Original Article

The Potential Protective Role of Alendronate Sodium Alone or in Combination with Ginger Against Fluvoxamine Maleate-Induced Osteoporosis in Adult Female Albino Rats (Histological and Immunohistochemical Study)

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ABSTRACT

Background: Selective serotonin reuptake inhibitors including fluvoxamine maleate (FM) have been shown to induce osteoporosis. Alendronate sodium (ALS) is anticatabolic agent used for osteoporosis treatment. Ginger (G) is strong antioxidant having beneficial effects on bone.

Aim: Evaluating the potential protective role of alendronate sodium alone or in combination with ginger against FM-induced osteoporosis in rats.

Materials and Methods: Sixty female rats were equally divided into six groups and they received their drugs daily for five weeks. Group I (Control), group II (ALS): received alendronate sodium 1 mg/kg, group III (G): received ginger 500 mg/kg, group IV (FM): received fluvoxamine maleate 2 mg/kg, group V: received FM+ALS with same doses as previous groups, group VI: received FM+ALS+G as previous groups. Femur bones were taken and processed for light and scanning electron microscopic inspection.

Results: Rats treated with FM only revealed signs of osteoporosis as osteoporotic cavities, irregularity and erosion of bone surface and decreased compact bone thickness. There were highly significant decline in area% of collagen fibers and highly significant rise in osteoclast count compared to control group. Mild reaction of osteopontin proteins in bone matrix was observed. Highly significant changes in serum calcium and alkaline phosphatase levels were detected. Administration of alendronate sodium with FM in group V minimized these changes, while administration of alendronate sodium and ginger with FM in group VI showed histological picture near to control group.

Conclusion: Alendronate sodium is useful in the protection against FM-induced osteoporosis and addition of ginger gives synergistic effect to it.

Key Words: Alendronate sodium, CD68, fluvoxamine maleate, ginger, osteopontin.

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INTRODUCTION

Osteoporosis in the last decades has become a universal problem of epidemic ratio affecting 10% of the world's population[1]. Osteoporosis is bone disorder characterized by bone density reduction leading to rise in bone fragility and fractures with mobility impairment[2]. It was found that osteoporosis happens silently and gradually[3].

Osteoporosis is a common illness occurring among the old people particularly postmenopausal women as a result of estrogen reduction[4]. Physicians are knowledgeable about osteoporosis correlated with old age, postmenopausal status, and secondary causes as chronic illnesses. However, they may not be aware of the adverse effects of many drugs that can decrease bone mineral density (BMD) leading to fracture. Some studies provide valuable data about drugs that put patients at risk for osteoporosis as glucocorticoids (GCs), selective serotonin reuptake inhibitors (SSRI), proton pump inhibitors, anticonvulsants, calcineurin inhibitors, anticoagulants and chemotherapies[5].

Depression is a very common psychological condition with high prevalence in females than males[6]. People with depression and anxiety problems are treated with selective serotonin reuptake inhibitors as a primary choice of treatment because these drugs are safer, well tolerated and with less adverse effects than other antidepressant drugs[7].

The usage of antidepressants, mainly serotonin reuptake inhibitors (SSRIs), increased recently all over the world. Low BMD and bone fractures were reported in individuals taking antidepressants and this raised the concern about the adverse impact of SSRIs on bone[8].

Fluvoxamine maleate (FM) is active SSRI which combines selectively with serotonin transporters, preventing reuptake of serotonin in nerves; consequently promoting the serotonergic effect in brain[9]. Fluvoxamine maleate is suggested as first line treatment for a lot of psychological problems including depression[10].

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Bisphosphonates are synthetic analogues of pyrophosphates which have been utilized in the management of different bone diseases. These agents perform anticatabolic action via changing osteoclast mobilization, differentiation and performance, and/or by inducing osteoclast apoptosis, consequently leading to a decline in bone resorption. Bisphosphonates can be classified according to their chemical structure into non-nitrogen–containing and nitrogen-containing bisphosphonates[11]. Bisphosphonates with nitrogen inside their side chain possess the strongest antiresorptive activity[12].

Alendronate sodium (ALS) is an oral bisphosphonate containing nitrogen utilized for osteoporosis treatment. About fifty percent of individuals treated with ALS reveal reduced fracture incidence in spine and hips[13].

Ginger (Zingeber officinle Rosc.) is one of Zingiberacae family. It grows in Asia and utilized in numerous areas as condiment and spice. In addition, the rhizome of ginger has many uses in herbal traditional medicine[14]. Ginger rhizome is usually used as dried powder, a fresh paste or for flavoring tea in a lot of countries[15]. The important efficient ingredients of ginger rhizome are gingerols (6-gingerol), shogoals, gingiberols, and zingerone. Ginger exhibited various biological activities including antiplatelet, antioxidant, antitumour, and antiarthritic activiy[16].

Ginger is a powerful anti-oxidant that is considered safe with minimal insignificant side effects[17]. Ginger was found to be effective with beneficial effects on bone-related diseases as osteoarthritis[18].

From the foregoing, it seems that fluvoxamine maleate (FM) has an adverse side effect on the bone. Thus, the purpose of this study was to investigate the effect of FM on the histological structure of femur bones of albino rats and to assess the potential protective role of alendronate sodium (ALS) alone or in combination with ginger through histological and immunohistochemical study.

MATERIALS AND METHODS

Matrials

Drug

• Fluvoxamine maleate (FM), a product of Pharco Pharmaceuticals; Alexandria, Egypt, available in a tablet form 50 mg with the trade name Faverin. The drug was dissolved in normal saline and given to the rats subcutaneously (We dissolved each tablet in 125ml normal saline to get solution containing 0.4mg /1ml).

• Alendronate sodium (ALS) was available in the form of tablets 70 mg with the trade name Fosamax (Merck & co. Inc., Whitehouse station, N.J., USA, M.O.H Reg. No. 195/2014). The distilled water was used to dissolve tablets which administrated orally to rats using gastric tube (We dissolved each tablet in 350ml distilled water to get solution containing 0.2mg /1ml).

• Ginger (G): ginger rhizome powder was available in the form of tablets (400 mg) with the trade name Ginger (produced by Arabic Company for Pharmaceuticals & Medicinal Plant MEPACO – MEDFOOD, Enshas El Raml, Sharkeia, Egypt). The tablets were dissolved in distilled water and administrated orally to rats using gastric tube (We dissolved each tablet powder in 4 ml distilled water to get solution containing 100mg /1ml).

Animals

Sixty adult female albino rats (about three months old) weighing 180–200g were utilized in this work. Animal care and hygiene were preserved to retain them in good and sanitary atmosphere. They were given balanced food with free access to water. All animal methods were carried out according to approved protocols and in accordance to the recommendations for suitable care and usage of experimental animals (Approval number: 10/2022 HIST28-2).

Experimental design

The animals were divided into 6 groups (10 rats in each group). In each group, the rats received their drugs once daily for five weeks.

Group I (control group): The animals of this group were subdivided into 3 subgroups:

Subgroup Ia: Included 4 animals, they were left without treatment through the experimental period.

Subgroup Ib: Included 3 animals, received 1 ml/day normal saline subcutaneously, for five weeks.

Subgroup Ic: Included 3 animals, received 1 ml/day distilled water orally using gastric tube, for five weeks.

Group II (ALS group): Received alendronate sodium 1mg/kg/day[19] orally using gastric tube, for five weeks. Each animal received one ml of ALS solution.

Group III (G group): Received ginger 500mg/kg/day[20] orally using gastric tube, for five weeks. Each animal received one ml of ginger solution.

Group IV (FM group): Received fluvoxamine maleate 2 mg/kg/day[21] subcutaneously, for five weeks. Each animal received one ml of FM solution.

Group V (FM+ALS group): Received fluvoxamine maleate concomitantly with alendronate sodium with the same doses as pervious groups.

Group VI (FM+ALS+G group): Received fluvoxamine maleate concomitantly with alendronate sodium and ginger using same doses as pervious groups.

Methods

Twenty four hours following last dose, blood samples were taken for biochemical study. Animals were then anesthetized by intraperitoneal injection of pentobarbital (35 mg/ kg)[22] and sacrificed. Femur bones of animals were dissected, washed and processed for light and scanning electron microscopic examination.

A- Biochemical study

Serum levels of calcium and alkaline phosphatase were estimated at clinical pathology lab of Menoufia University using spectrophotometer[23].

B- Light microscopic study

Samples were fixed in formalin10% and then underwent decalcification by ethylene diamine tetra-acetic acid (EDTA). After the specimens became decalcified, they were processed by the ordinary method to get paraffin blocks. 4µm thick sections were cut and the following studies were performed:

1. Histological study: Hematoxylin and Eosin (H&E) stain and Masson trichrome[24].

2. Immunohistochemical study: for detection of Osteopontin (matrix protein which is an indicator for bone formation)[25] and CD68 (a marker for macrophage and osteoclast)[26].

It was done on 4µm thick Paraffin sections by applying the technique of streptavidin–biotin complex. The sections were incubated with Primary rabbit polyclonal anti-osteopontin (OPN) antibody (Calbochem, San Dego, CA, USA), and mouse monoclonal anti CD68+ antibody (Lab Vision Laboratories, USA). The positive control of the primary antibodies was osteosarcoma for OPN and human tonsil for CD68+. The negative control was done by adding phosphate buffer solution instead of the primary antibody. Osteopontin reaction appeared as brown color in bone matrix around the lacunae, haversian canals and in cement lines, CD68 reaction appeared as cytoplasmic brown staining in the osteoclasts.

C- Scanning electron microscopic study[27]

Specimens were fixed into glutaraldehyde 2.5% and processed for examination by scanning electron microscope (JEOL,JSM-52500LV, Japan) at Faculty of Medicine, Tanta University, Egypt.

Morphometric measurements

1. Thickness of compact bone of the shaft in H&E stained sections at magnification of ×200.

2. Area percent of collagen fiber content in sections stained with Masson's trichrome at magnification of ×200.

3. The number of osteoclasts / high power field was counted in CD68 immunostained sections at magnification of × 400.

Ten sections of 5 animals for every group were examined and five non-overlapping fields / section were measured by image analyzer (Leica Q500 MC Progrem; Camabridge, UK)

Statistical analysis

SPSS program, version 17 (IBM Corporation, Somars, NewYork, USA) was used for statistical analysis of biochemical and morphometric results. The mean ± SEM (standard error of mean) was used to demonstrate data. ANOVA followed by "Tuckey" post hoc test was utilized to compare between the groups. Comparisons were considered highly significant in case of *P value<0.001*, significant in case of *P value<0.05* and non-significant if *P value>0.05*[28].

RESULTS

All subgroups of the control group showed the same histological, immunohistochemical and biochemical results. Therefore, subgroups (Ia, Ib & Ic) were referred to as the control group.

Light microscopic results

Histological study

• Hematoxylin and Eosin staining

H&E-stained sections of control group (group I) reveled the normal well-known histological structure. A transverse section of shaft of femur of control group showed compact bone surrounding bone marrow cavity. The compact bone exhibited homogenous deep acidophilic matrix. The outer surface of the bone was covered by smooth periosteum. The inner surface of bone was lined by endosteum formed of osteoblasts (Figures 1A, 1B, 1C). The compact bone is organized as external circumferential lamella located under the periosteum, Haversian system (osteon) composed of concentric lamellae around a central canal (haversian canal), interstitial lamellae in-between the osteons and inner circumferential lamella around the endosteum. Osteocytes appeared in between lamellae within their lacunae (Figures 1B, 1C). The compact bone showed sub-periosteal distinct basophilic cement lines reflecting new bone deposition and these lines acted as borders between newly added

bone matrix and the older one (Figure 1A). The head of femur of control group showed cancellous bone composed of branching and anastomosing bone trabeculae enclosing bone marrow spaces lined by smooth regular endosteum. Osteocytes appeared inside their lacunae (Figure 1D). Group II treated with alendronate sodium and group III treated with ginger showed the same histological picture as control group. In contrast, fluvoxamine maleate treated group (IV) revealed marked irregularity and erosion of compact bone surface with appearance of multiple osteoporotic cavities, some of them were elongated (Figure 2A). In some sections, the periosteum was very thick and in other sections, it appeared disrupted (Figures 2B, 2C). The endosteum appeared without osteoblasts (Figure 2A). Concerning the cells of bone, osteocytes were totally lost in some areas. Some osteocytes were degenerated with the appearance of empty lacunae, while others appeared shrunken (Figures 2B, 2C, 2D). Many osteoclasts with acidophilic cytoplasm and multiple nuclei were observed on the bone surface and inside osteoporotic cavities (Figures 2D, 2E1&E2). Less acidophilia of matrix and absence of cement lines were observed in many sections (Figures 2A, 2B, 2D). In some sections, cement lines appeared indistinct (Figure 2D). As regard the cancellous bone, it revealed discontinuous bone trabeculae having blind ends and wide interconnected bone marrow spaces. Some trabecular areas appeared with faintly stained matrix and others appeared devoid of osteocytes. Irregular eroded endosteal surface was seen in some areas (Figure 2F). Group V treated with both fluvoxamine maleate and alendronate sodium revealed a better histological picture than that of group IV. There were irregular basophilic cement lines and areas of faintly stained matrix. Also, widened osteocytes lacunae and few osteoporotic cavities were observed (Figure 3A). Concerning the cancellous bone, continuous bone trabeculae were observed but with some osteoporotic cavities (Figure 3B). Group VI treated with fluvoxamine maleate, alendronate sodium and ginger had a histological picture more or less similar to control group. Compact bone revealed normal periosteal thickness with underlying regular bone lamellae and homogenous deep acidophilic matrix. Endosteum was lined with many osteoblasts. Osteocytes appeared inside their lacunae. Multiple distinct basophilic cement lines were observed indicating new bone formation (Figures 4A, 4B, 4C). Regarding the cancellous bone, it showed branching and anastomosing bone trabeculae enclosing bone marrow spaces. Osteocytes appeared inside lacunae. Smooth endosteal surface was seen lining bone marrow spaces (Figure 4D).

• *Masson's trichrome staining*

The control group (I) revealed collagen fibers indicated by green color within the reddish bone matrix (Figure 5A). Also, groups II and III did not show difference in comparison with the control group. In contrast, fluvoxamine maleate treated group (IV) demonstrated apparently few amount of collagen fibers indicated by green color within reddish bone matrix. Thick fibrous periosteum and osteoporotic cavities were observed (Figures 5B). Group V treated with both fluvoxamine maleate and alendronate sodium revealed moderate amount of collagen fibers indicated by green color within the reddish bone matrix. Slight thickening of periosteum and small osteoporotic cavities were observed (Figure 5C). Group VI treated with fluvoxamine maleate, alendronate sodium and ginger showed nearly normal amount of collagen fibers indicated by green color within reddish bone matrix. Smooth periosteum was observed (Figure 5D).

Immunohistochemical study

• *Osteopontin immunostaining*

The control group (I) exhibited strong positive reaction of osteopontin protein in bone matrix around the lacunae, haversian canals and in cement lines (Figure 6A). Group II and group III had the same reaction of osteopontin protein in the bone matrix as the control group. Fluvoxamine maleate treated group (IV) revealed slight positive reaction of osteopontin proteins in bone matrix around an osteoporotic cavity and in cement lines (Figure 6B). Group V treated with both fluvoxamine maleate and alendronate sodium revealed moderate positive reaction of osteopontin proteins in bone matrix around the lacunae, haversian canals and in cement lines (Figure 6C). Group VI treated with fluvoxamine maleate, alendronate sodium and ginger showed strong positive reaction of osteopontin proteins in bone matrix around the lacunae, haversian canals and in cement lines (Figure 6D).

• *CD68 immunostaining*

The control group (I) showed mild positive CD68 cytoplasmic immunoreactivity in few osteoclasts lining haversian canals (Figure 7A). Groups II and III had the same CD68 cytoplasmic immunoreactivity as control group. Fluvoxamine maleate treated group (IV) revealed strong positive CD68 cytoplasmic immunoreactivity in many osteoclasts present in osteoporotic cavities and on the surface of bone (Figures 7B, 7C). Group V treated with both fluvoxamine maleate and alendronate sodium revealed moderate positive CD68 cytoplasmic immunoreactivity in some osteoclasts present on the bone surface (Figure 7D). Group VI treated with fluvoxamine maleate, alendronate sodium and ginger showed mild positive CD68 cytoplasmic immunoreactivity in few osteoclasts present on the bone surface (Figure 7E).

Scanning electron microscopic results

Scanning electron microscopic examination of femur of control rats (group I) revealed outer compact bone giving rise to continuous branching and anastomosing cancellous bone trabeculae which surrounded spaces of bone marrow

(Figure 8A). The surface of compact bone appeared smooth and regular containing haversian canals surrounded by lacunae of osteocytes (Figure 8B). Groups II and III had the same pictures as control group. In contrast, fluvoxamine maleate treated group (IV) revealed discontinuous broken bone trabeculae arising from compact bone. Also, Peeling off the surface of bone trabeculae was noticed. The surface of the compact bone appeared markedly irregular and destructed with cracks and osteoporotic cavities (Figures 9A, 9B, 9C). Group V treated with both fluvoxamine maleate and alendronate sodium revealed continuous bone trabeculae which surrounded spaces of bone marrow. However some bone trabeculae were seen broken and others appeared with osteoporotic cavities. Slightly irregular compact bone surface was also observed (Figures 10A, 10B). Group VI treated with fluvoxamine maleate, alendronate sodium and ginger showed a picture similar to control group with continuous bone trabeculae which surrounded spaces of bone marrow. Also, smooth regular compact bone surface was observed containing haversian canals (Figures 11A, 11B).

Statistical results

• Biochemical results

1- Serum Calcium Level

In comparison with control group, the serum calcium levels of groups II and III had non-significant difference. Fluvoxamine maleate treated group (IV) revealed highly significant decrease compared to control group. While fluvoxamine maleate and alendronate sodium treated group (V) showed highly significant increase compared to group IV. Fluvoxamine maleate, alendronate sodium and ginger treated group (VI) revealed non-significant difference compared to control. Significant increase was detected in group VI compared to group V (Table 1, Histogram 1).

2- Serum alkaline Phosphatase Level

The serum alkaline phosphatase levels of groups II and III revealed non-significant difference compared to control group. Fluvoxamine maleate treated group (IV) showed highly significant rise compared to control. Group V showed highly significant decrease compared to IV. While fluvoxamine maleate, alendronate sodium and ginger treated group (VI) did not show any significant difference in comparison with control group. Highly significant decrease was detected in group VI compared to group V (Table 1 and Histogram 2).

Morphometric results

1- Thickness of compact bone

In comparison with control group, the mean thickness of the compact bone of the shaft of groups II and III had non-significant difference. Fluvoxamine maleate treated group (IV) revealed highly significant decline in thickness of compact bone (p *value* <0.001) compared to control. In fluvoxamine maleate and alendronate sodium treated group (V), P was <0.001 reflecting highly significant increase in compact bone thickness compared to group IV. On the other hand, the compact bone thickness of fluvoxamine maleate, alendronate sodium and ginger treated group (VI) did not show any significant difference in comparison with control group. Comparing fluvoxamine maleate, alendronate sodium and ginger treated group (VI) to fluvoxamine maleate and alendronate sodium treated group (V), p was <0.05 reflecting significant increase in group VI (Table 2, Histogram 3).

2- Area% of collagen fibers

Area percentage of collagen fibers of groups II and III revealed non-significant difference compared to control group. Fluvoxamine maleate treated group (IV) revealed highly significant decrease ($P<0.001$) compared to control group. In fluvoxamine maleate and alendronate sodium treated group (V), the P value was < 0.001 reflecting highly significant increase in collagen fibers compared to group IV. In contrast, the mean area % of collagen fibers of fluvoxamine maleate, alendronate sodium and ginger treated group (VI) did not show any significant difference in comparison with control group. Comparing fluvoxamine maleate, alendronate sodium and ginger treated group (VI) to fluvoxamine maleate and alendronate sodium treated group (V), p was <0.001 reflecting highly significant increase in group VI (Table 2, Histogram 4).

3- Osteoclast Number

The mean osteoclast number of groups II and III revealed non-significant difference compared to control group. Fluvoxamine maleate treated group (group IV) revealed highly significant increase in mean osteoclast number compared to control group. Comparing group V to group IV, there was highly significant reduction in osteoclast number in group V. Non-significant change was detected between fluvoxamine maleate, alendronate sodium and ginger treated group (VI) and control group. Non-significant reduction in the number of osteoclasts was detected in the fluvoxamine maleate, alendronate sodium and ginger treated group (VI) compared to fluvoxamine maleate and alendronate sodium treated group (V) (Table 2, Histogram 5).

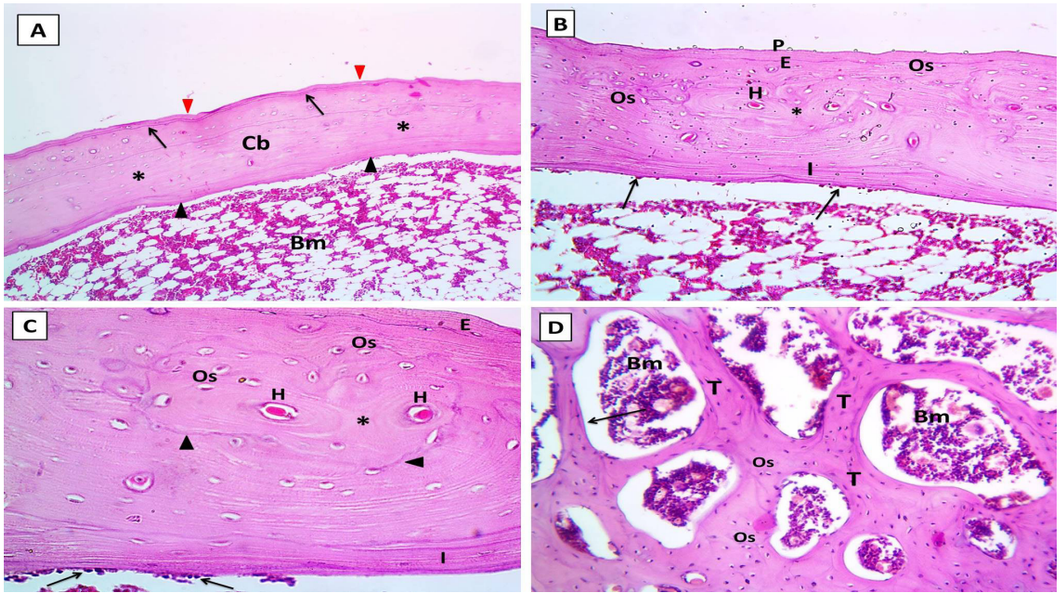


Fig. 1: A photomicrograph of H&E-stained sections of rat femur of control group (I): (A) transverse section of shaft of femur showing compact bone (Cb) surrounding bone marrow cavity (Bm). The compact bone appears with homogenous deep acidophilic matrix (*) and subperiosteal distinct basophilic cement lines (arrows). Notice: smooth periosteum (red arrowheads) and endosteum (black arrowheads). (B) transverse section of shaft of femur showing periosteum (P), external circumferential lamella (E), concentric lamella arranged around haversian canals (H), interstitial lamellae (*) and inner circumferential lamella (I). Osteocytes (Os) are observed in lacunae. Notice: Osteoblasts (arrows) lining the endosteum. (C) transverse section of shaft of femur showing haversian system (osteon) formed of concentric lamellae around haversian canals (H). In between lamellae, osteocytes (Os) are observed in lacunae. Basophilic cement line is noticed in some osteons (arrowheads). Notice: interstitial lamellae in between osteons (*), external circumferential lamella (E), and inner circumferential lamella (I). The endosteum is lined by osteoblast cells (black arrows). (D) longitudinal section of head of femur showing cancellous bone formed of branching and anastomosing bone trabeculae (T) enclosing bone marrow spaces (Bm). Osteocytes (Os) are seen in lacunae. Notice: Smooth endosteal surface (arrow) lining bone marrow spaces. H&E, (A) X 100, (B, D) X 200, (C) X 400.

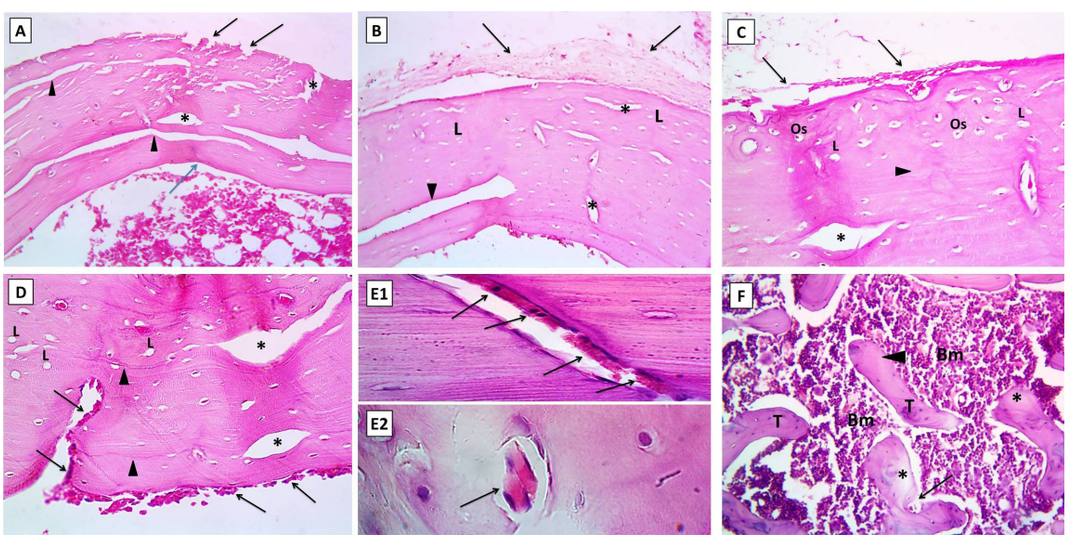


Fig 2: A photomicrograph of H&E-stained sections of rat femur of FM group (IV): (A) transverse section of shaft of femur showing marked erosion and irregularity of the bone surface (black arrows). Multiple osteoporotic cavities (*) are seen, some appear elongated (arrow heads). Less acidophilic matrix with absent cement lines was observed. The endosteal surface appears without osteoblasts (blue arrow). (B) transverse section of shaft of femur showing very thick periosteum (arrows). Multiple osteoporotic cavities (*) are seen, one of them appears elongated (arrowhead). Notice: Empty lacunae (L) and less acidophilic matrix with absent cement lines. (C) transverse section of shaft of femur showing disrupted periosteum (arrows), osteoporotic cavities (*) and empty lacunae (L). Some osteocytes (Os) appear shrunken. Areas of bone matrix appear without osteocytes (arrowhead). (D) transverse section of shaft of femur showing osteoclasts (arrows) with acidophilic cytoplasm and multiple nuclei lying on the bone surface and inside an erosion cavity. Osteoporotic cavities (*) and empty lacunae (L) are seen. Notice: less acidophilic matrix with indistinct cement lines (arrowheads). (E1 & E2) transverse section of shaft of femur showing osteoclasts (arrows) inside osteoporotic cavities. Osteoclasts appear with acidophilic cytoplasm and multiple nuclei. (F) longitudinal section of head of femur showing discontinuous bone trabeculae (T) enclosing wide interconnected bone marrow spaces (Bm). Areas of faintly stained matrix are seen (*). Some areas appear devoid of osteocytes (arrowhead). Notice: Irregular eroded endosteal surface (arrow). H&E, (A, B, F) X 200, (C, D) X 400, (E1 & E2) X 1000.

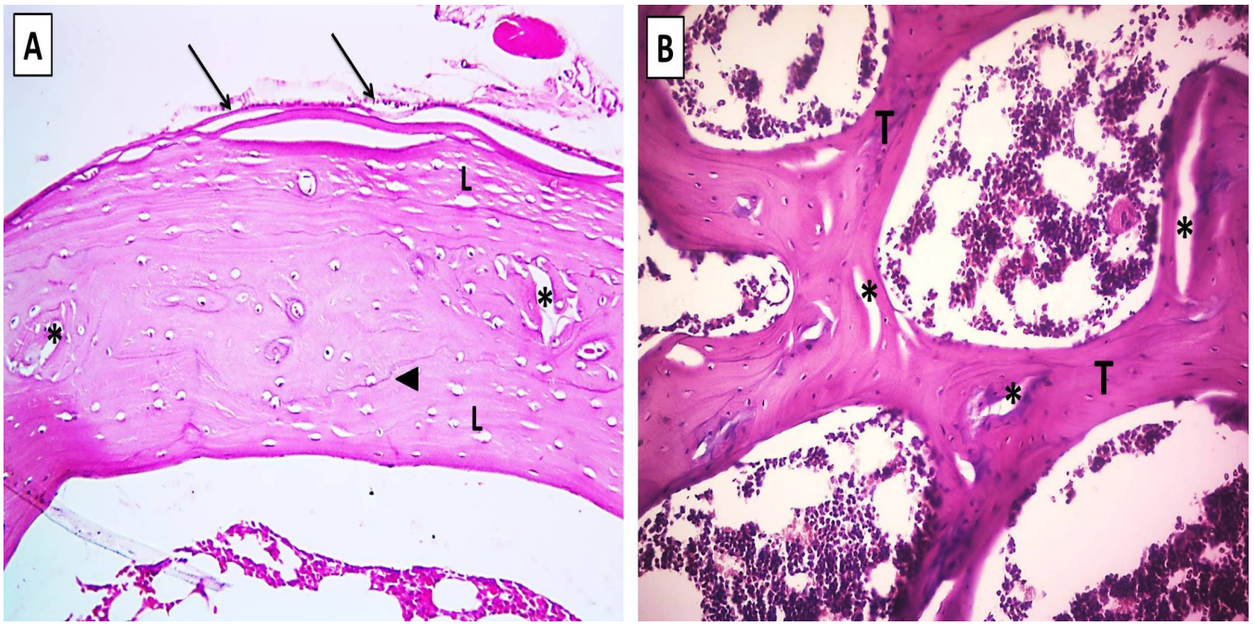


Fig. 3: A photomicrograph of H&E-stained sections of rat femur of FM+ALS group (V): (A) transverse section of shaft of femur showing faintly stained matrix, widened osteocytes lacunae (L), few osteoporotic cavities (*) and irregular cement lines (arrowhead). Notice: periosteum (arrows) appears nearly normal. (B) longitudinal section of head of femur showing continuous bone trabeculae (T) having some osteoporotic cavities (*). H&E, (A, B) X 200.

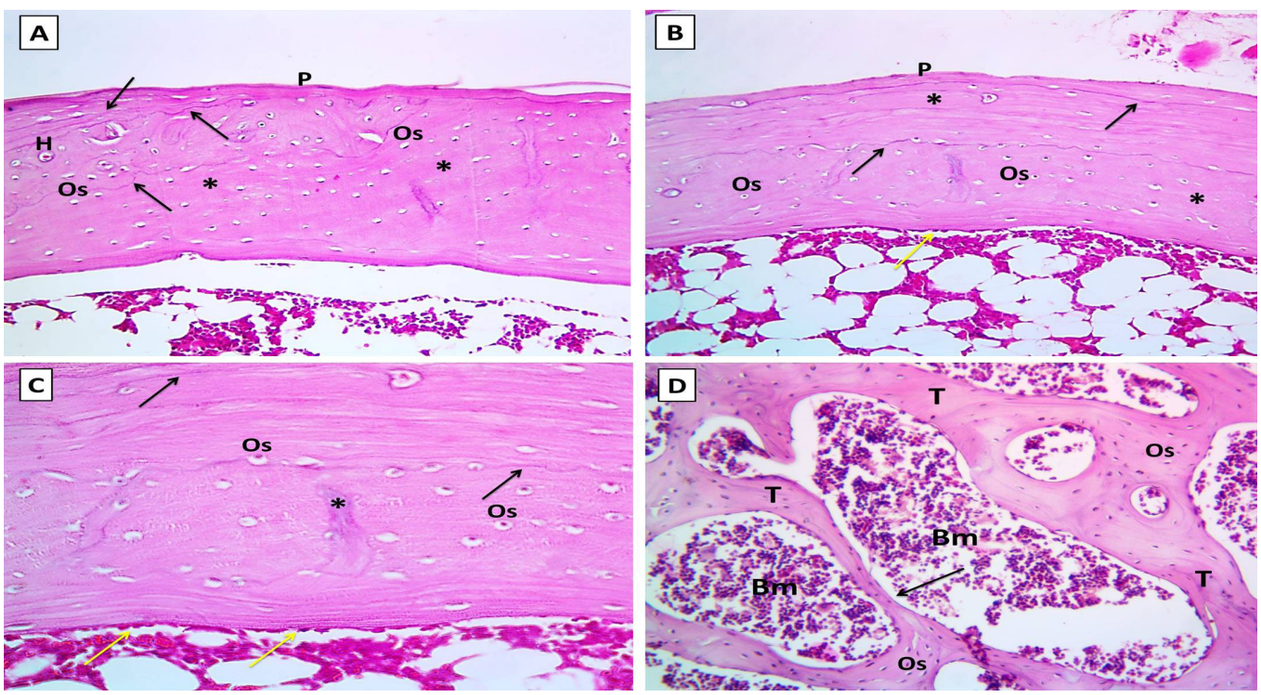


Fig 4: A photomicrograph of H&E-stained sections of rat femur of FM+ALS+G group (VI): (A) transverse section of shaft of femur showing apparently normal periosteal thickness (P), homogenous deep acidophilic matrix (*) and multiple distinct basophilic cement lines (arrows). Osteocytes (Os) are seen in their lacunae. Notice: Haversian canal (H) surrounded by osteocytes. (B) transverse section of shaft of femur showing regular bone lamellae with homogenous acidophilic matrix (*). Multiple distinct basophilic cement lines (black arrows) are observed. Osteocytes (Os) are seen in lacunae. Notice: smooth periosteum (P), the endosteum is lined with many osteoblasts (yellow arrow). (C) Higher magnification of (B) showing the inner part of the compact bone. Osteocytes (Os) are seen in lacunae. The endosteum is lined with many osteoblasts (yellow arrows). Notice: multiple cement lines (black arrow) and basophilic areas (*). (D) longitudinal section of head of femur showing branching and anastomosing bone trabeculae (T) enclosing bone marrow spaces (Bm). Osteocytes (Os) are seen in lacunae. Notice: Smooth endosteal surface (arrow) lining bone marrow spaces. H&E, (A, B, D) X 200, (C) X 400.

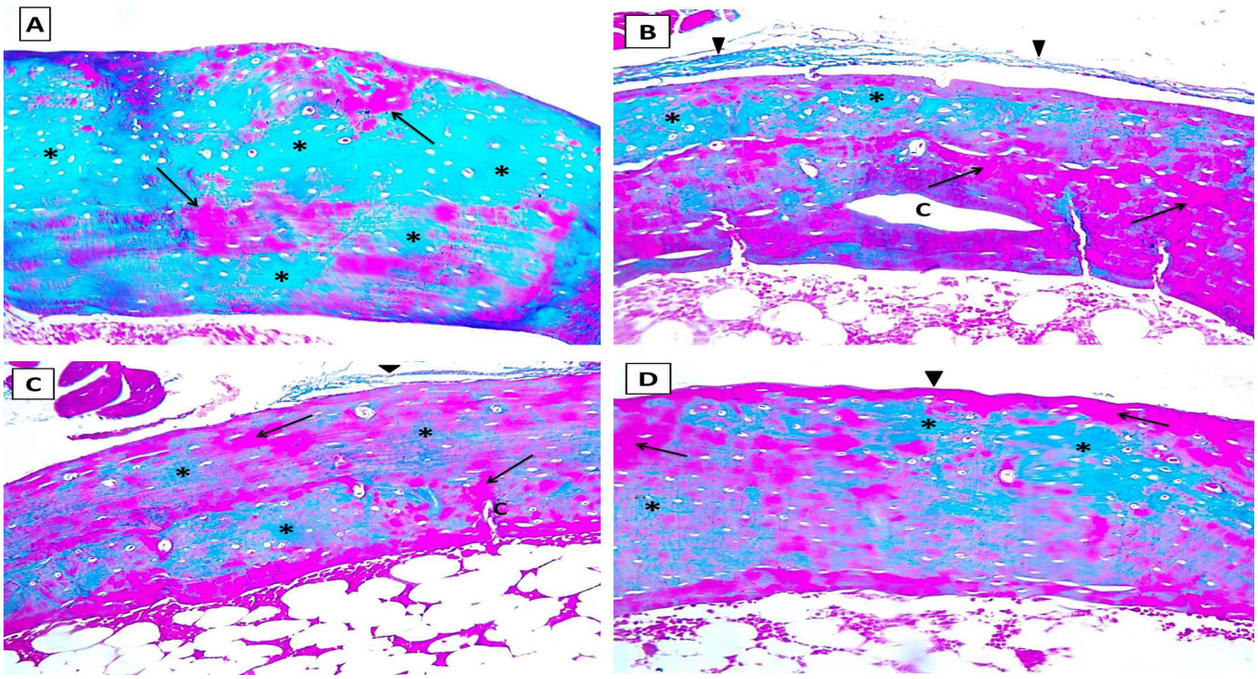


Fig. 5: A photomicrograph of Masson's trichrome-stained sections of the shaft of rat femur of the control and treated groups: (A) control group (I) showing collagen fibers indicated by green color (*) within the reddish bone matrix (arrows). (B) FM group (IV) showing apparently few amount of collagen fibers indicated by green color (*) within the reddish bone matrix (arrows). Osteoporotic cavities (c) and thick fibrous periosteum (arrowheads) are seen. (C) FM+ALS group (V) showing moderate amount of collagen fibers indicated by green color (*) within the reddish bone matrix (arrows). Small osteoporotic cavities (c) and slightly thickened periosteum (arrowhead) are seen. (D) FM+ALS+G group (VI) showing nearly normal amount of collagen fibers indicated by green color (*) within the reddish bone matrix (arrows). Smooth periosteum is noticed (arrowhead). Masson's trichrome, (A, B, C, D) X200.

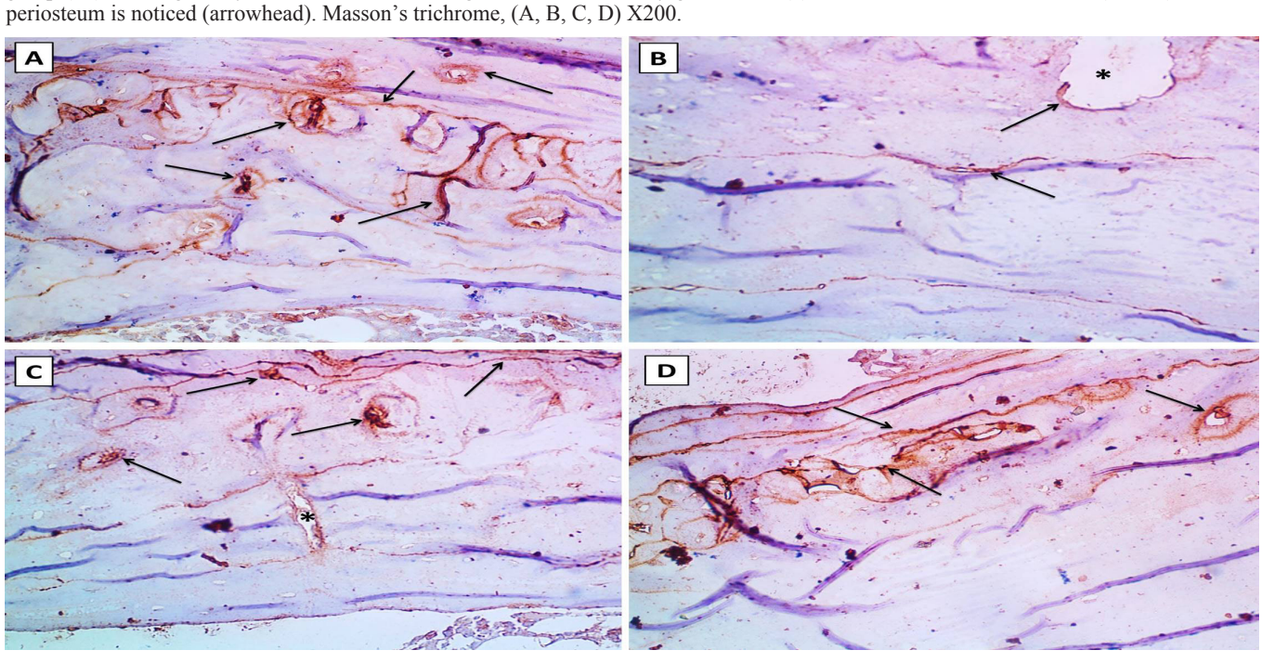


Fig. 6: A photomicrograph of OPN-stained sections of the shaft of rat femur of the control and treated groups: (A) control group (I) showing strong positive reaction of osteopontin proteins in bone matrix around the lacunae, haversian canals and in cement lines (arrows). (B) FM group (IV) showing mild positive reaction of osteopontin proteins in bone matrix around an osteoporotic cavity and in cement lines (arrows). Osteoporotic cavity (*) is observed. (C) FM+ALS group (V) showing moderate positive reaction of osteopontin proteins in bone matrix around the lacunae, haversian canals and in cement lines (arrows). Small osteoporotic cavity (*) is noticed. (D) FM+ALS+G group (VI) showing strong positive reaction of osteopontin proteins in bone matrix around the lacunae, haversian canals and in cement lines (arrows). OPN (A, B, C, D) X400.

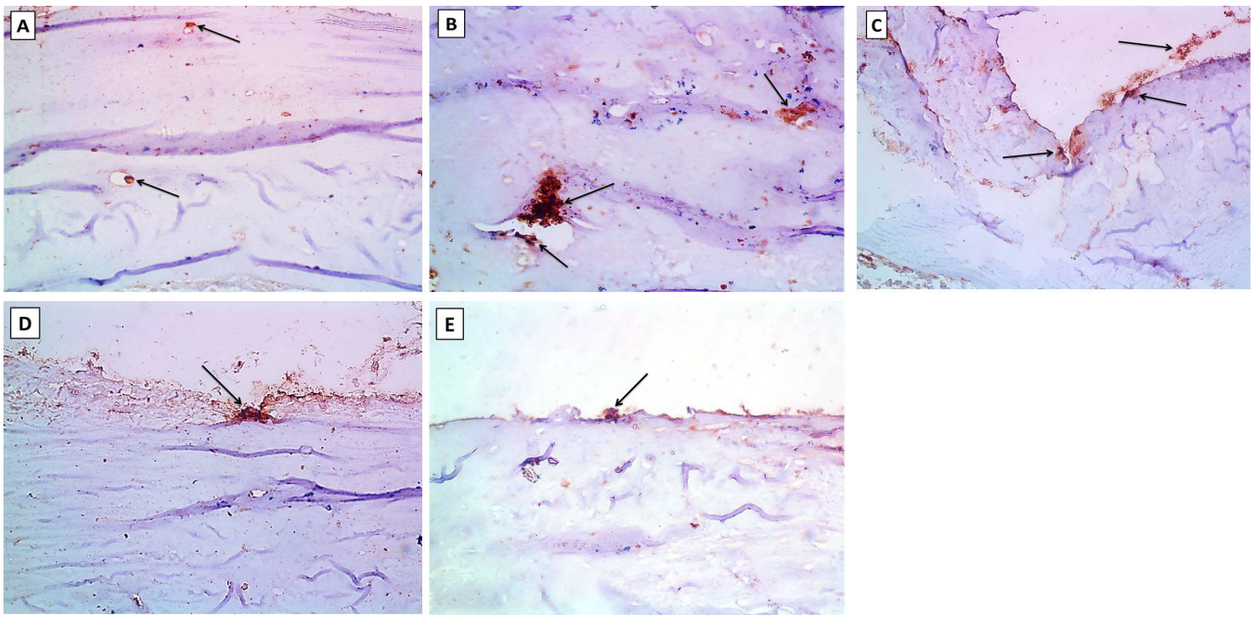


Fig. 7: A photomicrograph of CD68-stained sections of the shaft of rat femur of the control and treated groups: (A) control group (I) showing mild positive CD68 cytoplasmic immunoreactivity in few osteoclasts (arrows) lining haversian canals. (B) FM group (IV) showing strong positive CD68 cytoplasmic immunoreactivity in many or cluster of osteoclasts present in Osteoporotic cavities (arrows). (C) FM group (IV) showing moderate positive CD68 cytoplasmic immunoreactivity in many osteoclasts present on the surface of bone (arrows). (D) FM+ALS group (V) showing moderate positive CD68 cytoplasmic immunoreactivity in an osteoclast present on the bone surface (arrow). (E) FM+ALS+G group (VI) showing mild positive CD68 cytoplasmic immunoreactivity in an osteoclast present on the surface of bone (arrow). CD68 (A, B, C, D, E) X400.

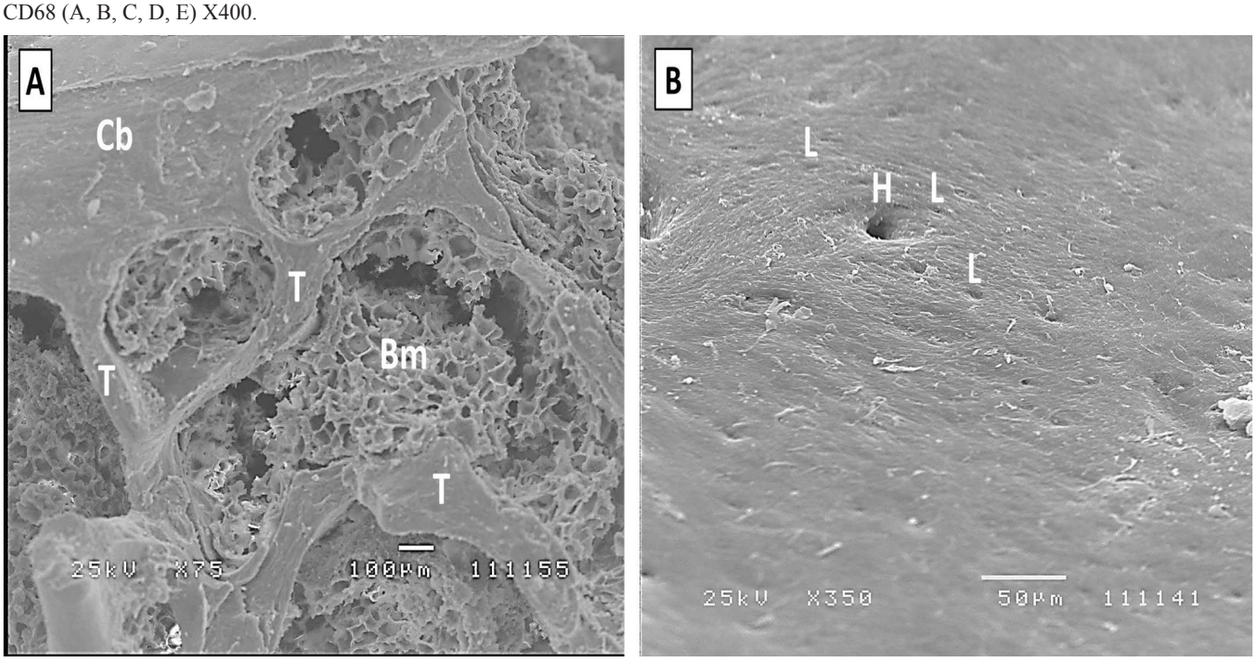


Fig. 8: A scanning electron micrograph of rat femur of control group (I): (A) showing the outer compact bone (Cb) giving rise to continuous branching and anastomosing cancellous bone trabeculae (T) with bone marrow spaces (Bm) in between. (B) showing the surface of the compact bone that appears smooth and regular. The haversian canal (H) is seen surrounded by lacunae (L) of osteocytes. A X 75, B X 350.

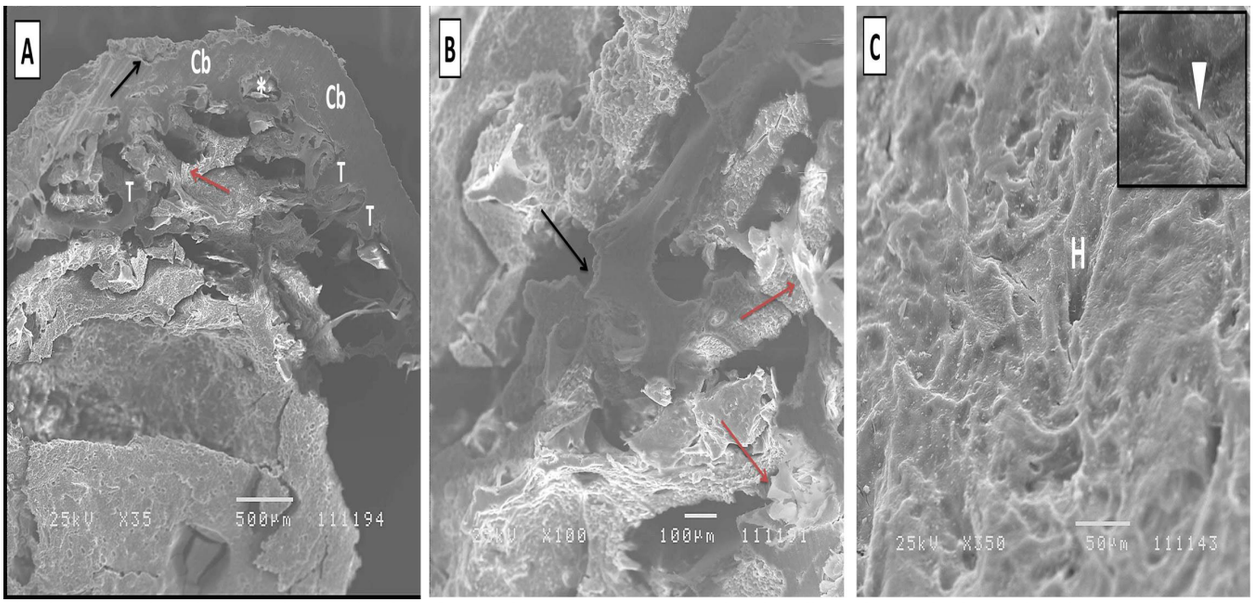


Fig. 9: A scanning electron micrograph of rat femur of FM group (IV): (A) showing discontinuous broken trabeculae (T) arising from compact bone (Cb). The compact bone appears markedly destructed with cracks (black arrow) and osteoporotic cavities (*). Peeling off the surface of bone trabeculae (red arrow) is noticed. (B) showing broken cancellous bone trabeculae (black arrow). Peeling off the surface of bone trabeculae (red arrows) is noticed. (C) showing the compact bone surface which appears with marked irregularity. Haversian canal (H) is seen. The inset shows cracks (arrowhead) in the compact bone surface. A X 35, B X 100, C X 350, inset X1000.

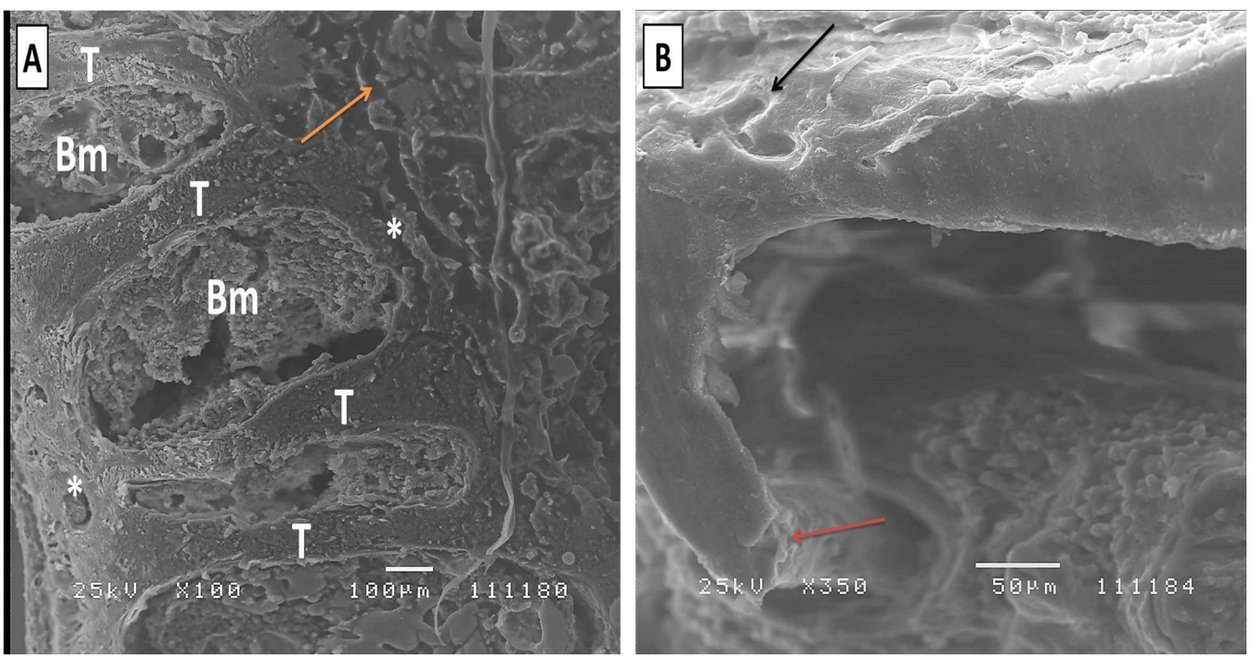


Fig. 10: A scanning electron micrograph of rat femur of FM+ALS group (V): (A) showing continuous bone trabeculae (T) surrounding bone marrow spaces (Bm). One of bone trabeculae is seen broken (orange arrow). Some osteoporotic cavities (*) are seen. (B) showing slight irregularity in the compact bone surface (black arrow). A broken trabecular bone is seen originating from the compact bone (red arrow). A X 100, B X 350.

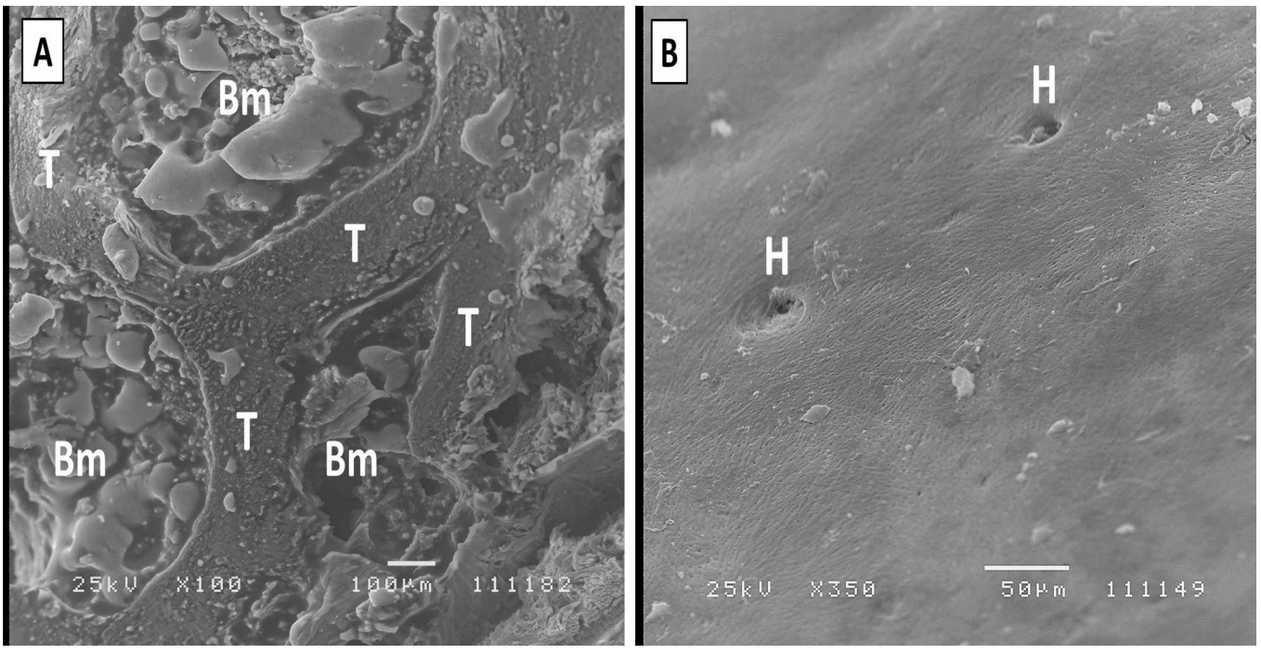


Fig. 11: A scanning electron micrograph of rat femur of FM+ALS+G group (VI): (A) showing continuous bone trabeculae (T) surrounding bone marrow spaces (Bm). (B) showing the surface of the compact bone that appears smooth and regular. Haversian canals (H) are seen. A X 100, B X 350.

Table 1: The biochemical results in the control and experimental groups

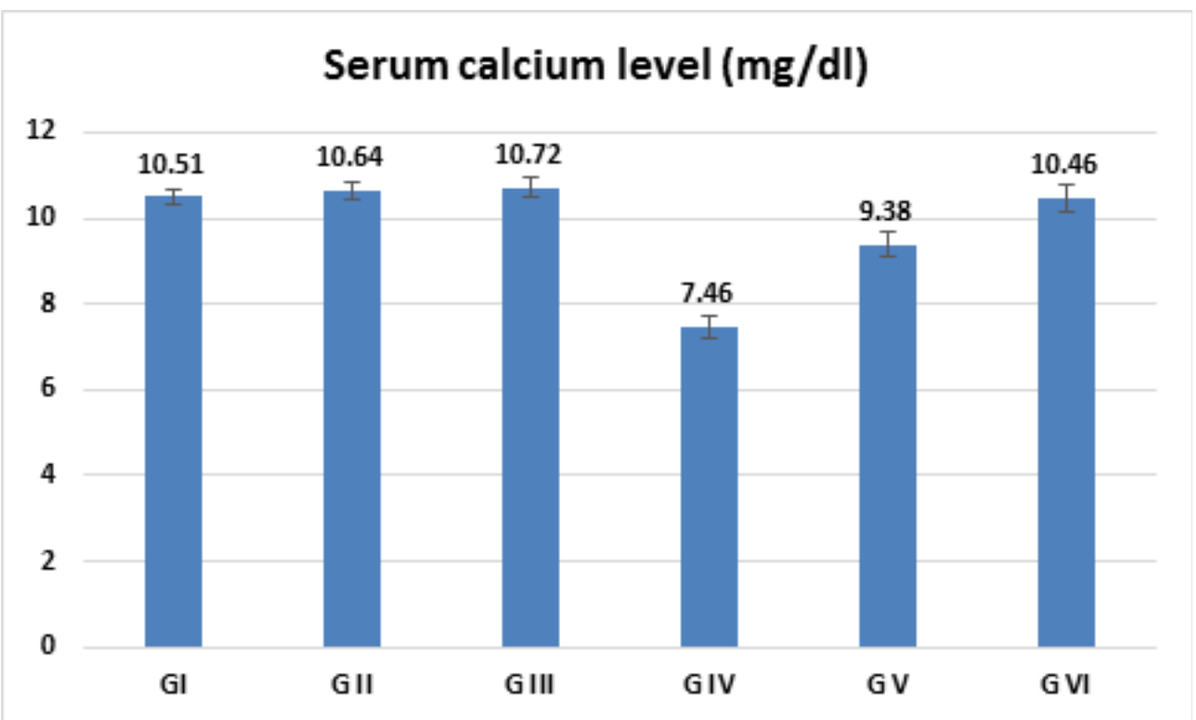
	Group I	Group II	Group III	Group IV	Group V	Group VI	*P.value*
Serum calcium level (mg/dl) Mean ± SD	10.51±0.40	10.64±0.49	10.72±0.54	7.46±0.55	9.38±0.66	10.46±0.69	*(P1>0.05)** *(P2>0.05)** *(P3<0.001)**** *(P4<0.001)**** *(P5>0.05)** *(P6<0.05)***
Serum ALP level (U/L) Mean ± SD	136.48±5.99	137.74±6.16	133.92±5.45	202.86±10.12	164.04±7.19	140.86±6.49	*(P1>0.05)* * *(P2>0.05)* * *(P3<0.001)**** *(P4<0.001)**** *(P5>0.05)** *(P6<0.05)* ***

P1: Group I V Group II P2: Group I V Group III P3: Group I V Group IV
P4: Group IV V Group V P5: Group I V Group VI P6: Group V V Group VI
Non-significant *(*P>0.05*) Significant **(*P<0.05*) Highly significant ***(*P<0.001*)

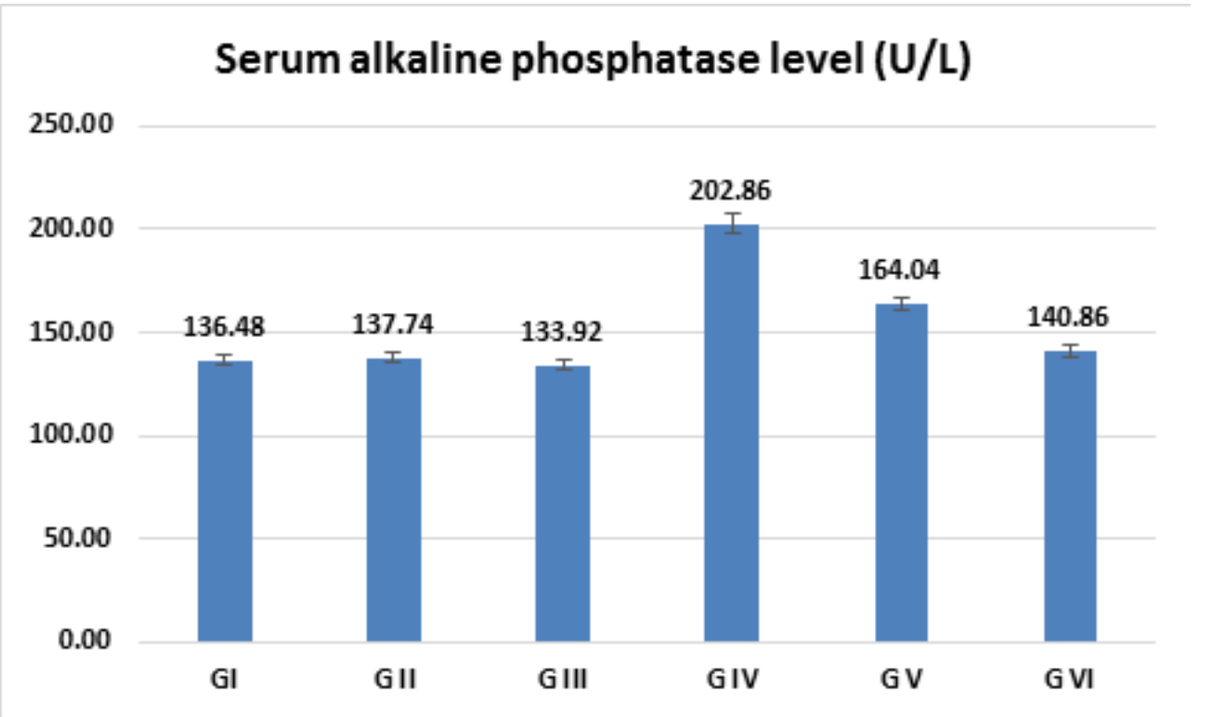
Table 2: The morphometric results in the control and experimental groups

	Group I	Group II	Group III	Group IV	Group V	Group VI	*P.value*
Thickness of compact bone (µm) Mean ± SD	559.68 ± 15.07	562.98 ± 18.00	564.06 ± 16.31	450.34 ± 22.50	523.68 ± 16.08	547.86 ± 15.69	*(P1>0.05)** *(P2>0.05)** *(P3<0.001)**** *(P4<0.001)**** *(P5>0.05)** *(P6<0.05)***
Area% of collagen fibers Mean ± SD	91.94 ± 2.81	92.78 ± 2.13	93.98 ± 2.80	35.64 ± 3.84	71.26 ± 4.34	88.02 ± 3.23	*(P1>0.05)** *(P2>0.05)** *(P3<0.001)**** *(P4<0.001)**** *(P5>0.05)** *(P6<0.05)****
Osteoclast number/high power field Mean ± SD	0.20 ± 0.45	0.00 ± 0.00	0.20 ± 0.45	3.40 ± 1.14	0.60 ± 0.55	0.40 ± 0.89	*(P1>0.05)** *(P2>0.05)** *(P3<0.001)**** *(P4<0.001)**** *(P5>0.05)** *(P6<0.05)****

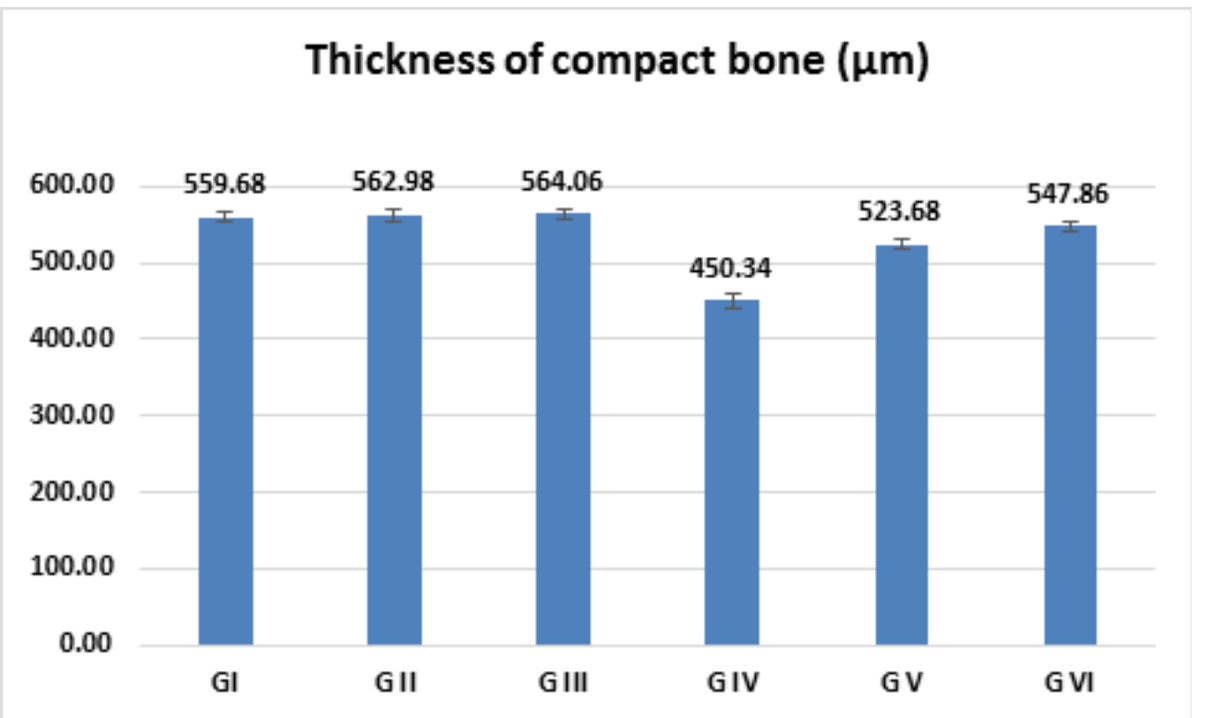
P1: Group I V Group II P2:Group I V Group III P3: Group I V Group IV
P4: Group IV V Group V P5: Group I V Group VI P6: Group V V Group VI
Non-significant*(*P>0.05*) Significant**(*P<0.05*) Highly significant***(*P<0.001*)



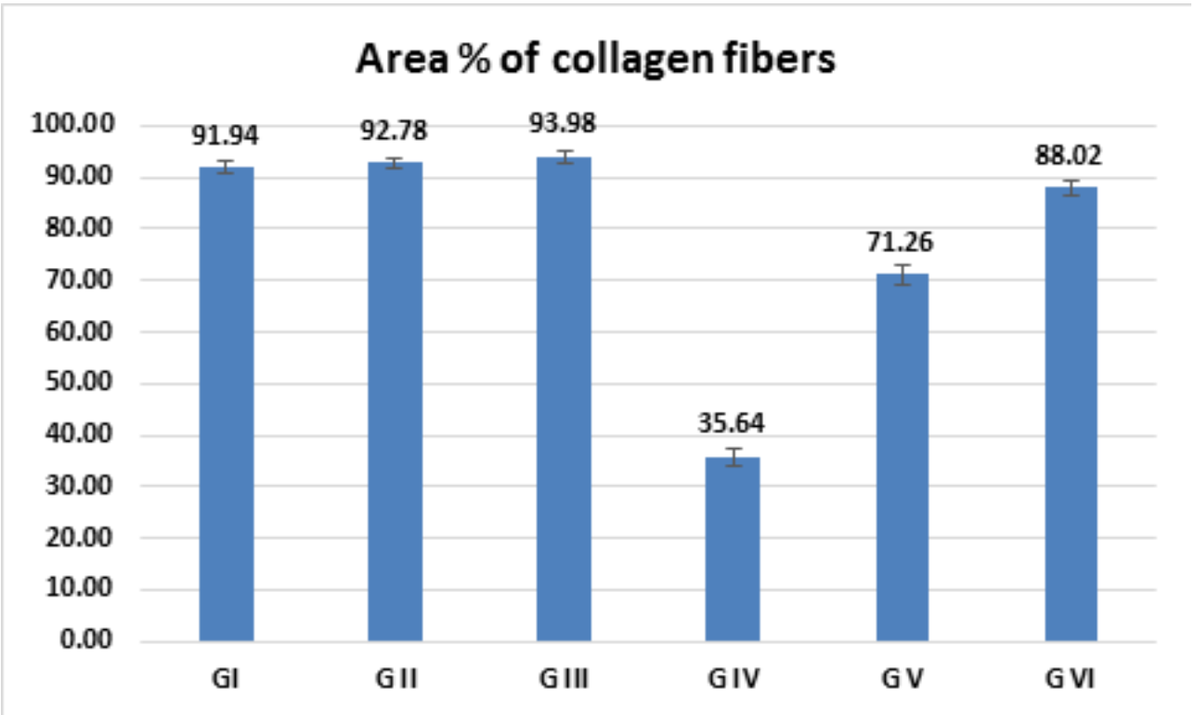
Histogram 1: The mean serum calcium level in the control and experimental groups



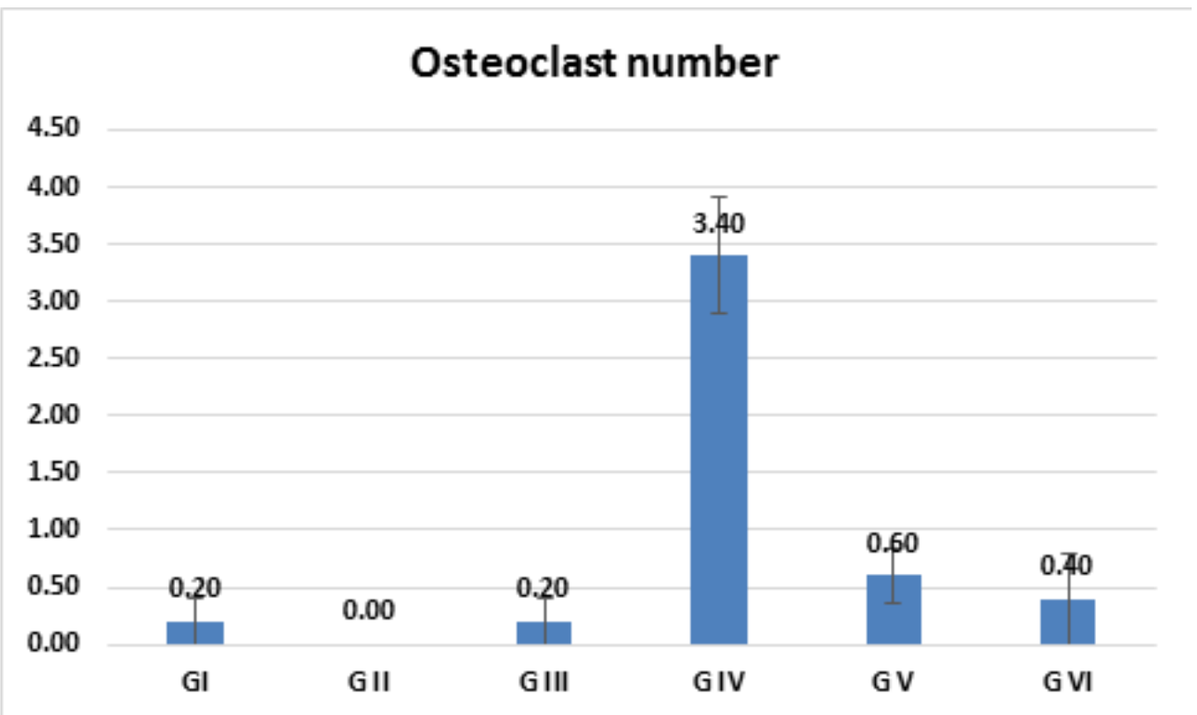
Histogram 2: The mean serum alkaline phosphatase level in the control and experimental groups



Histogram 3: The mean thickness of compact bone in the control and experimental groups



Histogram 4: The mean area% of collagen fibers in the control and experimental groups



Histogram 5: The mean osteoclast number in the control and experimental groups

DISCUSSION

Osteoporosis is reduction in bone mass resulting in decreased bone strength with fragility and fracture[29]. The bone undergoes constant remodeling, with repeated events in which bone is resorbed by osteoclasts followed by deposition of new bone matrix via osteoblasts[30]. These events ensure repair of repeated fractures and gets back the bone architecture. If bone resorption overtakes bone formation, osteoporosis takes place with fracture risk[31].

Antidepressant drugs are mainly used for anxiety and depression[32,33] and for other non-psychological disorders[34]. Antidepressants effect on bone was not searched deeply with controversial results of some studies. In some studies, usage of antidepressants has not been correlated with decreased BMD[35, 36] and in other studies the use of the antidepressant like lithium enhances bone regeneration[37]. On the other hand, there was a clear association between using antidepressants, mainly SSRI and reduced bone mineral density[38]. A previous study reported loss of bone as a result of using 5-hydroxytryptophan (precursor of serotonin) by triggering osteoclastogenesis[39].

Bisphosphonates are anti-resorptive medications used clinically for osteoporotic patients mostly by stimulating mature osteoclast apoptosis and preventing expression of RANK receptor on osteoclast precursors[40].

Ginger is a natural compound which was found to induce bone formation by osteoblasts[41].

Thus, this work was designed to investigate the effect of FM (as one of SSRIs) on the bone of adult female albino rats and to assess the potential protective role of alendronate sodium (as one of the bisphosphonates) alone or in combination with ginger by histological and immunohistochemical techniques.

Both light and electron microscope examination of the bone of FM group revealed numerous histopathological changes. Marked irregularity and erosion of the bone surface was detected. Also, there were cracks and multiple osteoporotic cavities in the compact bone with discontinuous broken cancellous bone trabeculae. Moreover, marked thickening of the periosteum and erosion of the endosteum with the absence of osteoblasts were seen. Less acidophilic or faintly stained matrix, absence of cement lines, degenerated osteocytes and many osteoclasts on the bone surface and inside osteoporotic cavities were observed. Similar findings were reported by previous histological studies on the effect of fluvoxamine maleate on the bone of the rats[42,21]. Moreover, our results were in line with recent clinical study which reported high risk of bone loss in women and men using SSRI[8]. Also, a previous clinical study on the usage of antidepressants SSRI on bone density revealed findings suggestive of osteoporosis[38].

Relationship between bone loss and SSRI was attributed to role of serotonin receptors in bone[8]. Many researches have revealed the existence of serotonin transporters and receptors in osteoclasts and osteoblasts that are main items in bone remodeling process, and proposed potential negative impacts, as decreased proliferation of osteoblast, by preventing reuptake of serotonin during bone remodeling[43, 44, 45, 46]. A previous work suggested that SSRI had the ability to decrease BMD via changing osteoclast differentiation and rising sympathetic tone[46].

Diem *et al*.[47], suggested that a reduced osteoblast activity or a disturbed osteoblast/osteoclast action as result of serotonin transporter prevention could be possible mechanism through it SSRI can affect BMD.

Gustafsson *et al*.[48] gave another explanation by stating that SSRI raise the activity of osteoclasts through triggering RANKL (receptor activator of nuclear factor kappa B ligand) and prohibiting osteoporotegrin. Also, RANKL is defined as osteoclast differentiation agent and is formed via marrow stromal cells and osteoblasts. When combines with RANK receptor of preosteoclasts and osteoclasts, it induces their differentiation & proliferation[49]. Osteoporotegrin is circulating receptor protein formed by marrow stromal cells and osteoblasts. It prevents formation of osteoclast by joining RANKL, so preventing the stimulation of preosteoclasts and inhibiting RANK/ RANKL interactivity[50]. Decreasing osteoblasts function with maintained osteoclasts activity results in a change in the bone remodeling process towards resorption and bone loss[51].

Our histological, immunohistochemical and morphometric results were in harmony with such explanations and confirmed them. There was highly significant decrease in thickness of compact bone of the FM-treated group compared to control group and this could be explained by imbalance between bone resorption and formation, leading to remodeling abnormality and consequent bone loss[52]. Also, cement lines separating the newly formed bone from the old one were absent in some sections and indistinct in others, and this was attributed to a rise in rate of bone resorption over bone synthesis[53].

In our study, marked thickening of the periosteum was observed and this could be explained as compensatory mechanism if bone loss occurs to preserve the strength of bone[54].

The appearance of the endosteum without osteoblasts was detected in our study and this was reported by previous authors in their study on the effect of SSRIs on bone and they attributed this finding to reduction in osteoblasts differentiation and proliferation[21, 55]. Such finding was confirmed by marked decrease in osteopontin (OPN) expression in bone matrix of the FM group compared to control group reflecting a decline in the activity of

osteoblasts and their maturation as osteopontin has been considered an indicator for bone formation. Osteoblasts form OPN which deposits in bone matrix[56].

Degeneration of osteocytes with empty lacunae, were noticed in this study. This was in agreement with prior research which stated that osteocytes loss or decrease in their numbers was main finding in osteoporosis pathogenesis[57].

In this study, sections stained with Masson's trichrome of FM group exhibited highly significant decrease in collagen content compared to control group and this was in agreement with prior research which attributed this to the decreased number of osteoblasts[58].

As regard the immunohistochemical study for CD68 and osteoclast number, we noticed highly significant rise in osteoclast number in FM group, this result is consistent with several studies stating that SSRI elevate osteoclast number[59, 42, 60].

Concerning the biochemical results of the FM group, they reflected accelerated bone turnover manifested through highly significant decline in serum calcium level and highly significant rise in serum ALP level compared with control group. These results coincided with previous study on the effect of FM on bone[21] and other studies on osteoporosis[31, 61, 62]. Elkalawy *et al*.,[21] attributed the increase in ALP to a compensatory reaction to bone destruction as ALP is component of cell membrane in various tissues having the highest density in the osteoblasts. Also, they attributed the reduction in serum Ca to the increase in bone resorption leading to hypercalcemia that induces compensatory process to decrease absorption of Ca from intestine and raise Ca excretion through the kidney.

In our study, the administration of Alendronate sodium with fluvoxamine maleate in group V protected the bone against histological and immunohistochemical changes induced by FM but this protection didn't reach the aimed expectations. There were irregular cement lines, areas of faintly stained matrix, widened osteocytes lacunae, broken bone trabeculae and few osteoporotic cavities. Morphometric and the biochemical results of this group (V) were supportive to the histological findings and demonstrated some improvement in their parameters in comparison to FM group (IV) but still less than those of control with significant difference. There were significant rise in compact bone thickness and the amount of collagen fibers, significant decline in osteoclast number, significant improvement of the serum calcium and ALP levels as compared to FM group (IV). Such findings were in agreement with previous researches on effect of alendronate on induced osteoporosis[63, 64, 12].

This protective effect of alendronate sodium against fluvoxamine maleate induced bone changes was in consistence with Badae *et al*.,[12] who stated that alendronate sodium is effective in the treatment and prevention of osteoporosis and increased bone formation. They attributed the protective effect of Bisphosphonates (including Alendronate) in induced osteoporosis, primarily to their antiresorptive effect through the inhibition of the enzyme of osteoclast (farnesyl pyrophosphate synthase) that is vital for osteoclast function and secondarily to inhibition of osteoblast apoptosis.

A previous study stated that bisphosphonates have the ability to prevent bone resorption mediated by osteoclasts and they trigger formation of bone[65]. Bitto *et al*.[66] demonstrated that alendronate treatment decreased osteoporosis in animals through increasing bone mineral density, strength and ameliorating histological bone damage.

In the present study, the administration of alendronate sodium and ginger with fluvoxamine maleate in group VI highly protected the bone against histological and immunohistochemical changes induced by FM as the results of this group were more or less similar to those of control group (I) and demonstrated much improvement in comparison with group V treated with fluvoxamine maleate and alendronate sodium only. These results indicated that ginger adding to alendronate sodium in FM-treated rats (group VI) may act with alendronate in synergistic way to decrease bone loss induced by FM.

Our results confirmed the protective role of ginger on bone as was reported by prior researches[67, 68, 69] in which the researchers explained their results by free radical scavenging and antioxidant activities of ginger. Moreover, this protective role of ginger could be explained by potent stimulating role of ginger on cardiac muscles in rats resulting in stimulation of blood circulation with improvement in blood supplies, bone cell activation and subsequently new bone formation[70].

In group VI, many osteoblasts were seen lining the endosteum with multiple cement lines indicating new bone formation and this was in harmony with Nabil *et al*.,[69] in their research on effect of ginger on alveolar bone, who detected high number of PCNA immunopositive bone cells and attributed this to osteoblast proliferation as a result of ginger treatment.

Also, Group VI showed strong positive reaction of osteopontin proteins in bone matrix and this was in line with previous study[68] which revealed significant rise in OPN expression in bone of rats treated with ginger. The authors stated that OPN combines strongly with hydroxyapatite crystals, so new bone formation progresses reflecting the positive role of OPN in repair of bone.

In our study, group VI demonstrated nearly normal amount of collagen fibers which did not show any significant difference with control group and this was in agreement with previous study[69] which revealed rise in the collagen content in ginger treated group. Fan *et al*.,[18] attributed this to 6-gingerol 'a main bioactive constituent of ginger' which stimulates formation of type I collagen.

A recent study[41] attributed the protective role of ginger on bone to its main compound, 6-gingerol which inhibits osteoclast differentiation by the reduction of RANKL expression in osteoblast precursor cells. Also, authors stated that 10-gingerol, another bioactive compound in ginger, inhibits osteoclastogenesis both in vivo and vitro.

CONCLUSION

From the histological and immunohistochemical results of the present study, Fluvoxamine maleate (a common SSRI) has been proved to trigger osteoporotic changes in compact and cancellous bone of the rats. Concomitant administration of alendronate sodium and ginger with it highly protected the bone against these changes.

RECOMMENDATION

Based on our results in this study, bone scanning for osteoporosis is recommended for all people who have been treated with any antidepressant for a long period. People taking SSRI who will be at danger to develop osteoporosis should be given protective therapy against osteoporotic changes at the onset of SSRI course. The combination between alendronate sodium and ginger may be unique treatment strategy to prevent bone loss in osteoporotic conditions. For future applications of this treatment strategy, further studies are recommended for proper clinical use for humans.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

الدور الوقائي المحتمل لأليندرونات الصوديوم بمفرده أو بالاشتراك مع الزنجبيل ضد هشاشة العظام المحدثة بفلوفوكسامين ماليات في إناث الجرذان البيضاء البالغة (دراسة هستولوجية و هستوكيميائية مناعية)

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الخلفية: لقد تبين أن مثبطات امتصاص السيروتونين الانتقائية بما في ذلك فلوفوكسامين ماليات (FM) تسبب هشاشة العظام. أليندرونات الصوديوم (ALS) هو عامل مضاد للتقويض يستخدم لعلاج هشاشة العظام. الزنجبيل (G) هو مضاد قوي للأكسدة له آثار مفيدة على العظام.

الهدف: تقييم الدور الوقائي المحتمل لأليندرونات الصوديوم بمفرده أو بالاشتراك مع الزنجبيل ضد هشاشة العظام الناجمة عن فلوفوكسامين ماليات في الجرذان.

مواد و طرق البحث: تم تقسيم ستون من اناث الجرذان بالتساوي إلى ست مجموعات وقد تلقوا أدويتهم يوميا لمدة خمسة أسابيع. المجموعة الأولى (المجموعة الضابطة) ، المجموعة الثانية (مجموعة أليندرونات الصوديوم): تلقت أليندرونات الصوديوم 1 مجم / كجم ، المجموعة الثالثة (مجموعة الزنجبيل): تلقت الزنجبيل 500 مجم / كجم ، المجموعة الرابعة (مجموعة فلوفوكسامين ماليات): تلقت فلوفوكسامين ماليات 2 مجم / كجم ، المجموعة الخامسة: تلقت فلوفوكسامين ماليات و أليندرونات الصوديوم بنفس جرعات المجموعات السابقة. المجموعة السادسة : تلقت فلوفوكسامين ماليات و أليندرونات الصوديوم و الزنجبيل كالمجموعات السابقة. تم الحصول على عظام الفخذ وتجهيزها للفحص بالمجهر الضوئى والإلكتروني الماسح.

النتائج: أظهرت الجرذان التي عولجت بفلوفوكسامين ماليات فقط علامات هشاشة العظام في شكل تجاويف ، عدم انتظام و تآكل سطح العظم ، وانخفاض سمك العظم القشري. كما وجد انخفاض ذو دلالة إحصائية كبيرة فى النسبه المئويه لمساحة ألياف الكولاجين وزيادة ذات دلالة إحصائية كبيرة في عدد الخلايا العظمية المرتبطة بتأكل العظام مقارنة بالمجموعة الضابطة. كما لوحظ وجود تفاعل طفيف للبروتين العظمى في مصفوفة العظام. لقد تم الكشف عن تغيرات ذات دلالة إحصائية كبيرة في مستوى الكالسيوم و الفوسفاتيز القلوي في الدم. وجد ان إعطاء أليندرونات الصوديوم مع فلوفوكسامين ماليات في المجموعة الخامسة قد قلل من هذه التغيرات ، بينما أظهر إعطاء أليندرونات الصوديوم والزنجبيل مع فلوفوكسامين ماليات في المجموعة السادسة صورة هستولوجية قريبة من المجموعة الضابطة.

الخلاصة: أليندرونات الصوديوم مفيد في الحماية من هشاشة العظام التي يسببها فلوفوكسامين ماليات وإضافة الزنجبيل يعطي تأثير تآزري له.