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Histological Effect of Bisphosphonate, Vitamin D and Olive Oil on Glucocorticoid Induced Osteoporosis (Gio) in Albino Rat

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ABSTRACT

Background: Previous studies demonstrated that the prevalence of osteoporosis was 4% in women aged 50 to 59 years compared to 44% in women aged 80 years and older. Osteoporosis may be primary or secondary. Glucocorticoid induced osteoporosis (GIO) is considered among the most common causes of secondary osteoporosis. The present study aimed to assess if vitamin D and olive oil could be useful in the treatment of GIO as bisphosphonate.

Materials and Methods: Fifty adult female albino rats weighing 180-220 grams and aged 16-19 weeks were divided into five groups (each consists of ten rats): the control group, osteoporotic group, bisphosphonate group, vitamin D group and olive oil group. The first group served as a negative control group. The other four groups were injected subcutaneously by methyl-prednisolone (0.5 mg/kg/day - three times a week) for 60 days to induce osteoporosis (glucocorticoid induced osteoporosis "GIO"). One of the four groups served as a positive control group while other three groups were treated with oral bisphosphonate (0.84 mg/kg/day - five days a week), oral Vitamin D_3 (0.1 ug/kg/day - five days a week) and oral Olive oil (0.1 ug/kg/day - five times a week) respectively for additional 60 days. At the end of the experiment, the right femur was removed from each rat and examined histologically after staining by hematoxylin and eosin stain as well as Masson's trichrome stain. The stained sections were photographed and analyzed to assess cortical bone thickness, osteocyte number and osteocyte lacunae. Results: By comparing the cortical thickness in all groups we detected a significant difference between bisphosphonate group and the control group, as well as between the GIO group and the control group. We also found a significant decrease in osteocyte number by comparing the GIO group to the control group. There was as a significant difference between vitamin D group and control group when we compared the number of osteocyte lacunae in all groups.

Conclusion: GIO affects mainly the cortical bone thickness as well as the osteocyte number. Bisphosphonate is possibly the drug of choice in the treatment of osteoporosis especially by increasing the cortical bone thickness. Although olive oil acts also on increasing cortical bone thickness as well as bisphosphonates but it was less effective. On the other hand, vitamin D increases both; the cortical thickness and the osteocyte number moderately and may be used as a prophylactic agent against osteoporosis.

Keywords: Osteoporosis – GIO – Bisphosphonates – Vitamin D – Olive oil.

INTRODUCTION

Osteoporosis is a systemic skeletal disorder characterized by low bone mass and micro-architectural deterioration of bone tissue with a consequent compromised bone strength and increased susceptibility to fracture. In the developed countries 12.6% of the population is elderly compared with 4.6% in the developing countries [1]. According to the national health and nutrition examination survey (NHANES) data, the prevalence of osteoporosis based on reduced hip bone density was 4% in women 50 to 59 years of age compared to 44% in women 80 years of age and older [2].

Ninety percent of hip fractures occur in persons aged 50 years or older, occurring most often in the eighth decade of life. A patient is considered osteoporotic when the dual-energy x-ray absorptiometry measurement of bone-mineral density is 2.5 standard deviations below the typical peak bone mass of young healthy women [2]. Often patients who have not sustained a fracture do not report symptoms that would alert the clinician to suspect a diagnosis of osteoporosis thus this disease is a "silent thief" that generally does not become clinically apparent until a fracture occurs [4].

The overall incidence of osteoporosis has a female-to-male ratio of 4:1 [5]. It occurs in both genders, at all ages and can be classified into three types: (1) Primary osteoporosis in which no underlying cause can be clearly identified, but often follows menopause in women and occurs later in life in men. (2) Secondary osteoporosis in which the underlying cause is known (e.g. hyperparathyroidism,

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hypophosphatemia, diabetes, alcoholism, gluco-corticosteroid use, etc.). (3) More rare forms of the disease, such as juvenile, pregnancy-related and postpartum osteoporosis [5].

Synthetic glucocorticoids are used in a wide variety of disorders including autoimmune, pulmonary and gastrointestinal diseases, as well as in patients following organ transplantation and with malignancies [6]. Many studies have shown that glucocorticoids decrease bone mass and thereby increase the risk of fractures, particularly fractures of the ribs, spine and forearm. Studies have shown that 30–50% of all fractures occur in hospital settings, usually associated with administration of high doses of glucocorticoids [8].

A wide variety of pharmacological interventions have been shown to decrease bone loss in glucocorticoid induced osteoporosis (GIO). The proposed treatments help to maintain or increase bone density include calcium supplementation, bisphosphonates, hormone replacement therapy, vitamin D in one of its many forms (cholecalciferol, calciferol, calcitriol, calcidiol, alfacalcidol), calcitonin, parathyroid hormone, fluoride, testosterone and anabolic steroids [9]. Olive oil also may increase bone mineral density (BMD) in osteoporotic glucocorticoid-treated animals [10].

The present study aimed to assess if vitamin D and olive oil could be useful in the treatment of GIO as bisphosphonate. The study compared the effectiveness of these compounds with the commonly prescribed bisphosphonate, which is identified now as the golden standard treatment for osteoporosis.

MATERIALS AND METHODS

Fifty adult female albino rats aged 16-19 weeks and weighing 180-220 grams were used through this study. The animals were housed in plastic cages with a metallic mish cover and dimension of 50×40×30 cm^3. Each cage contained five animals. The animals were fed ordinary laboratory diet, vegetables and bread with liberal supply of water.

The used rats were divided into five groups (each was formed of ten rats): control group, osteoporotic group, bisphosphonate group, vitamin D group and olive oil group. The first group served as a negative control group. The other four groups were injected subcutaneously by methyl-prednisolone, commercially known as "Depo Medrol" (0.5 mg/kg/day - three times a week) for 60 days to induce osteoporosis (glucocorticoid induced osteoporosis "GIO").

The osteoporotic group served as positive control group while other three groups were treated for another 60 days by oral bisphosphonate "Alendronate" (0.84 mg/kg/day - five days a week), oral Vitamin D_3 (0.1 ug/kg/day - five days a week) and oral olive oil (0.1 ug/kg/day - five times a week) respectively. Every rat was weighted every week and the dose was adjusted according to its weight. The doses were calculated according to the formula of [11] which equal:

$$\text{Animal dose} = \frac{\text{Human dose} \times 18 \times \text{animal weight in gram}}{1000 \times 200}$$

Groups:

1. **Control group:** ten rats were put under the same conditions as the other rats but were injected with saline subcutaneously for two months then sacrificed after another two monthes.
2. **Osteoporotic group:** ten rats were injected by methyl-prednisolone subcutaneously in a dose of 0.5 mg/kg three times a week for two months to induce osteoporosis then sacrificed.
3. **Bisphosphonate group:** ten rats were injected by methyl-prednisolone subcutaneously in a dose of 0.5 mg/kg three times a week for two months to induce osteoporosis and treated for additional two months by oral alendronate (one of the bisphosphonates) in a dose of 0.84 mg/kg five times a week by oro-gastric-tube [12] then sacrificed**.**
4. **Vitamin D group:** ten rats were injected by methyl-prednisolone subcutaneously in a dose of 0.5 mg/kg three times a week for two months to induce osteoporosis and treated for additional two months by oral vitamin D_3 in a dose of 0.1 ug/kg five times a week by oro-gastric-tube [12] then sacrificed**.**
5. **Olive oil group:** ten rats were injected by methyl-prednisolone subcutaneously in a dose of 0.5 mg/kg three times a week for two months to induce osteoporosis and treated for additional two months by oral olive oil in a dose of 10 ml/kg five times a week by oro-gastric tube [10] then sacrificed.

After scarification, the right femurs were removed from each rat and fixed in 10% formalin for histological examination.

Tissue processing:

After putting the bones in the fixative (10% formalin) for 24 hours, the process of decalcification was started by ethylene diamine

tetra-acetic acid (EDITA) 10% in 7-7.4 ph for four weeks and the end point of decalcification determined by manipulation and pending method [14].

After washing the fixed tissues in running tap water to remove the fixative from them, dehydration was done gradually in ascending grades of alcohol by putting the tissues in 50% alcohol then in 70% alcohol and finally in 100% alcohol. Clearing of the tissues with xylol was done. Each femur was cut transversely at the middle of the shaft.

The cleared fixed tissues were put in warm melted soft paraffin in an oven at 50°C for one day. The tissues were then transferred into melted hard paraffin in an oven at 57 °C for one hour. Then the specimens were transferred to casts filled with melted hard paraffin. The casts with their contents were cooled in ice until the paraffin was completely solidified forming blocks of hard paraffin with tissues in its centers. Each block of hard paraffin was cut into thin sections (5 micrometers thick) by rotatory microtome.

Paraffin sections representing all groups were then placed on clean glass slides smeared with glycerin-albumin, allowing few drops of glycerin-albumin, to flow beneath the section. The slides were warmed on a hot plate then left for several hours in the incubator to dry [14]. The slides were stained by hematoxylin and eosin stain and Masson's trichrome stain. The stained sections were examined histologically and photographed at the image analysis unit of Al-azhar Faculty of Medicine in Cairo. Images were analyzed by Optimas (Media Cybernetics, 1998 version 6.21.19); where three parameters were examined and assessed:

1. Cortical bone thickness: cortical thickness was measured from the periosteum to the endosteum in micrometers at three different points in each section.
2. Osteocyte number: the number of all osteocyte lacunae containing nuclei were counted in each section.
3. Number of osteocyte lacunae: the number of all osteocyte lacunae (with or without nuclei) were counted in each section.

RESULTS:

General results:

After injection of corticosteroids, the rats decreased in weight at a rate of 20 grams every month. After stopping of corticosteroids, they gained 20 grams per month and returned to their previous weight.

In the period of corticosteroid injection, mild redness in the skin around face were noted, which disappeared after stopping of corticosteroids.

The rats looked restless during the injection period, difficult to handle and biting each other. These signs disappeared after stopping of steroids.

Histological Results:

The following parameters were measured by computerized image analysis using Optimas (Media Cybernetics, 1998 version 6.21.19), then by Microsoft Excel 2010 to calculate average, standard deviation and P value in each group (table 1).

1. Cortical bone thickness (hematoxylin and eosin stain): By comparing cortical thickness in all groups there was a strong significant difference between the bisphosphonate group and the control group. Also there was a significant difference between osteoporosis group and control group. However, there was no significant difference between both vitamin D group and olive oil group when compared with the control group. Table (1) shows marked increase in the cortical bone thickness of bisphosphonate group and slight increase in vitamin D and olive oil groups when compared with control group. On the other hand, there was a decrease in the cortical thickness of osteoporosis group compared to the control group (Figure 1&2).

2. Osteocyte number (Masson's Trichrome stain): Comparing osteocyte number in all groups, there was a significant difference between the osteoporosis group and the control group. On the other side, there was no significant difference between other groups when compared with the control group.

Table (1) shows decrease in the osteocyte number of the osteoporosis group and minimal decrease in the osteocyte number of both bisphosphonate group and olive oil group. However, there was slight increase in osteocyte number of vitamin D group compared to the control group (Figure 1&2).

3. Number of osteocyte lacunae (Masson's Trichrome stain): Comparing osteocyte lacunae in all groups, there was a significant difference between vitamin D group and control group. However, there was no significant difference between other groups

when compared with the control group (Figure 1&2).

DISCUSSION:

Osteoporosis is a serious disease of bone that leads to increased risk of fracture. The bone mineral density is reduced. The bone microarchitecture is disturbed and the amount of non-collagenous proteins is altered. It is the result of years of bone loss, due to a "mismatch" between bone formation and resorption. Worldwide, osteoporosis causes more than 8.9 million fractures annually, resulting in an osteoporotic fracture every 3 seconds [14].

The risk of osteoporosis increase with the aging [16]. Worldwide, 1 in 3 women over age 50 will experience osteoporotic fractures, as will 1 in 5 men aged over 50 [17]. Osteoporosis is estimated to affect 200 million women worldwide, approximately one-tenth (10%) of women aged 60, one-fifth (20%) of women aged 70, two-fifths (40%) of women aged 80 and two-thirds (66.67 %) of women aged 90 [18].

Many studies have shown that glucocorticoids decrease bone mass and thereby increase the risk of fractures, particularly fractures of the ribs, spine and forearm. Studies have shown that 30–50% of all fractures occur in hospital settings, usually associated with administration of high doses of glucocorticoids [8]. The overall effects of glucocorticoids depend on a number of factors including the dose, the duration, the steroid type and the species tested [19].

The most important effect of glucocorticoids is suppression of bone formation by the following mechanisms:

First, glucocorticoids affect the differentiation and activity of many cell types.

Secondly, glucocorticoids modulate the transcription of many of the genes responsible for the synthesis of matrix constituents by osteoblasts, such as type 1 collagen and osteocalcin (OC).

Thirdly, Glucocorticoids inhibit the production of prostaglandins such as PGE2 which normally stimulate collagen and non-collagenous protein synthesis [20]. A wide variety of pharmacological interventions have been shown to decrease bone loss in GIO. Proposed treatments to help maintain or increase bone density include calcium supplementation, bisphosphonates, hormone replacement therapy, vitamin D in one of its forms (cholecalciferol, calciferol, calcitriol, calcidiol, alfacalcidol), calcitonin, parathyroid hormone, fluoride, testosterone and anabolic steroids [9].

The present study aims to assess if vitamin D or olive oil could be useful in the treatment of glucocorticoid induced osteoporosis as bisphosphonate. Also to compare the effectiveness of these compounds with a commonly prescribed bisphosphonate, the therapeutic class identified now as the golden standard treatment for osteoporosis.

Bisphosphonates stimulate transiently the proliferation of preosteoblast cells and increase their differentiation and may increase the production of the anti-resorptive protein osteoprotegerin by osteoblasts. If confirmed, this effect would synergize with the other effects described to reduce bone resorption [21].

Vitamin D metabolite 1, 25(OH)2 D3 acts as a hormone in the regulation of calcium and phosphorus metabolism, maintaining normal calcium and phosphorus concentrations in serum ensuring a normal mineralization of bone [21]. Olive oil shows improvement which may increase bone mineral density (BMD) in osteoporotic glucocorticoid-treated animals. It is an excellent source of gamma linoleic acid (GLA) which has been shown to reduce the excretion of calcium, inhibit bone reabsorption and at the same time increases the calcium content in the bone [10].

In our experiment, the glucocorticoid-treated animals showed weight reduction up to 20 gm compared with the control group. We expected weight gain as a result of glucocorticoid injection, according to Dallman et al. [22]. However, other studies agree with us and reported weight loss under glucocorticoid injection [23]. This might be due to glucocorticoid-induced anorexia in rats, as reported by Won Jahng et al. [25] or due to severe proteolysis and muscle loss [26] as a result of anxiety and excessive movements. After stopping of corticosteroid injection they started to gain weight.

Comparing cortical thickness in all groups there was a very significant increase in cortical bone thickness of bisphosphonate group and slight non-significant increase in cortical bone thickness of both vitamin D group and olive oil group when compared with control group. On the other side, there was a significant decrease in the cortical thickness of osteoporosis group when compared with control group. These results agree with Kozai et al. [27] who found that steroid treatment significantly decreased the bone mineral content and bone mineral density

in the femoral metaphysis. They also reported increased cortical bone mineral content and bone area in the femoral diaphysis.

When we compared osteocyte number in all groups we found a significant decrease in osteocyte number in GIO group when compared with control group. Our findings agreed with Derakhshanian et al. [27] who recorded a reduction in cortical and trabecular thickness accompanied by a significant decrease in the number osteoblasts in glucocorticoid-treated rats. These findings also agreed with other researches such as Sosa et al. [29] and Migliaccio et al. [30] who found that prednisolone administration induces apoptosis of both osteoblasts and osteocytes leading to suppression of bone formation and low BMD. There was also minimal non-significant decrease in the osteocyte number of both bisphosphonate group and olive oil group when compared with the control group. On the other hand, there was a slight non-significant increase in the osteocyte number of vitamin D group when compared with the control group.

By comparing osteocyte lacunae in all groups, there was a significant increase in the number of osteocyte lacunae of vitamin D group when compared with the control group. However, there was no significant difference between other groups when compared with the control group.

CONCLUSION

Osteoporosis affects mainly cortical thickness as well as osteocyte number. Bisphosphonates showed the best results and thus appear more helpful in the treatment of osteoporosis by increasing cortical thickness. Vitamin D and Olive oil act mainly by increasing number of osteocytes. However, vitamin D has a significant role in the treatment of osteoporosis but Olive oil may also have a role.

RECOMMENDATIONS

- Adequate intake of vitamin D is recommended to prevent osteoporosis.
- Bisphosphonate showed the best results; however, vitamin D also has an important role in its treatment. On the other side, olive oil may have a role in treatment of osteoporosis.
- We recommend further investigations to study the effect of corticosteroids on osteocytes where apoptosis may have an important role.

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Table (1)

1. Cortical bone thickness (in micrometers) - hematoxylin and eosin stain					
	Control	**Osteoporosis**	**Bisphosphonate**	**Vitamin D**	**Olive oil**
Average	291.4	242.9	608.2	308.4	306.8
S.D.	35.2	57.9	52.9	49.7	49.39
P value		0.036 *	5.54E-12 ***	0.387	0.432123
2. Osteocyte number per high power field (HPF) - Masson's Trichrome					
	Control	**Osteoporosis**	**Bisphosphonate**	**Vitamin D**	**Olive oil**
Average	9.4	6.8	8.1	10.7	7.2
S.D.	3.0	1.5	2.4	2.2	1.5
P value		0.026394 *	0.29955	0.28359	0.05559
3. Number of osteocyte lacunae per high power field (HPF) - Masson's Trichrome					
	Control	**Osteoporosis**	**Bisphosphonate**	**Vitamin D**	**Olive oil**
Average	13.7	14.3	13.2	16.9	17.2
S.D.	3.33	4.29	3.42	3.212	5.63
P value		0.731232	0.744667	0.042337 *	0.108138

Table (1): Average, standard deviation and P value of:

- Cortical thickness measured from periosteum to endosteum (in micrometer).
- Osteocyte number counted per high power field (HPF).
- Osteocyte lacunae counted per high power field (HPF).

P values:
: Less than 0.001 (very strong significant).
Less than 0.01 (highly significant).
Less than 0.05 (significant).
re than 0.05 (non-significant).

Figure (1) shows photomicrographs of femur sections from various groups of rats. The left column includes sections stained by H&E x40, while the right column includes sections stained with Masson's Trichrome x400.

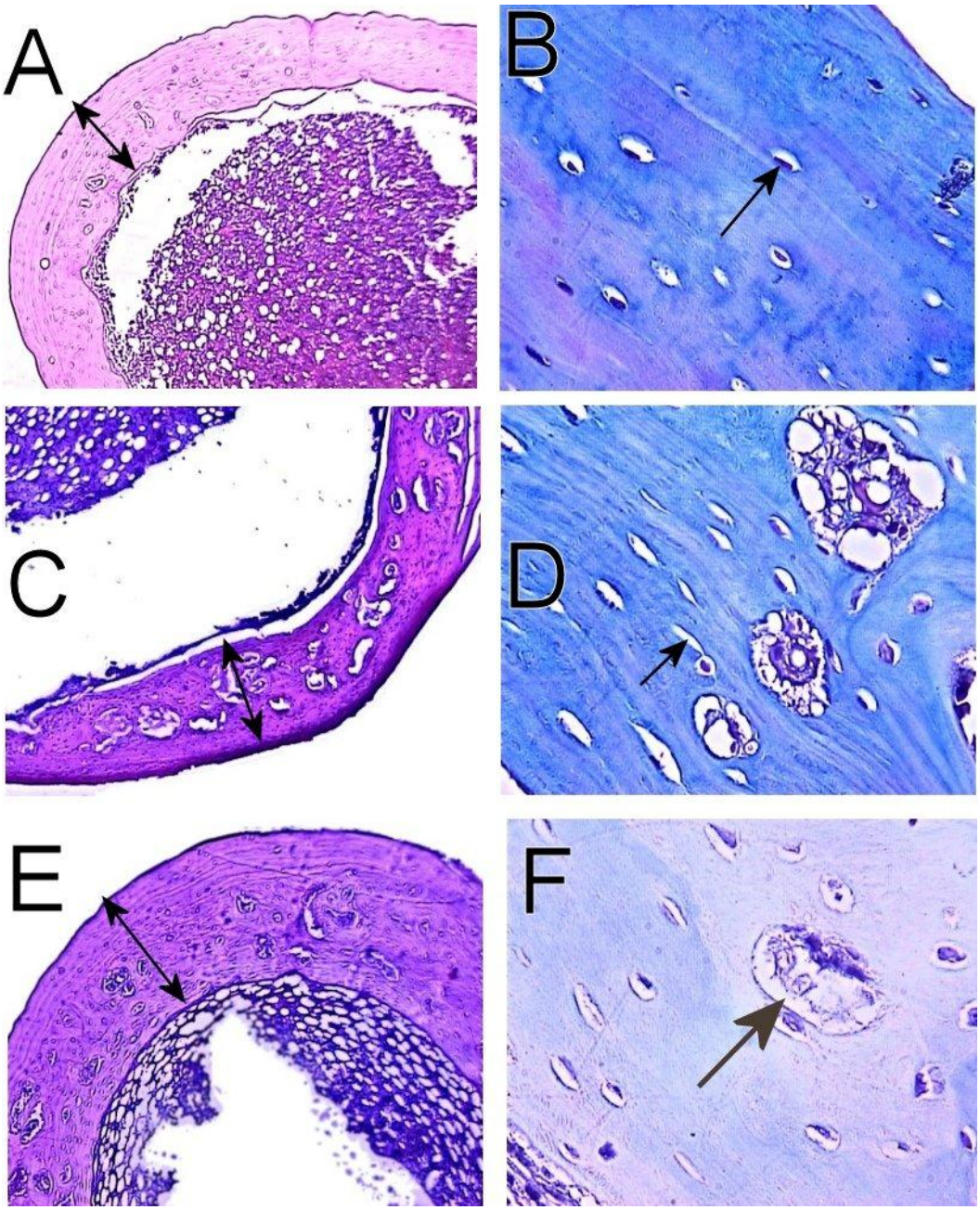

Figure A&B represent the control group. The normal cortical bone thickness (A) and numerous osteocytes with prominent nuclei that appear dark blue "arrow head" (B)**. Figure C&D** represent the osteoporosis group**.** Figure (C) shows reduced cortical bone thickness and wide resorbing areas. While (D) shows many osteocyte lacunae devoid of nuclei "arrow head" and adjacent three resorbing areas.
Figure E&F represent the bisphosphonate group. Note (E) shows increased cortical bone thickness and numerous osteocytes with some resorbing areas. While (F) shows osteocyte lacunae filled with nuclei. Note a resorbing area were seen "arrow".

Figure (2) shows photomicrographs of femur sections. The left column includes sections stained by H&E x40, while the right column includes sections stained with Masson's Trichrome x400.

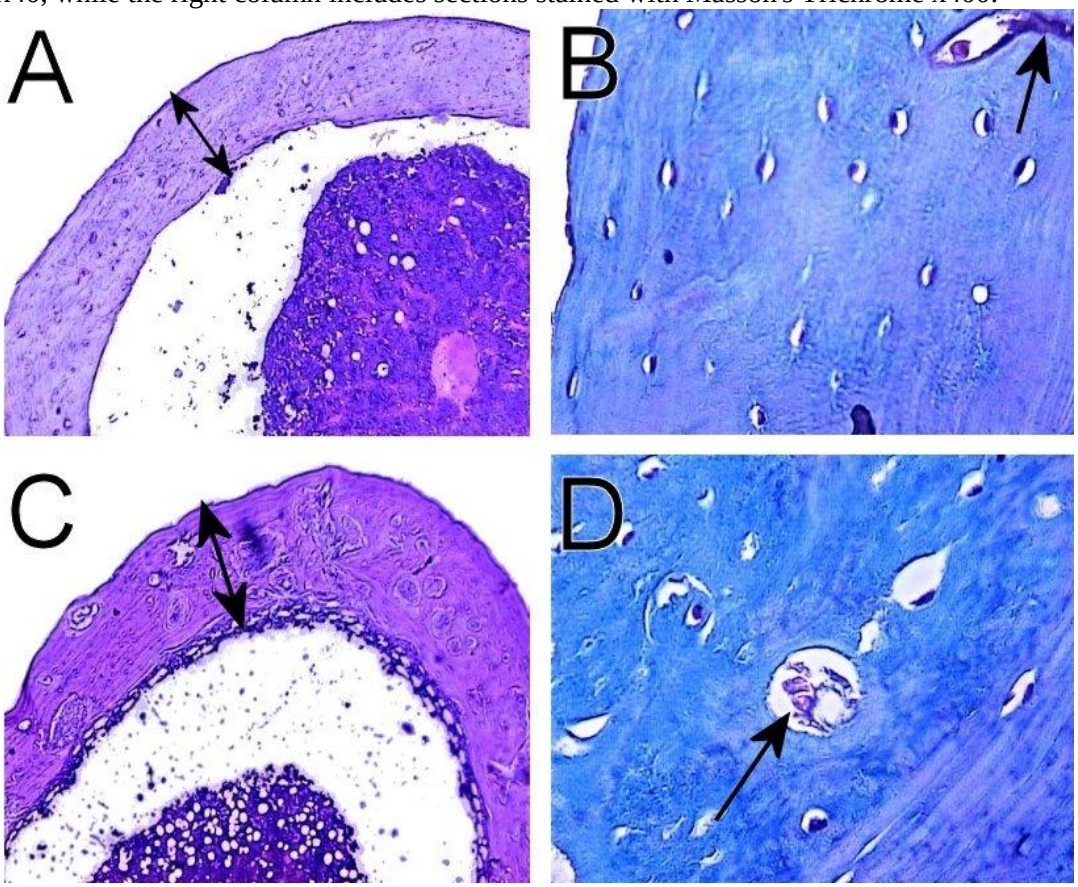

Figure A&B represents Vitamin D group. Figure (A) shows preserved cortical bone thickness and numerous osteocytes. Figure (B) shows numerous osteocytes with prominent nuclei and one Volkman's canal also appear "arrow".

Figure C&D represents Olive oil group. Figure (C) shows increased cortical bone thickness with numerous osteocytes and some resorbing areas. Figure (D) shows osteocyte lacunae were filled with nuclei and one area of resorption also seen "arrow".

Figure (3)

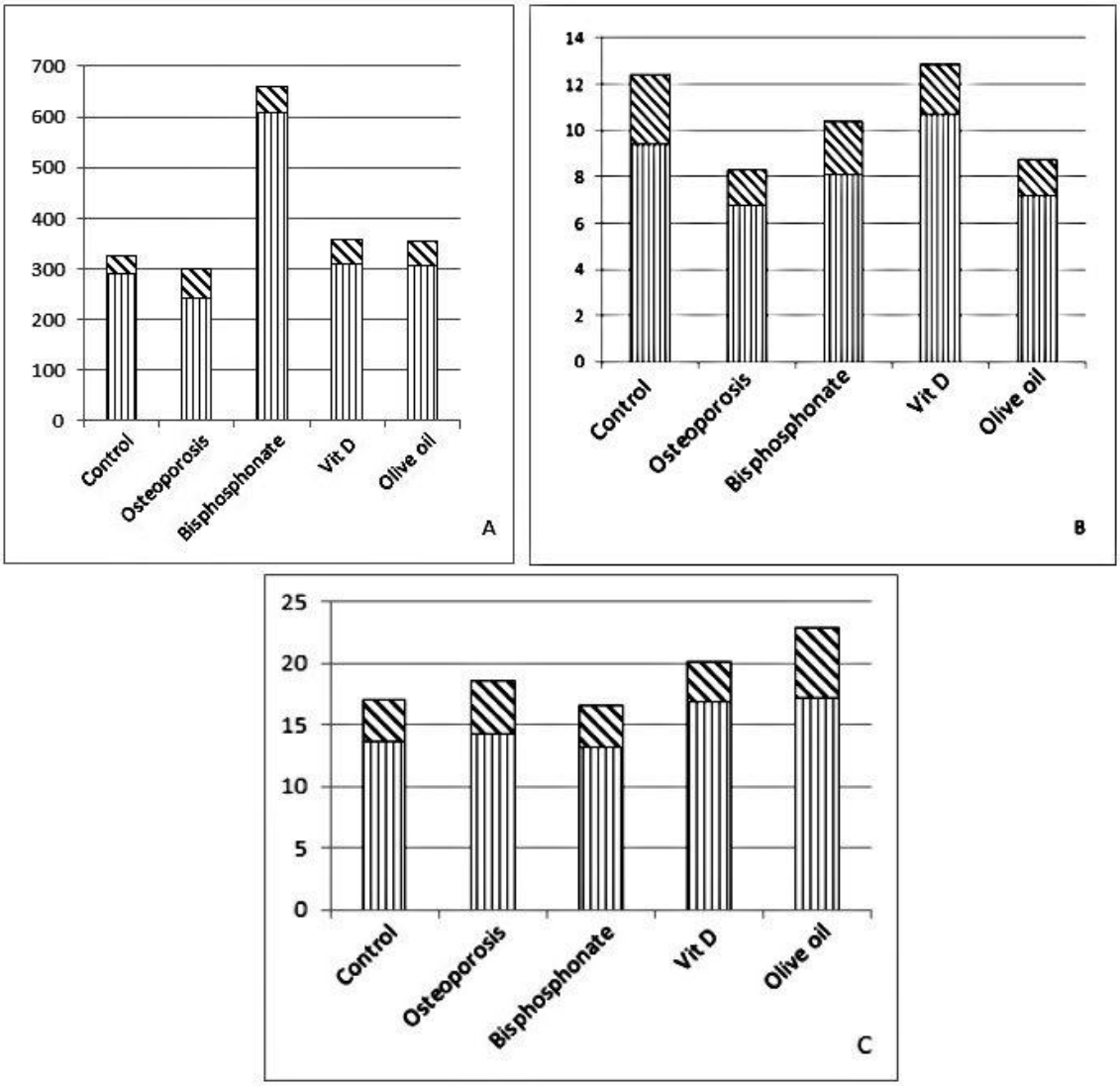

Figure (3): Comparison of data among all groups

* -Averages -Standard deviation

A. Cortical thickness in micrometers
B. Osteocyte number per HPF
C. Osteocyte lacuna number per HPF

In **figure 3A**, a marked increase in the cortical thickness of bisphosphonate group is shown and a decrease in the osteoporosis group. While both vitamin D and olive oil groups returned to nearly to the normal cortical thickness. In **figure 3B**, a decrease in the osteocyte number of osteoporosis group than other groups. **Figure 3C** demonstrates an increase in the number of osteocyte lacunae of the olive oil and vitamin D groups than other groups.