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Simvastatin Ameliorates the Glucocorticoid-Induced Osteoporosis in Adult Male Albino Rat

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Abstract: Background: Many studies have reported an action of statins on bone metabolism. **Aim:** The present study was performed to evaluate the effects of Simvastatin drug on femoral diaphysis of an adult male albino rat with glucocorticoid- induced osteoporosis (GIO). **Material and Methods:** Fourty adult male rats were equally divided as follow; group I (Control) received no medication, group II (Simvastatin group) received Simvastatin, group III (GIO group) received methyl prednisolone and group IV (GIO + Simvastatin group) received methyl prednisolone and simvastatin. Specimens of the middle shafts of femurs were processed for light and electron microscopic study. Morphometric study was done. Percentage of Ca content of bone was measured. Serumcalcium (Ca) & phosphorus (P) levels were measured. Statistical analysis for the collected data was performed. **Results:** In comparison with control group, group III showed indistinct cement lines, many osteocyte lacunae were empty and others contained very small peripheral nuclei. There was statistically highly significant decrease in the percentage of both Ca content and collagen content of bone with a statistically highly significant increase in the number of osteoclasts. Significant improvement was detected with simvastatin administration in group IV when compared with group III as regard all measured data. There were statistically insignificant changes in serum Ca and P levels among all groups. **Conclusion:** The administration of simvastatin, in concomitant with glucocorticoids, prevented the marked distortion in normal architecture of bone but not completely.
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Key words: Simvastatin, osteoporosis, methyl prednisolone, femur.

1. Introduction

Statins are competitive inhibitors of 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMG-CoA) which are widely used for the treatment of hyperlipidemia and improving clinical outcomes of patients with coronary artery disease and other cardiovascular conditions. They also have pleiotropic effects such as anti-inflammatory, antithromboticm, and immunomodulatory effects (Bauer, 2003 and Liao, 2005).

Many studies have reported that statins may have beneficial effects on the treatment of osteoporosis and fractures especially if selectively directed at bone (Anbinder et al., 2007).

Osteoporosis is a metabolic bone disease characterized by structural deterioration of bone tissue and trabecular spacing, leaving the bone tissue fragile with a high fracture risk (Marcu et al., 2011).

It was found that old and young, men and women and all ethnic groups studied have bone loss with glucocorticoids treatment, making this an important public health problem. Glucocorticoid-induced osteoporosis became the most common type of secondary osteoporosis (Caplan & Saag, 2009).

Although Simvastatin is not a new generation of the statin family, it is the most investigated statin family member in the field of bone research, particularly in animal models (Moshiri et al., 2016).

2. Materials and Methods

Animals:

Fourty adult male Sprague-Dawley albino rats, weighing (190-220 grams) were housed in suitable cages with controlled temperature and 12h light/dark cycles and had free access to water and food. They were acclimatized to their environment nearly one month before starting the experiment. All steps of the experiment were carried out according to the rules of ethical committee of Tanta faculty of medicine on the animal experiments.

Drugs:

Simvastatin drug (® Zocor 40 mg): the drug was purchased from the MSD Egypt company, the local subsidiary of Merck Company for pharmaceuticals, in the form of tablets (40mg/tab).

Methyl prednisolone sodium succinate drug (® Solu Medrol 500 mg): The drug was purchased from

Pfizer Company for pharmaceuticals, in the form of vial (500 mg/vial).

Experimental Design:

The animals were divided into four groups as follow:

Group I (control group):

Composed of 10 rats which housed under healthy conditions and received no medication.

Group II (simvastatin group):

Composed of 10 rats that received simvastatin drug orally at 10mg/kg by gavage daily for 8 weeks **(Shata et al., 2015 and Şipos et al., 2015).**

Group III (glucocorticoid induced osteoporosis group) (GIO):

This group was composed of 10 rats that received methyl prednisolone subcutaneously 0.1 mg/kg three times per week for 8weeks **(Nitta et al., 1999).**

Group IV (GIO + Simvastatin):

Composed of 10 rats that received methyl prednisolone (subcutaneously 0.1 mg/kg three times per week) in concomitant with simvastatin drug (10 mg /kg orally by gavage daily). Both drugs were given for 8weeks.

After 24 hours from the end of the experiment; all rats were anaesthetized by ether and sacrificed. Chest incision was done to expose the heart then blood samples were obtained by cardiac puncture.

Biochemical analysis:

The blood samples were centrifuged and serum stored immediately at -20°c for analysis. Serum calcium (Ca) and phosphorus (P) were measured by using automated analyser. The collected data were used for statistical analysis.

Tissue preparation:

The hind limbs were disarticulated at the hip joints. The femurs were carefully dissected and cleaned from adjacent muscles. The middle shaft of each femur was obtained by making section just under the trochanter and another one a little above the interchondylar fossa.

Light microscopic study:

The obtained specimens from the right femurs were fixed immediately in 10% buffered formol saline for 48 hours then washed by running tap water and incubated with 5 % nitric acid (5ml nitric acid +100ml distilled water) at 4°C under continuous shaking. The decalcifying solution was changed daily. The decalcification process was ended when the bone was easily penetrated through by a needle without any force ***(Callis, 2011 and Liu et al., 2017).***

Decalcified specimens were washed in running tap water then followed by routine dehydration and cleared before paraffin embedding.

Sections cut longitudinally at 5 µm and stained with Hematoxylin & Eosin and Masson's trichrome for light microscopic study ***(Bancroft & Stevens, 1996).***

Bone mineral content:

The middle shafts obtained from the left femurs were sectioned longitudinally then fixed in 2.5% glutaraldehyde in phosphate buffer (ph 7.4) at 4º c for 2 hours. Regional elemental bone composition and density was detected in the field area. The elements with higher atomic number such as calcium (Ca) and phosphorus (P) were determined and detected as peaks in graphs using energy-dispersive X-ray analysis (Oxford EDX, Jeol JSM-5300; Jeol, Tokyo, Japan). The mean values of data were used for statistical analysis.

Morphometric study:

The morphometric study was done using the image analyzer (Olympus BX41TF, Tokyo, Japan) at the cell biology department, medical research institute, Alexandria University.

The number of osteoclasts was estimated/high power field (HPF) in a fixed field size of nonconsecutive sections from each animal.

the color area percentage of blue-stained collagen fibers was measured by using an image J software analyzer. The area percentage of collagen fibers was recorded from 5 non overlapping fields per slide (at magnification x 200) from 5 slides selected randomly from each animal.

All quantitative data were tabulated and expressed as mean ± standard deviation (SD).

Statistical analysis:

Data were analysed as mean± standard deviation (SD) by using SPSS program version 22. ANOVA test was used for comparison among different groups. Scheffe test was used for comparison between the *P* values of different groups. Differences were considered significant when probability of difference (*P* value) $\leq$ 0.05. ***(Dawson-Saunders and Trapp, 2001).***

3. Results

A- Light microscopic results:

1-Haematoxylin and eosin stained sections:

In group I (the control group), the longitudinal sections from the middle shaft, diaphysis, of the femur showed shell of cortical bone, formed of bone lamellae appeared as distinct cement lines with the osteocytes inside their lacunae in between these lamellae. The endosteal surface appeared regular and smooth separating the bone marrow inside the cavity from the cortical bone. The osteoblasts and osteoprogenitor cells were seen lining the endosteal surface with some osteoclasts within the cavity nearby the surface **(figures1-A & B).**

The Longitudinal sections from the middle shaft of femur in group II (Simvastatin group) showed nearly similar results as the control group with normal architecture of the compact bone.

In group III, longitudinal sections of the middle shaft, diaphysis, showed evident histological changes in the form of indistinct cement lines, many osteocyte lacunae were devoid of their nuclei and others contained very small peripheral nuclei. Multiple Howship's lacunae with large multinucleated oesinophilic osteoclasts inside them were seen on the endosteal surface **(figures 2- A & B).**

Longitudinal sections of the femoral shaft of group IV showed marked improvement in comparison with the GIO group. The outer surface of the cortical bone was covered with normally appeared periosteum. The Haversian canals showed dilated blood vessels. The osteocytes in their lacunae were seen within the bone matrix in between the cement lines. Some empty lacunae were still seen. The endosteal surface lining of the cortical bone showed some small resorption areas with osteoclasts inside but some cuboidal osteoblasts were seen around **(figures3-A, B & C)**.

2- Masson's trichrome stained sections:

Masson's trichrome stained sections from the middle shaft of femur of group I & II showed normal heavily blue-stained collagen fibers arranged in the form of lamellae. **(figure4-A).** Sections from group III **(figures 4- B)** showed lightly stained collagen fibers of bone lamellae with loss of its lamellar arrangement. Some resorption cavities were seen within the bone matrix.

In group IV, sections of shaft showed areas of normally stained collagen fibers and others of decreased intensity of collagen fiber staining **(figure4-C)**.

B - Bone mineral content:

The calcium (Ca) and phosphorus (P) contents of the middle shaft of left femur of animals of different groups were detected as peaks in the graphs. Group I revealed the highest peak intensity for Ca **(figure5-A).** The group II was nearly similar to the control group **(figure5- B)**. The Ca peak intensity in group III (GIO – group) showed the lowest peak for Ca with elevated one for P **(figure5- C).** Group IV showed nearly normal peak intensity for Ca, which is elevated as compared with that in group III **(figure5-D).**

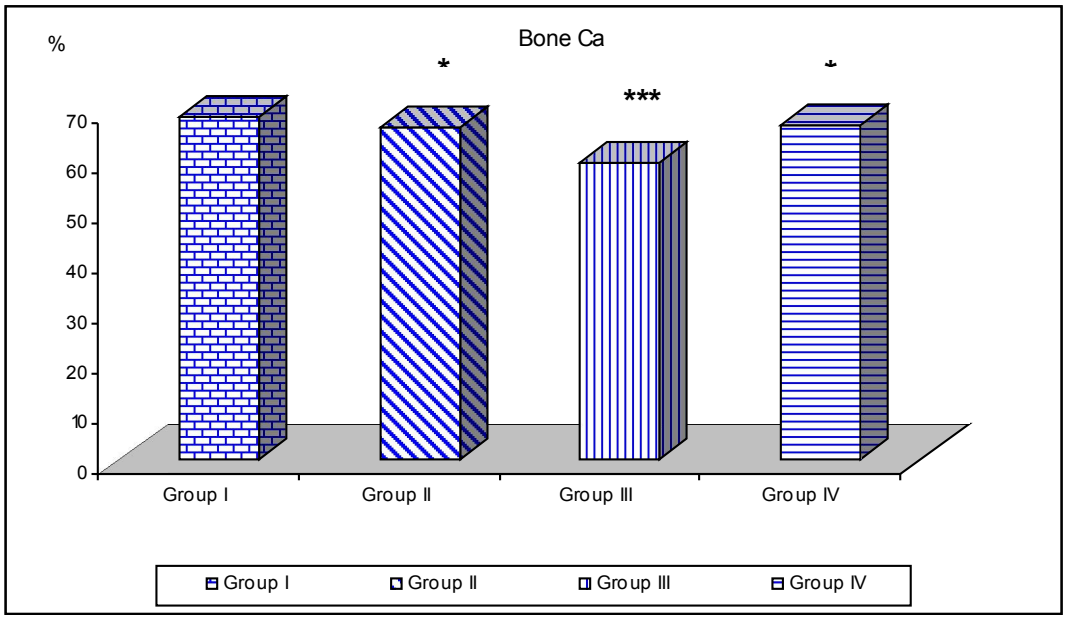
C - Statistical analysis:

There was no statistically significant difference between both group I & group II in all measured data.

The mean percentage of Ca content of bone showed statistically highly significant decrease in group III ($P = 0.001$) in comparison with other groups, which showed no statistically significant difference when compared with each other **(table 1, Histogram 1).**

The mean serum Ca and P in all groups showed no statistically significant difference when compared with each other **(table 2, Histogram 2).**

The mean number of osteoclasts/ HPF in group III showed a statistically highly significant increase ($P = 0.001$) in comparison with group I **(table 3)**. The mean color area percentage of collagen of bone in group III showed a statistically highly significant decrease (P value = 0.001) when compared with the control group **(table 4)**. Significant improvement was detected in group IV compared to group III as regard all measured data **(tables 3 & 4).**



Histogram (1): The mean percentage of calcium content in different groups.

Table (1): The mean percentage of calcium content of bone ± SD and statistical comparison of different groups.

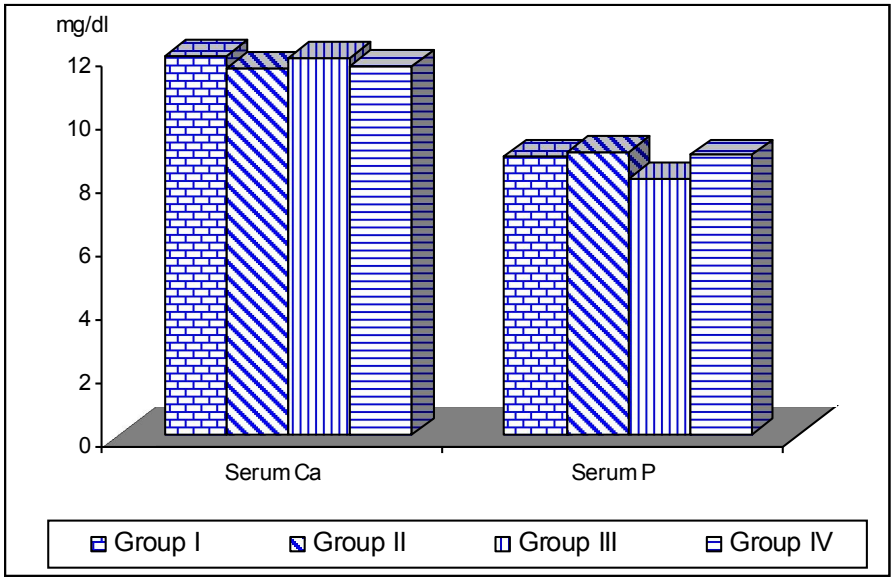
Bone Ca	Range	Mean ± SD	F. test	*p*. value		
Group I (GI)	62.3 – 80.8	68.43 ± 6.35	7.727	0.001*		
Group II (GII)	60 – 71.2	66.23 ± 3.48			*P* 1	0.239
Group III (GIII)	56.5 – 60.9	59.18 ± 1.51			*P* 2	0.001*
Group IV (GIV)	62.2 – 72.2	66.66 ± 3.34			*P* 3	0. 342

SD: standard deviation. *P*1: *P* value between GI & GII. *P*2: *P* value between GI & GIII. *P*3: *P* value between GI & G IV *: significant *P* value

Table (2): The mean serum Ca ± SD and the mean serum P± SD and their statistical comparisonin different groups.

		Range	Mean ± S. D	F. test	*p*.value
Serum P	**Group I**	7.4 – 10.9	8.80 ± 1.28	1.563	0.200
	Group II	7.9 – 10.9	8.89 ± 0.95		
	Group III	6.7 – 9.4	8.05 ± 0.78		
	Group IV	7.5 – 10.3	8.81 ± 0.95		
Serum Ca	**Group I**	11.7 – 12.2	11.93 ± 0.18	1.094	0.371
	Group II	10.8 – 12.7	11.54 ± 0.53		
	Group III	11.3 – 12.9	11.90 ± 0.52		
	Group IV	10.9 – 12.4	11.63 ± 0.55		

SD: standard deviation



Histogram (2): The mean serum Ca ± SD and the mean serum P± SD in different groups.

Table (3): The mean number of osteoclasts/ HPF and statistical comparison ofdifferent groups ± SD.

Number of Osteoclasts / HPF	Range	Mean ± SD	F. test	*P*. value		
Group I (GI)	0 – 4	2.13 ± 1.2	35.113	0.001*		
Group II (GII)	1 – 4	2.63 ± 0.72			*P*1	0.056
Group III (GIII)	2 – 6	4.84 ± 1.19			*P*2	0.001*
Group IV (GIV)	0 – 4	2.59 ± 1.01			*P*3	0.073

SD: standard deviation. *P*1: *P* value between GI & GII. *P*2: *P* value between GI & GIII. *P*3: *P* value between GI & GIV *: significant *P* value

Table (4): The mean color area percentage of collagen ± SD and statistical comparison of different groups.

Color Area %	Range	Mean ± SD	F. test	p. value		
Group I (GI)	29.16 – 46.11	36.30 ± 6.95	46.771	0.001*		
Group II (GII)	29.67 – 37.92	35.08 ± 2.40			*P*1	0.578
Group III (GIII)	0.35 – 12.67	6.44 ± 4.54			*P*2	0.001*
Group IV (GIV)	24.25 – 38.71	31.24 ± 4.47			*P*3	0.025*

SD: standard deviation. *P*1: *P* value between GI & GII. *P*2: *P* value between GI & GIII. *P*3: *P* value between GI & GIV **:* significant *P* value

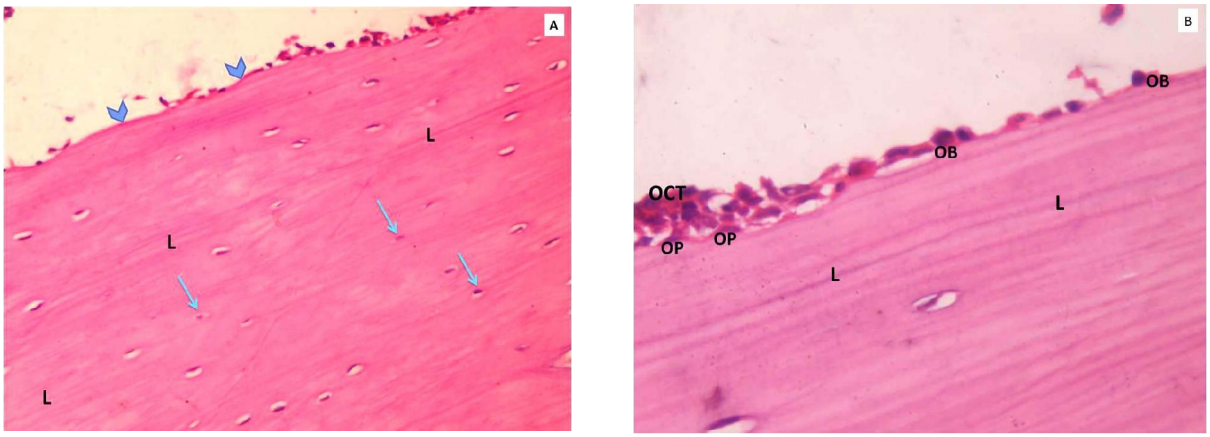


Figure 1: H & E (A, × 400-B, × 1000).

Photomicrographs of longitudinal sections in the middle shaft of femur from group I & II showing nearly similar results with normal architecture of the compact bone. The cement lines (L) are distinct in the bone matrix. The endosteal surface (arrow heads) is regular and smooth, separating the cortical bone from the bone marrow (BM). The osteocytes in their lacunae (arrows) are seen within the matrix. (figure A). The osteoblasts (OB), osteoprogenitor cells (OP) and some osteoclasts (OCT) are seen lining the endosteal surface (figure B).

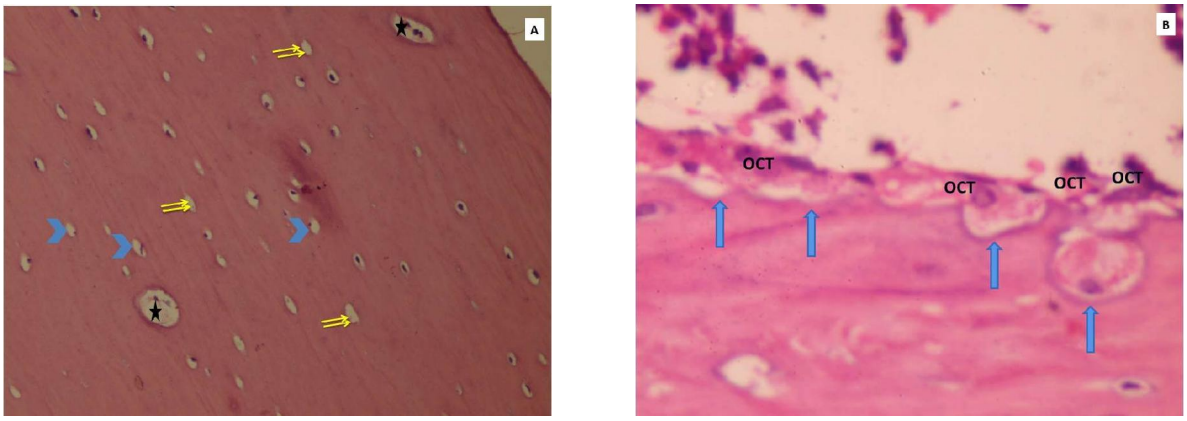


Figure 2: H & E (A, × 400-B, × 1000).

Photomicrographs of longitudinal sections in the middle shaft of femur from group III (GIO group), showing many osteocyte lacunae devoid of nuclei (double arrows). Other lacunae contain very small peripheral nuclei (arrow heads). The bone matrix is homogenous without distinct cement lines with some osteoporotic cavities (★) (figure A). Multiple Howship's lacunae (arrows) with many multinucleated osteoclasts (OCT) are seen on the endosteal surface (figure B).

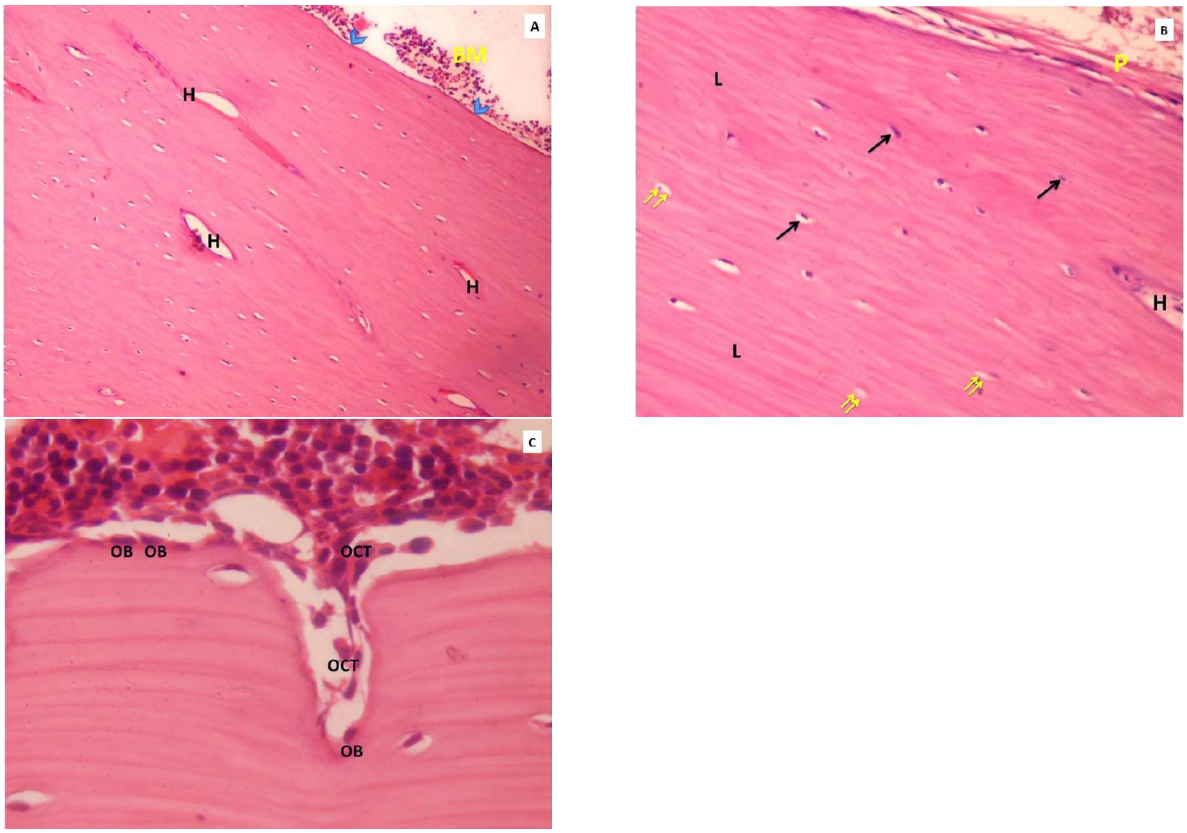
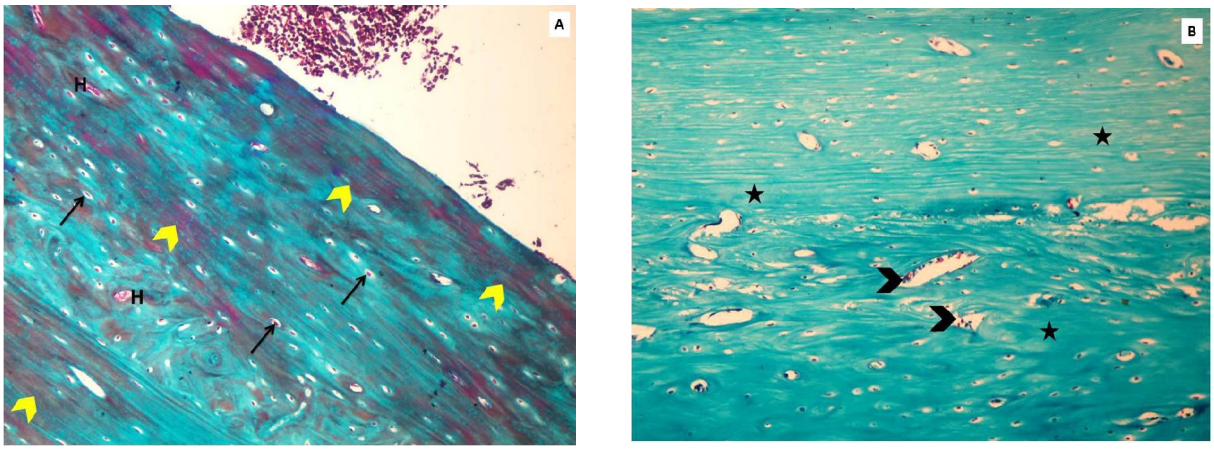


Figure 3: H & E (A, x200-B, x400-C, x1000).

In group IV (GIO + simvastatin group) sections, the endosteal surface is regular and continuous (arrow heads), separating the cortical bone from the bone marrow (BM). The Haversian canals (H) with dilated blood vessels are seen within the bone matrix (Figure A). Osteocytes are seen in their lacunae (arrows) within the bone matrix. Some lacunae are empty (double arrows). Also the cement lines (L) are seen within the matrix. The outer surface of the bone is covered with normally appeared periosteum (P). Haversian canal (H) is seen within the bone matrix (figure B). In (figure C) osteoclast cells (OCT) within a small resorption area and some cuboidal osteoblasts (OB) are seen lining the endosteal surface.



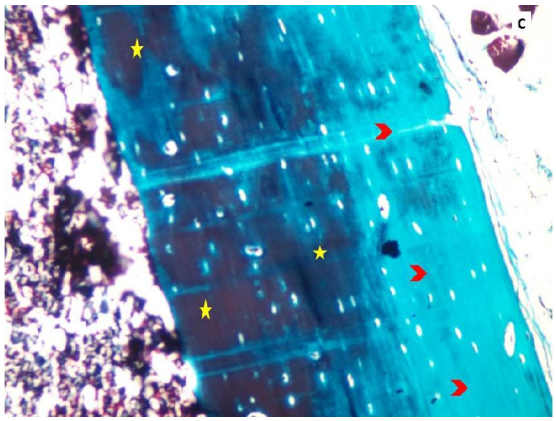


Figure 4: Masson's trichrome X 200.

Photomicrographs of longitudinal sections in the middle shaft of femur from all groups. Groups I & II (figure A), showing lamellar arrangement of abundant heavily blue-stained collagen fibers (arrow heads). Haversian canals (H) can be seen within the bone matrix. Osteocytes with their dark nuclei can be seen within their lacunae (arrows). The sections of group III (figure B) show marked decrease in collagen fiber staining of bone lamellae (★) with loss of its lamellar arrangement. Some resorption cavities (arrow heads) are seen within the bone matrix. After simvastatin administration (figure C) there are areas of normally stained collagen fibers (★) and others (arrow heads) of decreased intensity of collagen fiber staining.

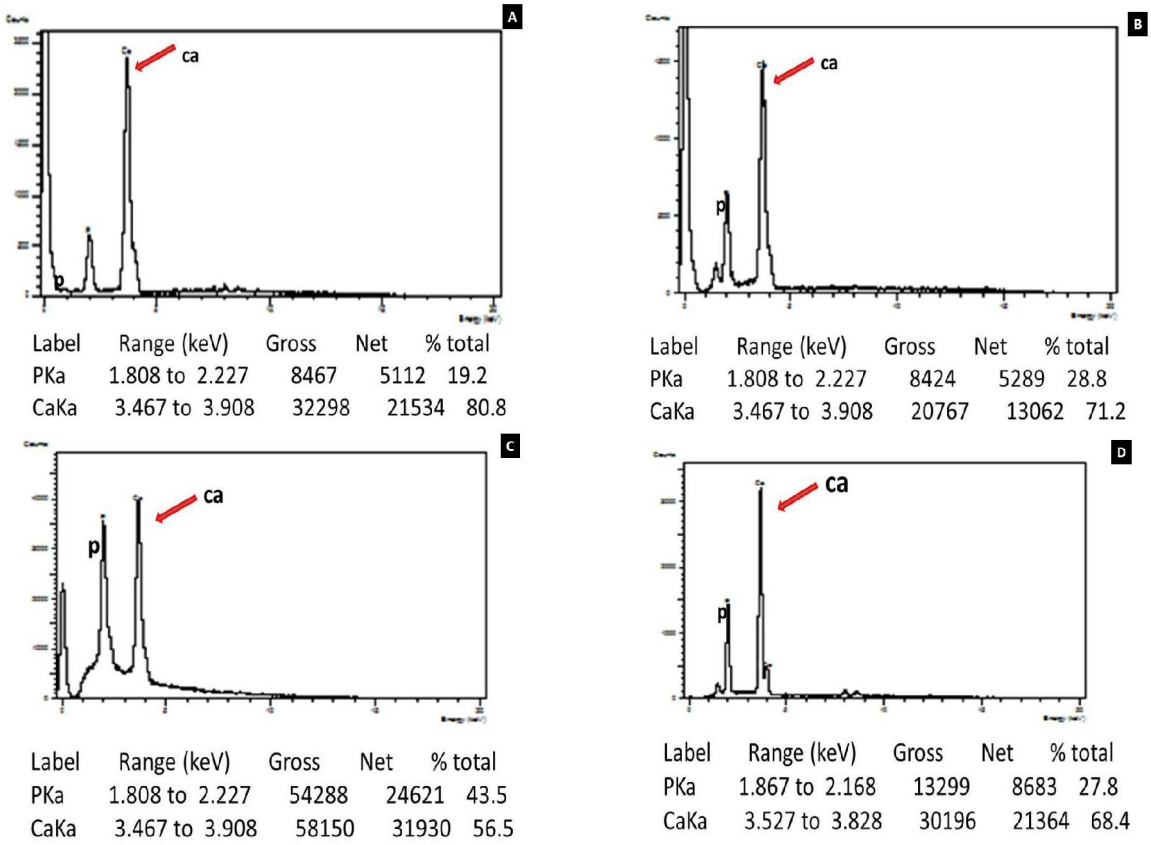


Figure 5: EDX: Graphs for energy-Dispersive X-ray line scan showing (Ca) and (P) contents of the middle shaft of femur from all groups

4. Discussion:

Statins are lipid lowering drugs and are routinely administered in the treatment of hyperlipidemia. The scientists have found that statins have other pleiotropic effects that may be beneficial in bone regeneration ***(Oryan et al., 2015).***

Osteoporosis and other diseases of bone loss are a major public health problem. Glucocorticoid-induced osteoporosis is the most common type of secondary osteoporosis. They are widely used as a treatment for numerous clinical disorders for long periods but their adverse effects on bone metabolism are seriously damaging ***(Mazziotti et al., 2006 and Mc-Donough et al., 2008).***

The present study was performed to evaluate the effects of oral simvastatin administration on

osteoporosis induced by methyl prednisolone injection in adult male albino rat. The middle shaft of femur or diaphysis was chosen as an example for the cortical or compact bone.

In the present study it was found that the histological pattern of the compact bone in the shaft of the femurs of rats after administration of simvastatin orally at dose 10 mg/kg for 8 weeks (in group II) revealed nearly similar results as the control group (group I). This is in agreement with Elsaid & Sadek, (2017) who demonstrated that administration of simvastatin orally to healthy rats for 2 months produces nearly no effect on bone structure.

In group III, after administration of methylprednisolone there were evident histological changes in the form of osteoporotic cavities within the matrix in addition to irregularly eroded endosteal surface. Multiple Howship's lacunae with osteoclasts inside them were seen on the endosteal surface. Many osteocyte lacunae were devoid of their nuclei and others contained pyknotic nuclei.

Similar findings were found by Omara et al., (2009) who found marked decrease in mid-diaphyseal cortical bone thickness and increased bone resorption in dexamethazone- treated rats compared with controls. He stated that the cortical bone loss is due to increased activation of Haversian remodeling systems with subsequent increased Haversian canal diameter. He added that dexamethasone inhibit the production of osteoprotegerin, which is a protein produced by osteoblasts to inhibit osteoclast differentiation, leading to increased osteoclast differentiation with subsequent increased bone resorption.

Bitto et al., 2009; Cui et al., 2012 and Zhou et al., 2017 stated that GIO results from impaired bone formation as well as exaggerated bone resorption. There are many possible pathological mechanisms of GIO for example impairing osteoblast function, decreasing the apoptosis of mature osteoclasts, secondary hyperparathyroidism, increased renal excretion and decreased intestinal absorption of calcium in addition to impairment of hypothalamus hypophysis adrenal cortex system.

O'Brien et al., (2004) and Yao et al., (2013) stated that osteocytes exposed to GCs for a prolonged period of time showed apoptosis, which appeared in the form of empty lacunae. This agreed with the findings in the present study.

In this experiment, the administration of simvastatin in concomitant with cortisone showed marked improvement of bone microarchitecture in comparison with group III. The endosteal surface was regular and continuous. The bone matrix showed distinct cement lines with the osteocytes in their lacunae in between. Osteoclast cells inside Howship's lacunae of bone resorption were seen but surrounded by new bone deposition. Some empty lacunae were still seen. The endosteal surface showed some small resorption areas. These results were in accordance with Oxlund & Andreassen, (2004) who revealed that simvastatin administration to ovarictomized rats increased cortical bone formation at the periosteal bone surface. The new cortical bone exhibited a normal lamellar structure. Also the osteoclast number became significantly less in group IV. The previous findings indicated that simvastatin increased bone formation and decreased bone resorption but this improvement not complete like normal. Small osteoporotic spaces still present with some discontinuous trabeculae.

These findings are in agreement with Moshiri et al., (2016) who stated that Simvastatin reduces bone resorption and bone loss, where it (i) enhances osteogenesis by increasing number of osteoblasts via increasing bone morphogenic protein-2 (BMP-2) expression (ii) reduces osteoblast apoptosis thus increasing the population of osteoblasts in the healing bone (iii) reduces osteoclastogenesis by decreasing the differentiation of macrophages or monocytes into osteoclasts.

Also Wang et al., (1995) and Cui et al., (1997) showed that concomitant use of lipid clearing agentswith steroids have the potential to decrease the severity of steroid induced osteoporosis.

In the current experiment, the GIO model (group III) showed marked increase in fatty tissue in bone marrow spaces. On the other hand, the bone marrow spaces became narrower and more cellular with little fatty tissue in group IV following administration of simvastatin.

Canalis et al., (2007) and Lin et al., (2014) showed that glucocorticoid therapy not only produce bone loss but also accumulate large amounts of fat in bone marrow (fatty marrow). This indicates that glucocorticoids alter lineage of mesenchymal stem cells (MSC) to adipocytes at the expense of osteoprogenitor cells and hence osteoblasts.

Also Chen et al., (2016) stated that the diminution of the medullar cellularity with its enrichment in adipose cells has negative consequences on the bone.

Song et al., (2003) and Liu et al., (2009) demonstrated that simvastatin inhibited adipocytic differentiation of mesenchymal stem cells from rats and promoted osteoblastic differentiation in vitro. They explained that this effect is partially mediated by inducing BMP-2 expression in bone mesenchymal stem cells.

Masson's trichrome stain used in the current work revealed a highly significant decrease in the mean color area percentage of blue stained collagen in GIO group when compared with other groups.

These findings indicate decreased collagen content of bone with glucocorticoids treatment. Romas, (2008) and Payer et al., (2010) stated that glucocorticoids affect the function of the differentiated mature cells, where they inhibit the synthesis of bone extracellular matrix components by osteoblasts, such as type 1 collagen and osteocalcin. This results in decrease in bone matrix available for mineralization. They explained that glucocorticoids mediate this action by affecting the transcription of many of genes in addition to the inhibition of the synthesis and activity of many local acting factors that affect osteoblasts, including cytokines like interleukins 1 & 6 and many growth factors.

Ho et al., (2009) demonstrated that simvastatin treatment significantly increased the osteoblastic cells with immuno-stained BMP-2, collagen type I and osteocalcin in vertebral bones in ovarictomized rats. This increase in osteogenic proteins in bone may explain the improvement in collagen content and maturity found in group IV.

In this experimental study, the mean percentage of calcium content of bone in group IV showed significant increase in comparison with group III. On the other hand the means of the serum values of calcium and phosphorus in the different groups showed no statistically significant differences when compared with each other. At the same time the mean percentage of calcium content in group III (GIO group) revealed a highly significant decrease when compared with the control.

The previous finding can be explained by Wang et al., (2002) and Fukumoto & Martin, (2009), who stated that the methylprednisolone has negative effect of on calcium metabolism. It reduces the gastrointestinal absorption of calcium and increases urinary excretion of calcium, which leads to calcium deficit. low serum ionized calcium levels results in hyperparathyroidism, which may, in turn, cause a change in calcium levels resulting in calcium release from skeleton, thus causing bone loss. So in group III the serum calcium was normal and its content in the bone specimens was significantly low.

Pytlik et al., (2003) stated that simvastatin administered to ovariectomized rats showed a statistically significant increase in mineral content in the tibia, intensified bone formation processes and decreased bone resorption processes induced by bilateral ovariectomy. Moreover, Horecka et al., (2016) observed decrease of calcium plasma level in simvastatin-treated group of post-menopausal women. Although it did not reach statistical significance, he explained that by the intense use of calcium for bone recovery.

Conclusion:

It could be concluded that:

The administration of simvastatin drug to healthy rats produces no significant effect on bone microarchitecture and mineral content. Its administration in concomitant with glucocorticoids prevented the marked distortion in the normal architecture of bone but not completely.

Recommendations:

According to the results of the present work, simvastatin drug may be a new treatment strategy for preventing bone loss and reserving bone mass in osteoporotic disorders.

More researches should be done, especially on the clinical level, to evaluate the possibility of using of simvastatin in the treatment and prevention of osteoporosis.

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