover, high fat diet causes hyperlipidaemia-induced osteoclastogenesis and free fatty acid (FFA)-induced adipogenesis instead of osteoblastogenesis (13).

Osteoblast cells isolated from HFD-fed rats exhibited the impairment of osteoblastic insulin signaling as well as reduction of cell proliferation and survival. (Insulin increases bone anabolic markers, including collagen synthesis, and alkaline phosphatase production and promotes osteoblast proliferation and differentiation) (13). Free fatty acids degenerate insulin receptors present on the outer surface of the osteoblast thus decrease the osteocalcin secretion by the osteoblast itself. Osteocalcin acts as an important non-collagen protein present in the bone matrix which regulates glucose metabolism in the body as well (13).

Raloxifene belongs to a class of drugs called selective estrogen receptor modulators (SERMs). It is FDA-approved for the prevention and treatment of osteoporosis in postmenopausal women (14). Raloxifene reduces the expression of bone turnover markers, increases bone mineral density, reduces vertebral fractures, decreases the breast cancer incidence and decreases the lipid concentration in blood stream. Raloxifene effects on bone formation are likely mediated by multiple mechanisms, including a prolongation of osteoblast lifespan , as well as effects on osteoblast differentiation and function (15). Furthermore, estrogen treatment inhibits osteocyte apoptosis (16). since osteocyte is involved in mechanosensing and transducing loading responses(16), these effects of estrogen deficiency could potentially impair the skeletal response to loading. Moreover, Raloxifene decreases bone resorption through its clear effects on osteoclast development, activity, and apoptosis. (17).

Most of the previous researches (18, 19, and 20) investigated the effect of Raloxifene on ovariectomy induced osteoporosis in rats. To the best of our knowledge, the literature contains few data on the effect of Raloxifene on the treatment of osteoporosis caused by high fat diet and this is why this study was carried out to evaluate the efficiency and safety of Raloxifene in the prevention of osteoporosis induced by high fat diet in rats.

MATERIALS AND METHODS

Thirty female rats weighing 150-200 grams were used in this study. These animals were obtained from and caged in Faculty of Agriculture, Alexandria University, after gaining the approval of the Research Ethics committee of the faculty, they were caged in specially designed wire mesh cages.

The animals were divided randomly into three equal groups (10 rats each) as follows:

Group I: Control group. Rats were injected with vehicle (saline) to control the influence of any injection stress.

Group II: High fat diet group. Rats were fed high fat diet (Normal rat chow containing 20% was raised to 59.28% daily) (21)

Group III: High fat diet with Raloxifene. Rats were fed high fat diet and at the same time were given Raloxifene daily at a dose of 1 mg/kg using oral gavage syringe for 12 weeks. (13)

Raloxifene (Eli Lilly chemical company, Indiana US) is supplied in form of tablets, each tablet contains 60mg. Each tablet was dissolved in 100ml distilled water so that each ml contained 0.6 mg Raloxifene. The volume of suspension given orally was calculated according to the weight of each rat 1mg/kg. (13)

At the end of the 12 weeks, all rats were scarified. The right mandible of each rat was dissected out. The right first and second molar region segments were prepared for light microscopic examination and histomorphometric analysis.

As, the rat's dental formula is: I $\frac{1}{1}$, C $\frac{0}{0}$, P$\frac{0}{0}$, M$\frac{3}{3}$. Rats have 8 teeth on the lower jaw and 8 on the upper, a total of sixteen teeth.

Histological procedure:

The mandibles were fixed in 10 % neutral-buffered formalin, washed, decalcified for 2 weeks with formic acid, dehydrated in ascending concentrations of ethanol, cleared with xylene, and embedded in paraffin wax blocks. Sections were cut mesiodistally at a thickness of 5 µm and stained with Haematoxylin and Eosin then examined by light microscope. (22).

Histomorphometric:

Computer assisted histomorphometry were performed in order to measure the percentage of total bone surface area in the different groups. From each animal tissue block, three sections were obtained from standardized depths with a total of thirty sections from each group. The bone surface area was calculated using a software "Image J 1.46" Measurements were obtained from each slide then the mean values were calculated. The total bone surface area of the formed bone was measured using an objective lens of magnification 10. The total magnification was 100. (21).

Statistical analysis: (22).

The collected data of the percentage of bone surface area were statistically analyzed by using ANOVA test to reveal the difference in bone surface area between the three groups by means of IBM SPSS software (Armonk, New York: IBM Corporation)

RESULTS

Light microscope results:

Control group: interdental and inter-radicular alveolar bone revealed the normal structure features which included thick bone trabeculae, numerous resting lines and regular smooth bone surface facing the periodontal ligaments, (figures 1 and 2)

High fat diet group: Bone resorption was evident at the interdental and interradicular regions. The bone surfaces appeared irregular with the presence of How ship's lacunae containing osteoclasts. Wide bone marrow spaces were seen in between the thin bone trabeculae. (Figures 3 and 4)

High fat group with Raloxifene: A generalized appearance of restoration of the normal structural features of the alveolar bone was the main finding. The alveolar bone revealed a normal appearance comparable to that in the control group as regarding

the appearance of the inter radicular and interdental regions, (figures 5 and 6)

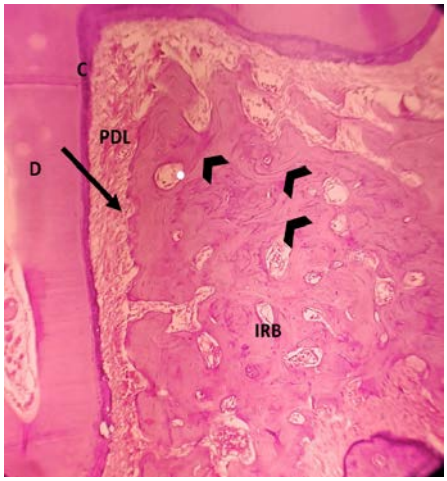


Fig1: Light micrograph (**LM), control group** showing well-formed inter radicular septum of the alveolar bone with a relative smooth boundary (arrow) at the interface with PDL. Resting lines could be seen clearly between bone segments (arrow head). Note the dense cancellous bone trabeculae enclosing bone marrow tissue of variable width. (H&E×100)

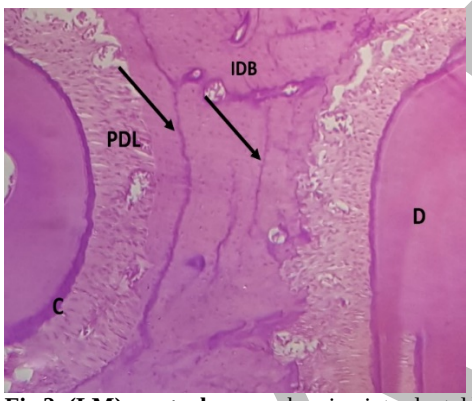


Fig 2: (LM), control group showing interdental bone with adequate width and smooth boundary facing the PDL which exhibit well organized fibres. Resting lines are seen at variable distances between bone segments (arrows). D: dentine C: cementum IDB: interdental bone (H&E) ×100

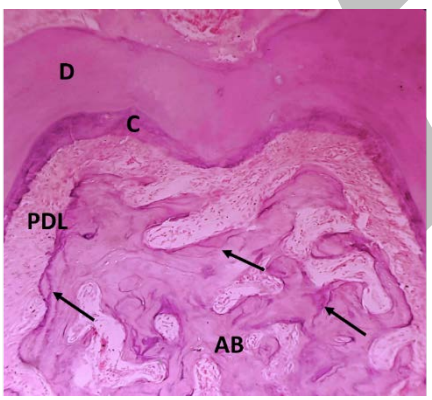
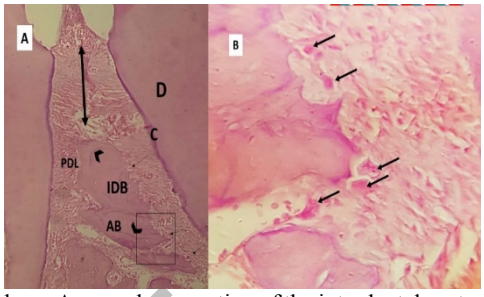


Figure 3: (LM), high fat diet group showing areas of resorption of inter radicular region of the alveolar bone . Note the decreased density of the bone trabeculae and prominent deeply stained reversal lines (arrows) D: dentine C: cementum
AB: alveolar bone. (H&E×100)

Fig 4: LM High fat diet group showing a part of the interdental



bone A: reveals resorption of the interdental septum (↕). Numerous resting lines (arrow heads) are also seen B: higher magnification of the inset in figure A showing irregular bone surface seen facing PDL and osteoclasts in Howship's Lacunae at a little distance from the bone (arrows) D : dentine C: cementum IDB: interdental bone AB: alveolar bone (H&E A×100 , B×400)

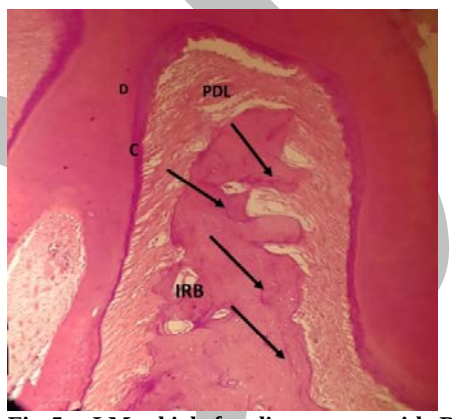


Fig 5: LM , high fat diet group with Raloxifene showing restoration of the interradicular region of the alveolar bone with smooth and regular surface and a number of resting lines (arrows). The bony mass surrounding narrow marrow spaces appears well organized D: dentine C: cementum IRB: interradicular bone (H&E×100)

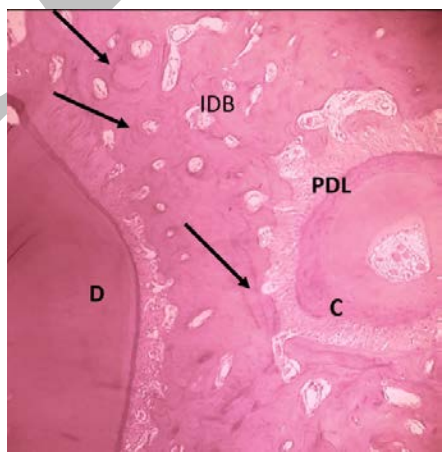


Fig 6: LM high fat diet group with Raloxifene showing regular bone surface of the interdental bone. Which shows adequate density with many resting lines (arrows). D: dentine C: cementum IDB: interdental bone (H&E) ×100

Histomorphometric results

Table (1) shows comparison between the three groups included in the study (control, high fat diet, high fat diet with raloxifene) regarding the percentage of bone surface

area (%), where the mean values were 93.7±2.17, 68.7±7.82, , 81.5±6.225 respectively.

It was noted that the high fat diet group exhibited the lowest percentage of bone surface area followed by high fat diet group with raloxifene, where the values were 68.7±7. 82, 81.5±6.225, There was a statistically significant difference between high fat diet and control group where the P1 value was <0.0001. After Raloxifene administration there was a statistically significant increase in percentage of bone surface area in relation to high fat diet alone. Moreover, the difference between high fat diet group with raloxifene and control group was statistically significant p1<0.0001

Table 1: Showing the difference between the three groups regarding percentage of mean bone surface area.

	CONTROL	HFD	HFD+RALOX	F VALUE	P VALUE
MAX-MIN	96.2-90	81-51	90-70	54.5192	<0.0001*
MEAN	93.7	68.7	81.5		
MEDIAN	94.2	69	82		
SD	2.176025	7.82357	6.225004		
P 1		<0.0001*	<0.0001*		
P2	0.00015*				

f= anova
p1= p value of post hoc test to compare each group with control
p2= p value of post hoc test to compare between both treated and untreated group
*Significant difference p≤0.05

DISCUSSION

High fat diet is one of the main causes of osteoporosis and has adverse effects on the alveolar bone structure. As Roy B et al (2016) (6), Lee RH (2004) et al (7) and Fantuzzi G et al (2005) (8) mentioned in their studies.

Raloxifene belongs to a class of drugs called selective estrogen receptor modulators (SERMs). It is FDA-approved for the prevention and treatment of osteoporosis in postmenopausal women. (14). The current study is most probably the first to use Raloxifene as a prophylactic for the management of osteoporosis caused by high fat diet, so it aimed at evaluating the efficiency and safety of Raloxifene in the prevention of osteoporosis caused by high fat diet in rats.

The histological results of the current study revealed that the bone trabeculae of the high fat diet rats were less dense than in the control group and the Cancellous bone showed relatively widened marrow spaces and thin bony trabeculae. This was proved by Wauquier F et al (2009)(23) and Liu A-L et al (2004)(24) as they found a selective increase in the production of reactive oxygen species (ROS) in the adipose tissues of high fat diet rats which inhibits osteoblastogenesis .

Beside the inhibitory effect of high fat diet on osteoblasts, it also has an inductive stimulatory effect on osteoclasts ,S Pramojanee, et al (2013) (13) proved that HFD causes hyperlipidaemia that induces osteoclastogenesis and free fatty acid (FFA) induces adipogenesis instead of osteoblastogenesis ,Moreover, they investigated that the osteoblastic cells isolated from HFD-fed rats exhibited the impairment of insulin signalling as well as reduction of cell proliferation and survival of osteoblast. (Insulin increases bone anabolic markers, including collagen synthesis, and alkaline phosphatase production and promotes osteoblast proliferation and differentiation).

Fantuzzi G et al (2005) (25) revealed in their study that the circulating and tissue proinflammatory cytokines released from the adipocytes promotes osteoclast activity and bone resorption through modifying the receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (RANK/RANKL) ligand/osteoprotegerin pathway (IL-1,IL-6 ,tumor necrotizing Factor-α) thus increasing bone resorption which may lead to osteoporosis

The quantitative histomorphometric analysis in this current study confirmed the histological results. The Histomorphometric analysis revealed that the percentage of bone surface area of the high fat diet group was much lower than that of the control group .This is in agreement with Patsch JM et al (2011)(26) who found a severe decrease in the bone surface area in mice fed HFD.

Moreover Macri EV et al (2012)(27) worked on male Wistar rats fed HFD for 8 weeks .They found that these animals exhibited the lowest total skeleton bone surface area and bone mineral content. They explained that the bone mineral alterations might be the result of the formation of intestinal soaps with calcium that reduce its absorption.

The histological and histomorphometric findings of the deteriorated bone tissue of high fat diet group, showed remarkable differences from those of raloxifene treated and control groups. In the present work Raloxifene maintained the normal trabecular width and thickness with a sufficient amount of resting lines that prove a continuous bone formation and a sufficient amount of bone surface area. Those findings were supported by Park SB et al (2016)(28) who concluded that selective estrogen receptor modulator treatment improved the trabecular quality of the vertebral body, enhanced spinal fusion, and increased the amount of compact bone mass within the fusion bed in rats that had went through an ovariectomy

In addition , F Syed et al(2005)(29) found that Estrogen inhibits bone resorption via its effects on the receptor activator of NF-κB ligand (RANKL/RANK) osteoprotegerin system, as well as by reducing the production of a number of pro-resorptive cytokines, along with a direct effects on osteoclast activity and their lifespan. They added that, estrogen effects on bone formation are also likely mediated by multiple mechanisms, including a prolongation of osteoblast lifespan, as well as its effect on osteoblast differentiation and function.

Also, the present histological results showed that the bone marrow of bones of the animals which received raloxifene were highly cellular with few fat cells. This was explained by the findings of Lacey D et al (1998)(30) who revealed that Raloxifene regulates progenitor cell differentiation into osteoblast instead of adipocyte .

The obtained histomorphometric results of the Raloxifene added groups showed that their bone surface area was similar to the control group. This may be emphasized by the results of Luvizuto ER et al (2010)(31) who worked on

female rats with extracted tooth that were given Raloxifene (1mg/kg) for 60 days after ovariectomy, , they showed that raloxifene treated group had got the highest mean bone formation value in the post-extraction period. In 2010 , Stuermer E et al (32) studied the effect of Raloxifene on tibia metaphyseal fracture healing in osteoporotic rats . They found that the trabecular width was significantly enhanced in Raloxifene treated animals compared to their width in ovariectomized animals without raloxifene administration where the percentage of bone in the whole callus area (callus density) was significantly higher.

CONCLUSION

From the previous findings, it could be concluding that high fat diet has detrimental effects on alveolar bone structure. In addition, Raloxifene can counteract these effects mainly by its stimulatory effect on osteoblast function and inhibition of osteoclast formation and function.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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