Original Article

Collagen Hydrolysate Against Fluvoxamine Maleate-Induced Osteoporosis in Albino Rats: A Histological and Immunohistochemical Study

Seham Abd Elhamid Mohamed Elkalawy[1], Maha Mohamed Abo Gazia[2], Sally Ahmed Abd Elaziz Elekhtiar[2] and Dalia Ibrahim Ismail[1]

Histology Department, Faculty of Medicine, [1]Cairo University, [2]Kafrelsheikh University, Egypt

ABSTRACT

Background: Osteoporosis (OP) is a prevalent degenerative bone disease among patients receiving selective serotonin reuptake inhibitors as fluvoxamine maleate (FM). Collagen hydrolysate (CH) is a nutritional component that has antiresorptive effect.

Aim of Work: Evaluate the possible protective effect of CH against FM-induced OP in adult male albino rats.

Material and Methods: Thirty six rats were divided into 4 groups; group I (control), group II (OP group): injected with FM daily for 4 weeks, group III (CH group): received FM concomitant with oral CH for 4 weeks, group IV (recovery group]: received only FM for 4 weeks & were left without taking any drugs for another 4 weeks. Total serum alkaline phosphatase (ALP) and calcium (ca^{+2}) were measured. Bone specimens from the right femurs and first lumbar vertebrae were processed for H&E stain, Mallory's trichrome stain and immunohistochemical staining for osteopontin (OPN) and proliferating cell nuclear antigen (PCNA). This was followed by morphometric & statistical analysis.

Results: Both groups II & IV showed significant elevation in ALP & reduction in Ca^{+2} compared to control. Bone sections revealed evident histological changes; osteocytes with pyknotic nuclei inside widened lacuna, widened haversian canals. Bone matrix showed fain areas, cavitations & multiple resorption cavities with osteoclasts. There was significant reduction in the mean thickness of compact bone, the mean area of trabecular bone, area % of OPN & mean number of PCNA +ve cells compared to control. Group III exhibited significant reduction in ALP & elevation in Ca^{+2}. The bone showed preserved histological architecture almost as the control. There was significant increase in the mean thickness of compact bone, the mean area of trabecular bone, area % of OPN & mean number of PCNA +ve cells compared to groups II & IV.

Conclusion: CH has a potential osteoprotective effect against FM-induced osteoporosis.

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Key Words: Collagen hydrolysate; fluvoxamine maleate; Osteoporosis; Osteopontin; PCNA.

Corresponding Author: Dalia Ibrahim Ismail, MD, Department of Histology, Faculty of Medicine, Cairo University, Cairo, Egypt, **Tel.**: +20 1005050870, **E-mail:** drdaliaibrahim@hotmail.com

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INTRODUCTION

Osteoporosis (OP) is a globally growing health problem affecting over 200 million people worldwide and the annual incidence of hip fracture due to this disease is about 1.7 million worldwide[1]. It is characterized by deterioration of bone tissue and low bone mass because of enhanced bone resorption that is not compensated by enhanced bone formation, increasing fracture risk[2]. Primary OP is associated with aging and is more common in women especially postmenopausal due to decreased estrogen. Secondary OP occurs as a result of medical conditions as rheumatoid arthritis, kidney failure, Cushing syndrome, hormonal causes as diabetes and hyperparathyroidism, or due to long-term usage of anticonvulsant, corticosteroid, antidepressant and antipsychotic drugs[1,3].

The prevalence of OP has been increased among patients with depression due to treatment with antidepressants especially selective serotonin reuptake inhibitors (SSRIs)[4], which are frequently used due to better patient's compatibility and less anticholinergic adverse effects[5], nevertheless, they have been found to exert negative effects on bone density and increase fracture risk within the first eight months[6,7,8]. Serotonin 5-hydroxytryptamine (5-HT) receptors have been recognized on osteoblasts, osteocytes and osteoclasts, and the effect of SSRIs on bone appears to be controlled by the activation of these receptors via autocrine, paracrine, endocrine and neuronal pathways; so SSRIs are considered as a secondary cause of OP[9].

Fluvoxamine (FM) is an effective specific SSRI available since 1983. It binds to serotonin transporter selectively, blocking serotonin reuptake into the pre-synaptic neurons; thus enhancing the serotonergic outcome in the brain[10]. It is recommended as a first line therapy for a number of psychotic disorders as depression that is a common major disorder affecting 20% of the individuals within their lifetime[11], delirium[12], anxiety disorders which are the most prevalent

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psychiatric disorders and women are twice as commonly affected as men[13], obsessive-compulsive disorder which is the fourth most common mental disorder[14], traumatic brain injury that is extensively followed by disorders of mood, attention, sleep and anxiety[15].

Current treatments for OP are dominated by drugs that suppress bone formation and may contribute to the pathogenesis of osteonecrosis. To restore the extensive bone loss, there is a great need for anabolic treatments that induce osteoblasts to build new bone[16].

Collagen type I is the main structural protein dispersed throughout the body; forming 25% of the total body protein and 80% of the total connective tissue in humans. Also, it is an important bone component; being the main extracellular matrix protein and plays a role in osteoblast differentiation as well[17]. Nowadays; researches are directed at naturally occurring agents that possess a potential antiresorptive effect and collagen hydrolysate (CH), hydrolyzed collagen type I, is one of these agents currently under analysis. It results from partial hydrolysis of gelatins (bovine, pocrine or fish) to enhance their solubility and absorption[18]. CH consists of small peptides with low molecular weight (collagen peptides, CP) enriched in specific amino acids: proline, hydroxyproline and glycine[19]. CH has been receiving attention as a probable oral supplement for the recovery of osteoarticular tissue[20]; as it has a better outcome on bone health when administered orally in combination with calcium and vitamin D[21].

Osteopontin (OPN), a highly phosphorylated sialoprotein, is a major constituent of mineralized extracellular matrices of teeth and bones. It plays a significant role in the attachment and mineralization of osteoblasts; so it marks mature osteoblasts and the beginning of their bone mineralization activity and also indicates osteoblastic differentiation[22,23,24]. Proliferating Cell Nuclear Antigen (PCNA) is an auxiliary protein of DNA-polymerase enzymes, essential for DNA synthesis and is used as a marker for proliferating cells[25].

Although many studies have addressed the effect of collagen peptides in osteoporotic models, there is no experimental or clinical data concerning its role when accompanied with SSRIs therapy especially FM (the most commonly used one), and whether this combination will protect the bone tissue or not. Moreover, no studies were done on the affection of trabecular bone following FM administration. Accordingly, the aim of the present work was to examine if consuming CH concomitantly with fluvoxamine maleate could protect the bone (both compact and spongy) against OP. This was done in adult male albino rat using laboratory, histological, immunohistochemical and statistical methods.

MATERIALS AND METHODS

Materials

Drugs

- Fluvoxamine maleate (FM), with trade name Faverin (Pharco Pharmaceuticals, Alexandria, Egypt), was supplied in the form of tablets 50 mg each and was dissolved in 50 mL normal saline (0.9% Nacl); so that each rat receives 0.4 mL saline containing 0.4 mg FM.

- Collagen hydrolysate (CH) (Great Lakes Gelatin Company, Illinois, USA), was supplied in the form of sachets each contains 12g CH powder that was dissolved in 12 mL distilled water; so that each rat receives 0.5 mL distilled water containing 0.5 g CH.

Animals

This study included 36 adult male albino rats about 3 months old and with average weight (180- 200 grams). They were housed in the Animal House of Kasr Al-Ainy Faculty of Medicine, Cairo University. They were kept in stainless steel cages under standard environmental conditions with free access to standard diet and water throughout the experimental period. All the experimental procedures were done according to guidelines approved by the Animal Use Committee of Cairo University.

Experimental design

Rats were divided into 4 groups

• **Group I:** Control group included 9 rats that were subdivided equally into:

- Subgroup IA: rats were injected with 0.4 mL normal saline subcutaneously (SC) daily for 4 weeks (corresponds to group II).

- Subgroup IB: rats were injected with 0.4 mL normal saline SC with concomitant oral administration of 0.5 mL distilled water via gastric gavage daily for 4 weeks (corresponds to group III).

- Subgroup IC: rats were injected with 0.4 mL saline SC daily for 4 weeks then stopped, and the rats were left without taking any injections for another 4 weeks (corresponds to group IV).

• **Group II:** Osteoporosis group (OP group) included 9 rats that were injected SC with FM dissolved in normal saline in a dose of 2mg/kg/day for 4 weeks[26].

• **Group III:** Collagen hydrolysate group (CH group) included 9 rats that received FM as in group II concomitant with oral CH in a dose of 2.5 g/kg/day for 4 weeks[27].

• **Group IV:** Recovery group included 9 rats that received FM as in group II for 4 weeks only and they were left without taking any drugs for another 4 weeks.

Methods

Laboratory investigations

At the end of experimental duration for each group and just before scarification, blood samples were taken from the tail veins in heparinized capillary tubes. They were analyzed for alkaline phosphatase (ALP) and calcium (Ca^{+2}) in Medical Biochemistry Department, Faculty of Medicine, Cairo University.

Histological examination

According to experimental duration, rats were sacrificed by intraperitoneal injection of phenobarbital (80mg/kg[28]. Specimens from the upper end of the right femur just below the greater trochanter (compact bone) and from the first lumbar vertebra (trabecular bone) were dissected and excised, fixed in 10% buffered formalin solution for 24 hours, then were decalcified by the daily exchange of ethylene-diamine tetra-acetic acid (EDTA) for 4 weeks[29]. After decalcification, specimens were dehydrated in ascending grades of ethanol (70%, 95%, and 100%), cleared in xylol and embedded in paraffin wax. Serial sections of 5-7 µm were cut using Leica rotator microtome (Germany) and were subjected to the following:

1. Hematoxylin & eosin (H&E) stain[30].
2. Mallory's trichrome stain[31] for detection of mineralized (mature) collagen fibers.
3. Immunohistochemical stain[31] using:
    - Anti osteopontin antibody (OPN): a ready to use mouse monoclonal antibody (Lab Vision Corporation, CA, USA, catalogue number SAB4200018). Positive cells showed brown cytoplasmic reaction
    - Anti Proliferating Cell Nuclear Antigen antibody (PCNA): a ready to use mouse monoclonal antibody (Lab Vision Corporation, CA, USA, catalogue number MS106P). Positive cells show brown nuclear deposits.

Sections were counter stained with hematoxylin stain and the negative control sections were prepared by omitting the primary antibody.

Morphometric study

This was done using ″Leica Qwin 500″ software image analyzer computer system (Leica image system Ltd; Cambridge, England). In 10 non-overlapping randomly chosen fields for each section and using an objective lens of x10 magnification, the following parameters were measured:

1. Thickness of compact bone in H&E stained sections.
2. Area of trabecular bone in H&E stained sections.
3. Area percent of collagen fiber content in Mallory's trichrome-stained sections.
4. Area percent of osteopontin immunoreaction.
5. Number of PCNA immunoreactive osteoblasts.

Statistical Analysis

Laboratory and morphometric results were statistically analyzed using SPSS package version 21 (SPSS Inc., Chicago, USA). Comparisons between groups were done using ANOVA (analysis of variance) followed by post hoc Tukey test. Data were expressed as mean and standard deviation (SD) for the quantitative variable. Results were considered significant when $p<0.05$[32].

RESULTS

Laboratory results

Laboratory investigation results are illustrated in (Table 1)

Histological Results

Hematoxylin and Eosin stained sections

Femur sections

Transverse sections of femurs from the control rats (group I) showed regular bone lamellae with homogenous deep eosinophilic matrix and multiple distinct cement lines. Osteocytes inside their lacunae were seen in between these lamellae surrounding haversian canals and the endosteum revealed many osteoblasts (Figure 1a). Whereas sections from group II (OP group) displayed evident histological changes; the bone matrix showed faintly stained areas with multiple cavities and indistinct cement lines. Osteocytes were seen with small dark nuclei inside widened lacunae and around widened haversian canals. The endosteal surface was interrupted with no apparent osteoblasts and showed several resorption cavities with multiple large osteoclasts

having eosinophilic cytoplasm and multiple nuclei (Figures 1b, 1c). Femur sections from group III (CH group) showed preservation in bone microstructure similar to the control; regular bone lamellae with multiple distinct cement lines, deep eosinophilic homogenous bone matrix, osteocytes inside their lacunae around haversian canals and multiple osteoblasts lining smooth endosteum (Figure 1d). Group IV (recovery group) showed no improvement; there were the same histological alterations seen in group II (Figures 1e, 1f).

Lumbar vertebrae sections

Transverse sections of lumbar vertebrae from the control group (group I) revealed multiple thick bone trabeculae enclosing bone marrow cavities that contained hematopoietic tissue, scattered adipocytes and blood sinusoids, and the bone matrix was homogenously eosinophilic. Osteocytes resided in their lacunae and the endosteal surfaces displayed multiple osteoblasts (Figure 2a). Regarding group II (OP group), there were thin interrupted bone trabeculae with wide marrow cavities filled with numerous fat cells. Few osteocytes were seen and the endosteal surfaces showed multiple resorption cavities with no apparent osteoblasts (Figure 2b). Group III (CH group) demonstrated trabecular bone almost preserving its normal histological architecture; thick branching and anastomosing trabeculae with homogenous eosinophilic matrix and highly cellular marrow cavities containing blood sinusoids. Multiple osteocytes were seen inside their lacunae and the endosteal surfaces were smooth and lined with multiple cuboidal osteoblasts (Figure 2c). Group IV (recovery group) showed the same histological changes as group II (Figure 2d).

Mallory trichrome stained sections

Transverse sections of femur and lumbar vertebrae from the control rats (group I) displayed bone formed mostly of mineralized bone matrix with very small areas of unmineralization and osteocytes were seen inside their lacunae (Figures 3a, 4a). As regards group II (OP group), bone sections exhibited unmineralized bone matrix in most areas with the presence of resorption cavities. There were small areas of mineralized bone matrix (Figures 3b, 4b). Sections from group III (CH group) revealed histological picture typical to the control (Figures 3c, 4c). Whereas group IV (recovery group) showed unmineralization in most areas of bone matrix with small mineralized areas (Figures 3d, 4d).

Immunohistochemical results

OPN immunostained sections

Femur and lumbar vertebrae sections of the control rats (group I) exhibited positive OPN immunoreaction that appeared as brown cytoplasmic deposits in osteoblasts lining the endosteum and in bone matrix (Figures 5a, 6a). Whilst group II (OP group) displayed negative OPN immunoreactivity within both the osteoblasts and bone matrix (Figures 5b, 6b). Sections from group III (CH group) showed brown positive OPN immunoreaction in the cytoplasm of osteoblasts and in bone matrix (Figures 5c, 5d). Regarding group IV (recovery group); there was negative OPN immunoreaction in both osteoblasts and bone matrix (Figures 6c, 6d).

PCNA immunostained sections

Control sections from femur and lumbar vertebrae (group I) revealed positive PCNA immunoreactivity, which appeared as brown nuclear deposits in the osteoblasts (Figures 7a, 8a). Concerning sections from group II (OP group); there was negative PCNA immunoreaction in the osteoblasts (Figures 7b, 8b). PCNA immunostained sections from group III (CH group) presented brown nuclear PCNA immunoreactivity within the osteoblasts (Figures 7c, 8c). Whilst group IV (recovery group), showed osteoblasts with negative PCNA immunoreaction (Figures 7d, 8d).

Morphometric results

Morphometric results are illustrated in (Table 2).

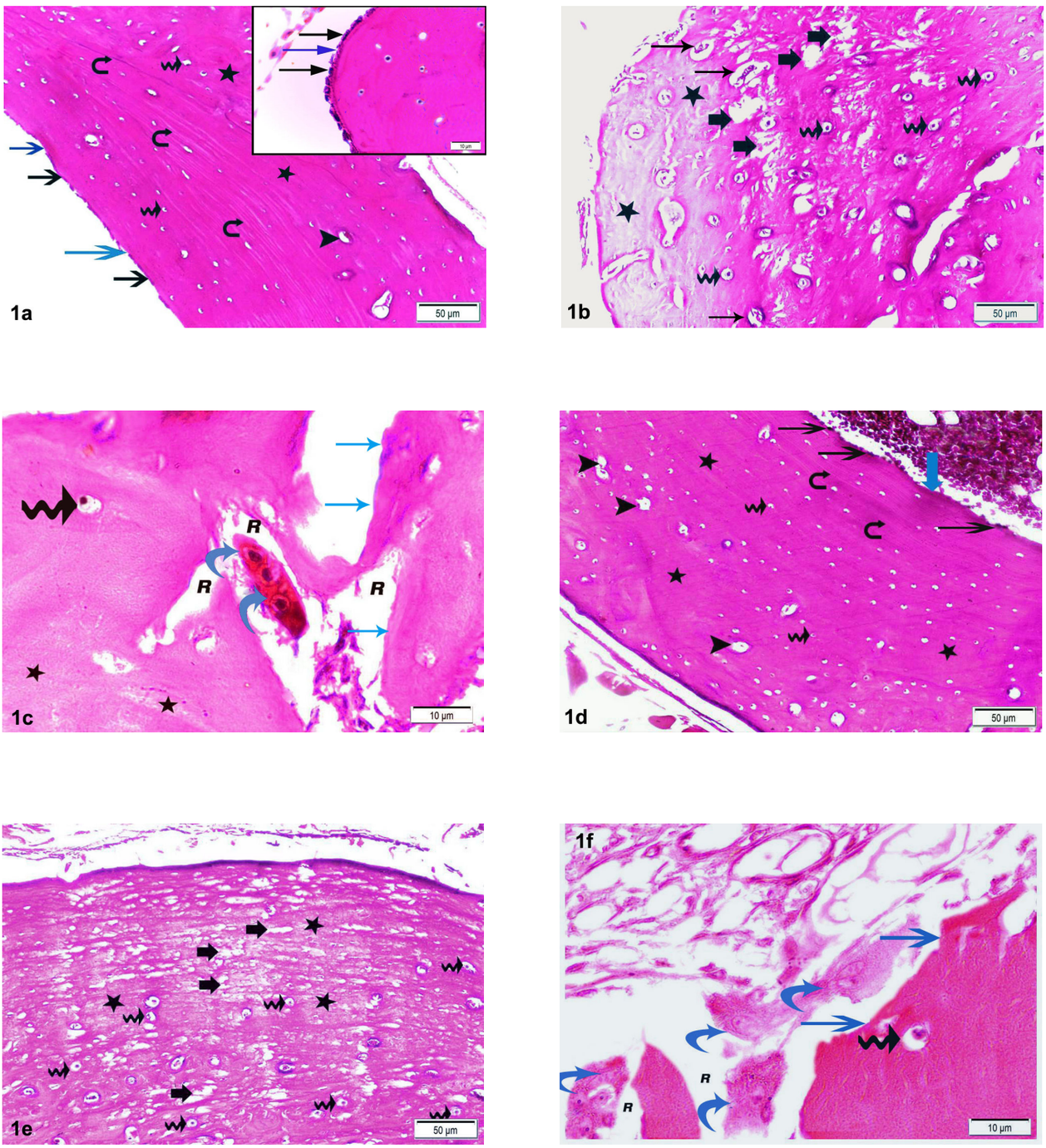

Fig. 1: Photomicrographs of transverse sections from rats' femurs stained with H&E. 1a) Group I (control group) shows regular bone lamellae with homogenous deep eosinophilic matrix (black stars) and multiple distinct cement lines (curved arrow). Osteocytes are seen in their lacunae (wavy arrows) around haversian canals (arrowheads) and the endosteum (blue arrow) is lined with numerous osteoblasts (black arrow) (x200). The inset is a higher magnification of the endosteal surface (blue arrow) with multiple osteoblasts (black arrows) (x1000). 1b) Group II (OP group) displays multiple cavities (thick arrows) within faint bone matrix (black stars), widened osteocytes lacunae (wavy arrows) and haversian canals (thin arrows) (x200). 1c) Higher magnification shows an osteoclast (blue curved arrows) in a large resorption cavity (R) and the endosteal surface with no osteoblasts (blue arrows). The bone matrix exhibits faintly stained areas (black stars) and an osteocyte appears with small dark nucleus (wavy arrow) (x1000). 1d) Group III (CH group) presents regular bone lamellae with homogenous deep eosinophilic matrix (black stars) and multiple distinct cement lines (curved arrows). Osteocytes inside their lacunae (wavy arrows) surround haversian canals (arrowheads). The endosteum (blue arrow) is lined with multiple osteoblasts (black arrow) (x200). 1e) Group IV (recovery group) reveals faintly stained matrix (black stars), multiple cavities (thick arrows) and widened osteocytes lacunae (wavy arrows) (x200). 1f) Higher magnification shows multiple osteoclasts (blue curved arrows) within large resorption cavities (R). No osteoblasts lining the endosteum (blue arrows) and an osteocyte with a karyolytic nucleus is seen (wavy arrow) (x1000).

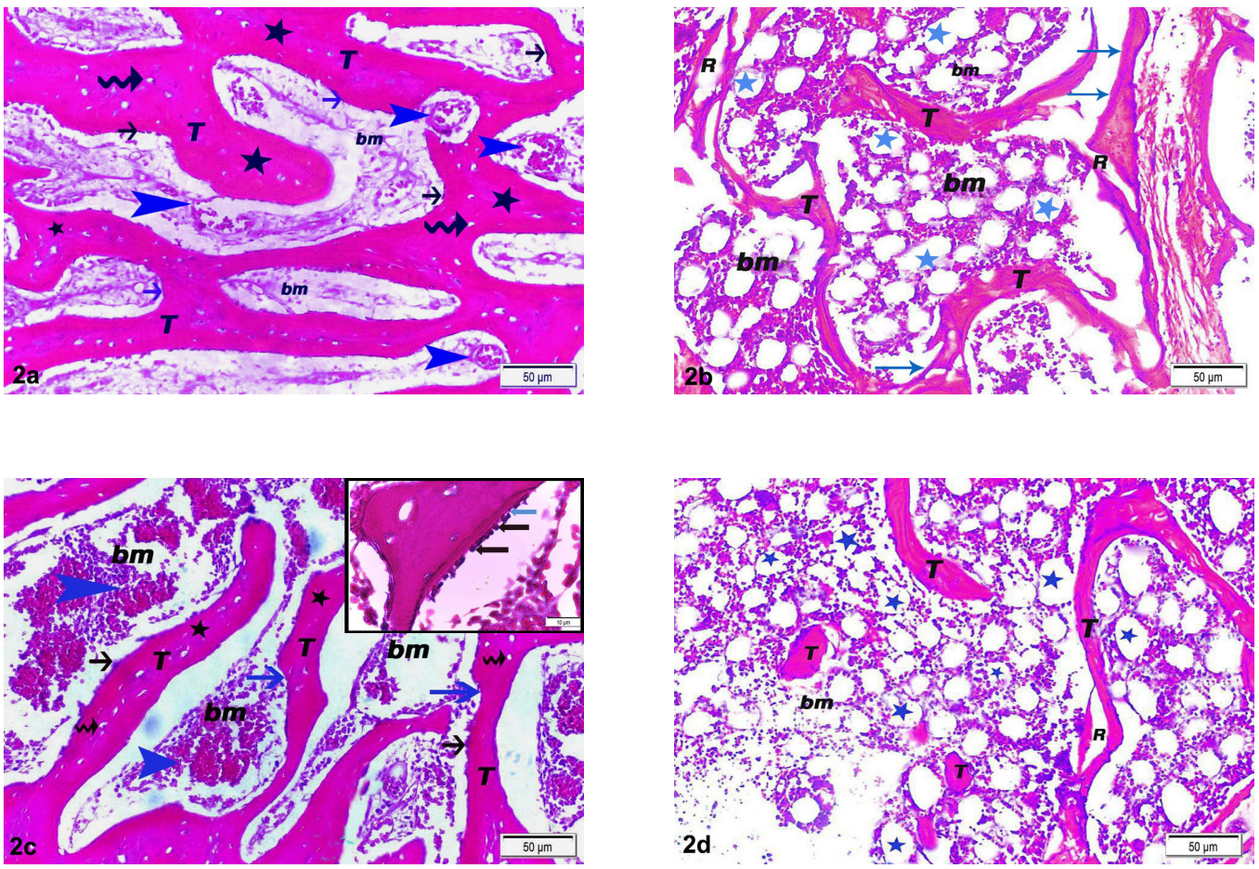

Fig. 2: Photomicrographs of transverse sections from rats' lumbar vertebrae stained with H&E. 2a) Group I (control group) displays multiple thick bone trabeculae (T) enclosing bone marrow cavities (bm) with many blood sinusoids (blue arrowheads). Osteocytes inside their lacunae (wavy arrow), homogenous eosinophilic bone matrix (black stars) and smooth endosteal surfaces (blue arrows) lined by osteoblasts (black arrows) are seen. (x200). 2b) group II (OP group) shows thinning of bone trabeculae (T) with resorption cavities (R), no osteoblasts lining the endosteum (blue arrows), and wide marrow cavities (bm) filled with numerous fat cells (blue stars) (x200). 2c) Group III (CH group) presents thick branching and anastomosing bone trabeculae (T) with homogenous eosinophilic matrix (black stars). They enclose highly cellular marrow cavities (bm) containing blood sinusoids (blue arrowheads). Osteocytes reside in their lacunae (wavy arrows) and the endosteal surface is smooth (blue arrows) and lined with osteoblasts (black arrows) (x200). The inset is a higher magnification for the smooth endosteal surface (blue arrow) lined with multiple cuboidal osteoblasts (black arrows) (x1000). 2d) Group IV (recovery group) shows thin interrupted bone trabeculae (T) with resorption cavity (R) and wide bone marrow cavities (bm) filled with numerous fat cells (blue stars) (x200).

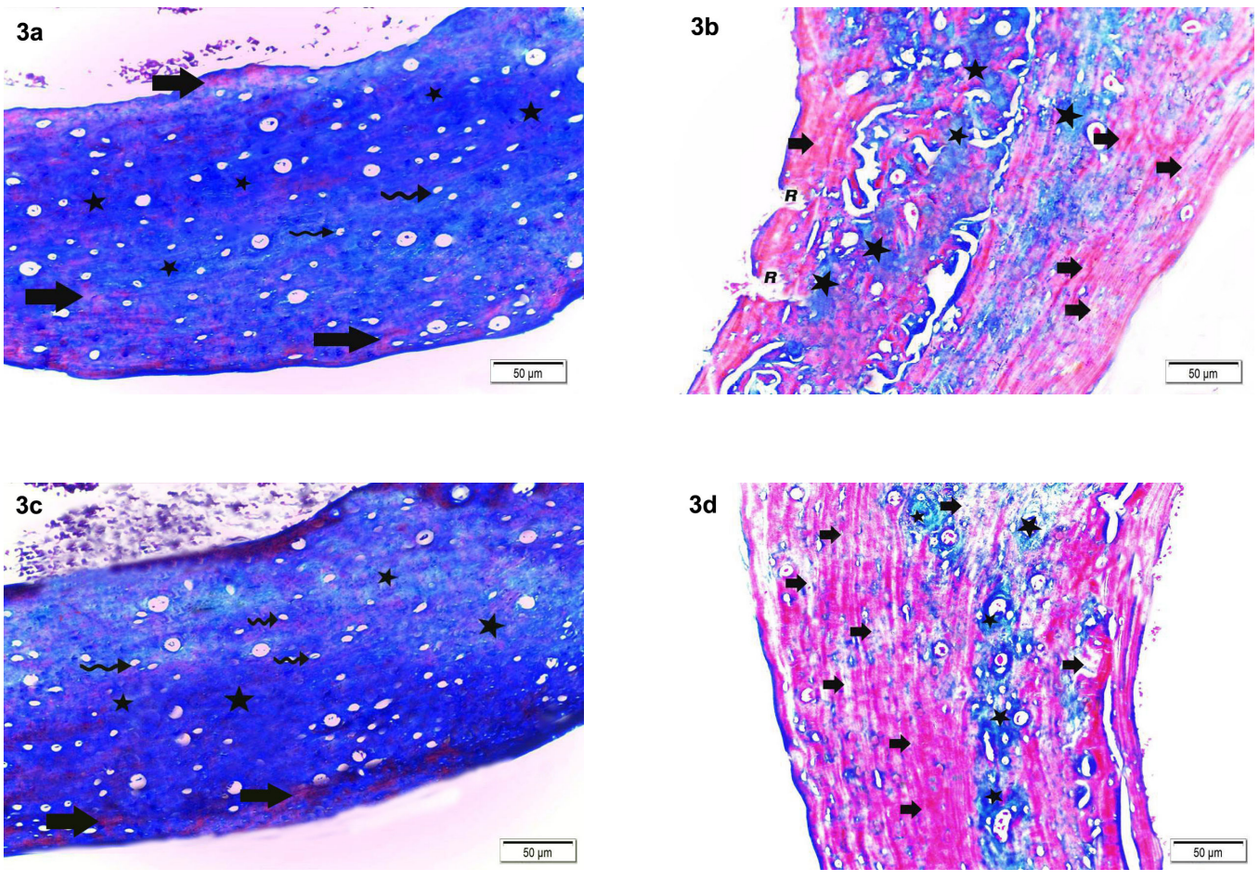

Fig. 3: Photomicrographs of transverse sections from rats' femurs stained with Mallory's trichrome stain x200. 3a) Group I (control group) reveals bone formed mostly of mineralized bone matrix (stars) with very small unmineralized areas (thick arrows). Osteocytes are seen inside their lacunae (wavy arrows). 3b) group II (OP group) shows mainly unmineralized bone matrix (thick arrows) with resorption cavities (R).There are small mineralized areas (stars). 3c) Group III (CH group) displays mostly mineralized bone matrix (stars) with osteocytes (wavy arrows) in between. Very small areas of unmineralization are seen (thick arrows). 3d) Group IV (recovery group) exhibits unmineralized bone matrix (thick arrows) with the presence of small areas of mineralization (stars).

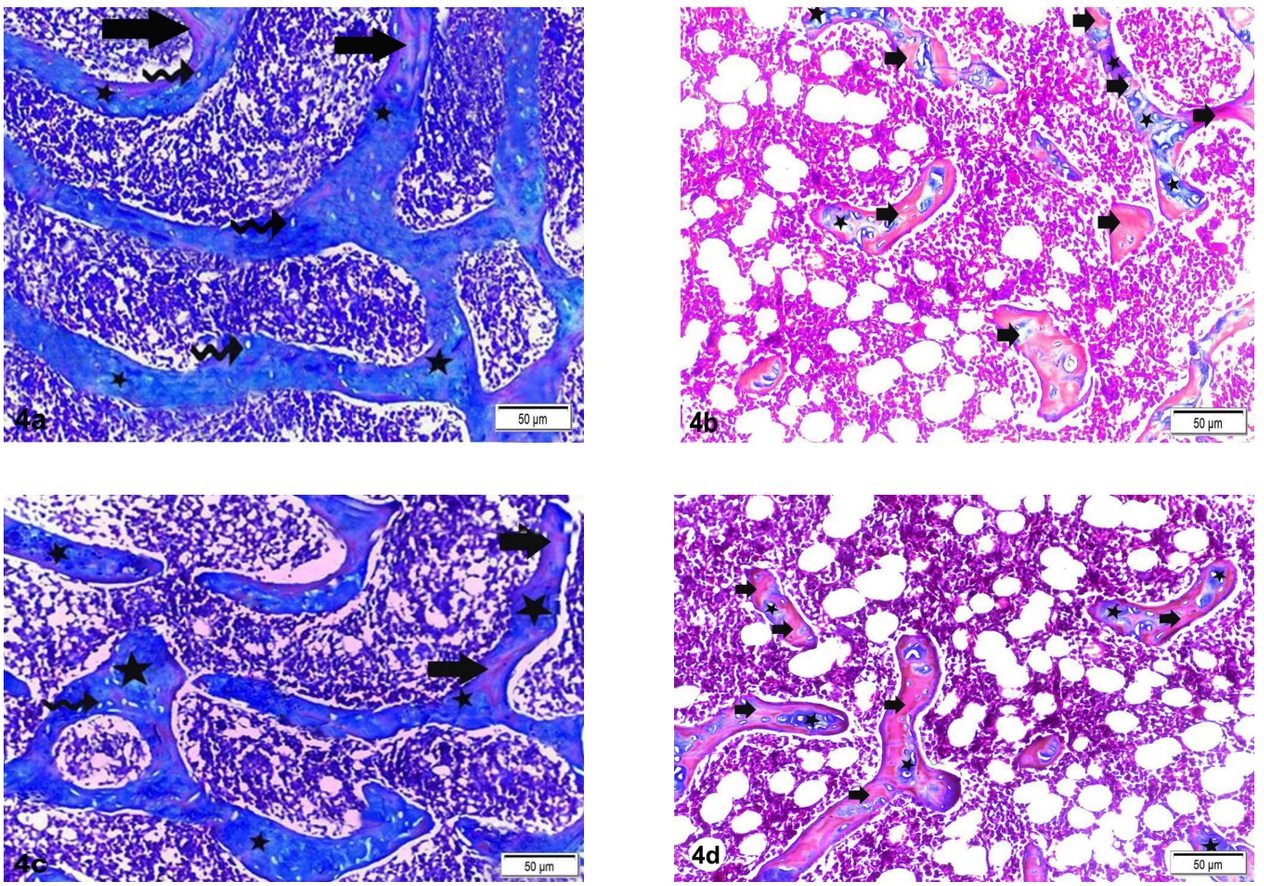

Fig. 4: Photomicrographs of transverse sections from rats' lumbar vertebrae stained with Mallory's trichrome stain x200. 4a) Group I (control group) shows branching and anastomosing bone trabeculae formed mostly of mineralized bone matrix (stars) with osteocytes in their lacunae (wavy arrows). Very small unmineralized areas (thick arrows) are seen. 4b) Group II (OP group) reveals mostly unmineralized bone matrix (thick arrows) with small areas of mineralization (stars). 4c) Group III (CH group) displays branching and anastomosing bone trabeculae formed mostly of mineralized matrix (stars) with osteocytes inside their lacunae (wavy arrows). There are small areas of unmineralization (thick arrows). 4d) Group IV (recovery group) shows mostly unmineralized matrix (thick arrows) with small areas of mineralization (stars).

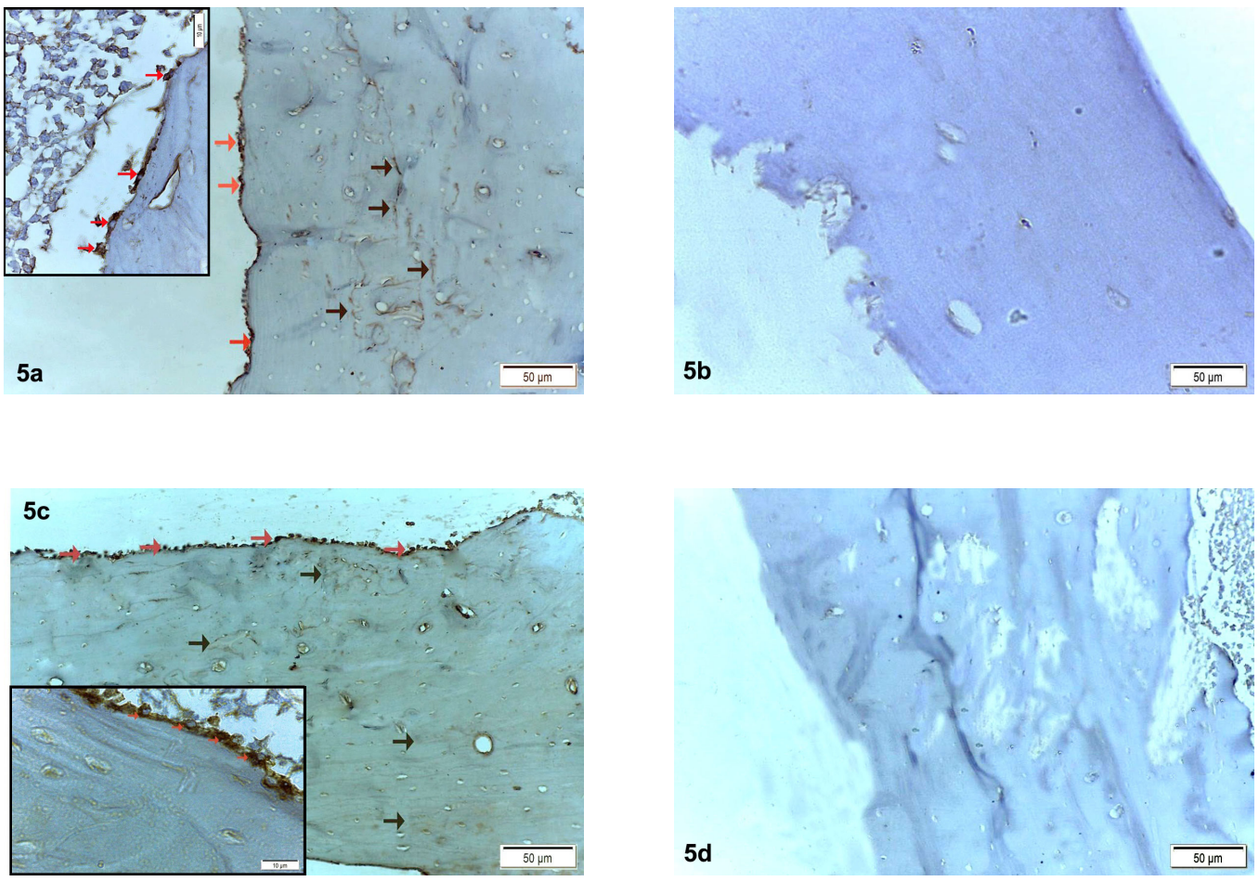

Fig. 5: Photomicrographs of transverse sections from rats' femurs immunostained with anti OPN antibody. 5a) Group I (control group) presents +ve OPN immunoreactivity within the osteoblasts (red arrow) and the bone matrix (black arrow) (x200). The inset is a higher magnification for +ve OPN immunoreactive osteoblasts with brown cytoplasmic deposits (red arrow) (x1000). 5b) Group II (OP group) shows -ve OPN immunostaining. 5c) Group III (CH group) reveals +ve OPN immunoreactivity within the cytoplasm of osteoblasts (red arrow) and in the bone matrix (black arrow) (x200). The inset is a higher magnification for +ve OPN immunoreactive osteoblasts (red arrows) (x1000). 5d) Group IV (recovery group) shows -ve OPN immunoreaction (x200).

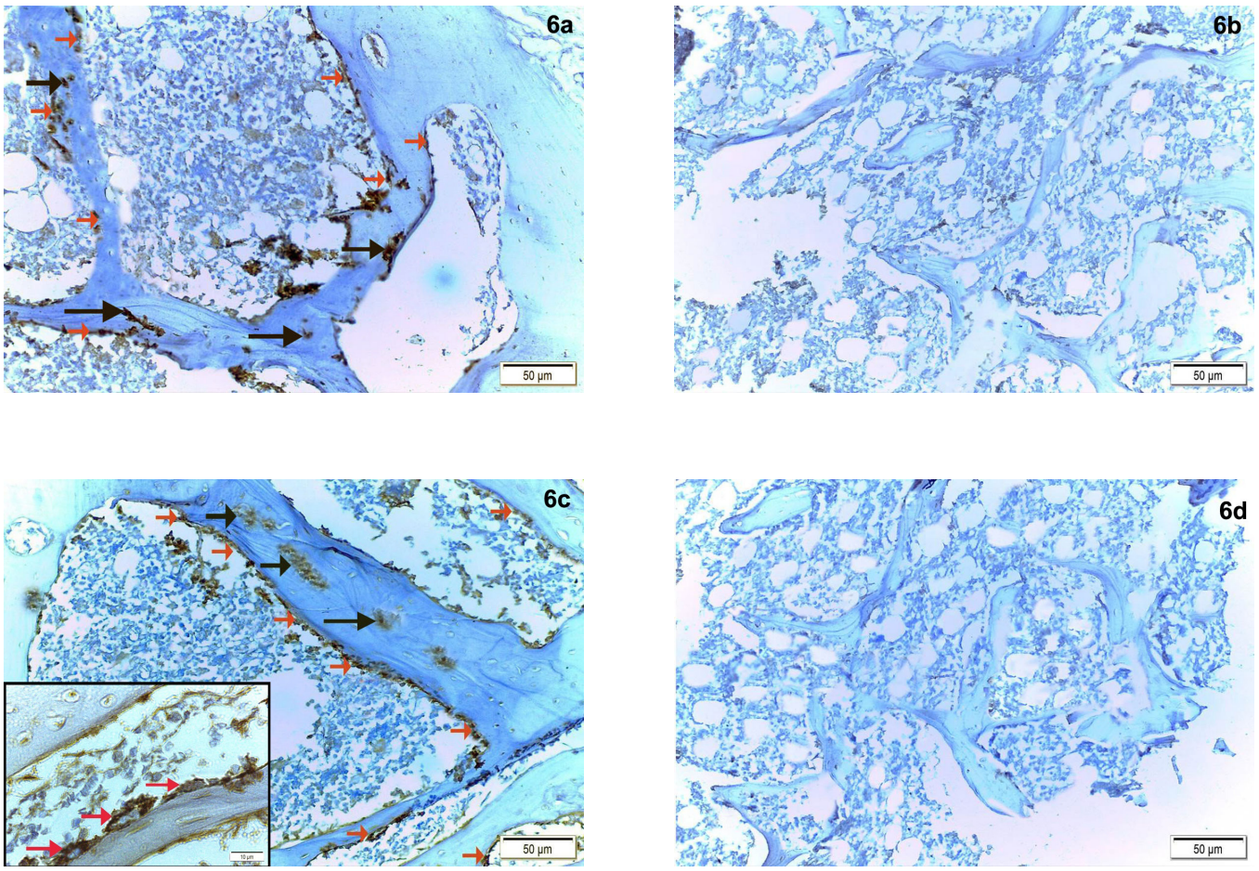

Fig. 6: Photomicrographs of transverse sections from rats' lumbar vertebrae immunostained with anti OPN antibody. 6a) Group I (control group) exhibits +ve OPN immunoreactivity appearing as brown cytoplasmic deposits in the osteoblasts (red arrow) and bone matrix (black arrow) (x200). 6b) Group II (OP group) shows -ve OPN immunoreaction (x200). 6c) Group III (CH group) displays +ve OPN immunostaining within the cytoplasm of osteoblasts (red arrow) and in the bone matrix (black arrow) (x200). The inset is a higher magnification showing osteoblasts with +ve OPN immunoreaction (red arrow) (x1000). 6d) Group IV (recovery group) shows -ve OPN immunoreaction (x200).

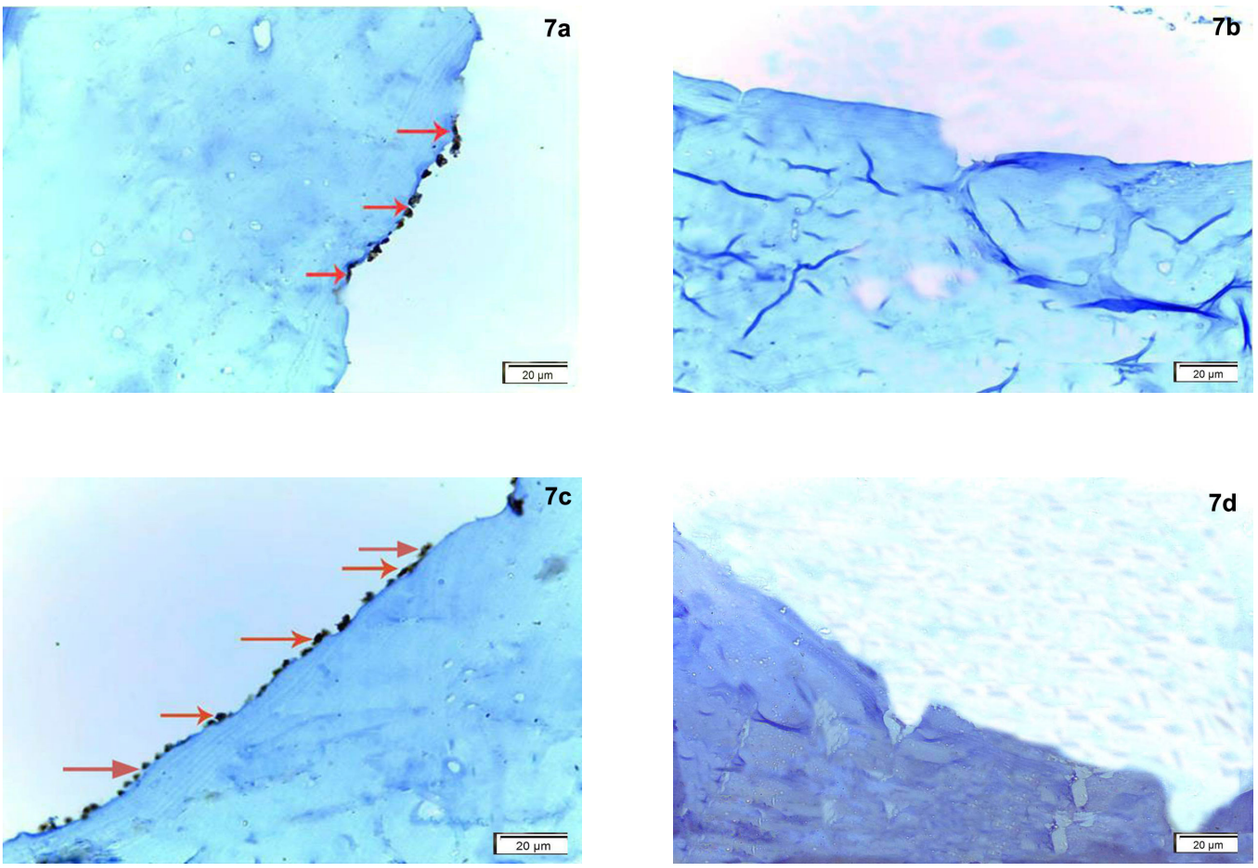

Fig. 7: Photomicrographs of transverse sections from rats' femurs immunostained with anti PCNA antibody (x400). 7a) Group I (control group) presents +ve PCNA immunoreactivity appearing as brown nuclear deposits in the osteoblasts (red arrows). 7b) Group II (OP group) shows -ve PCNA immunoreaction. 7c) Group III (CH group) exhibits numerous +ve PCNA immunostained osteoblasts (red arrow). 7d) Group IV (recovery group) displays -ve PCNA immunoreactivity.

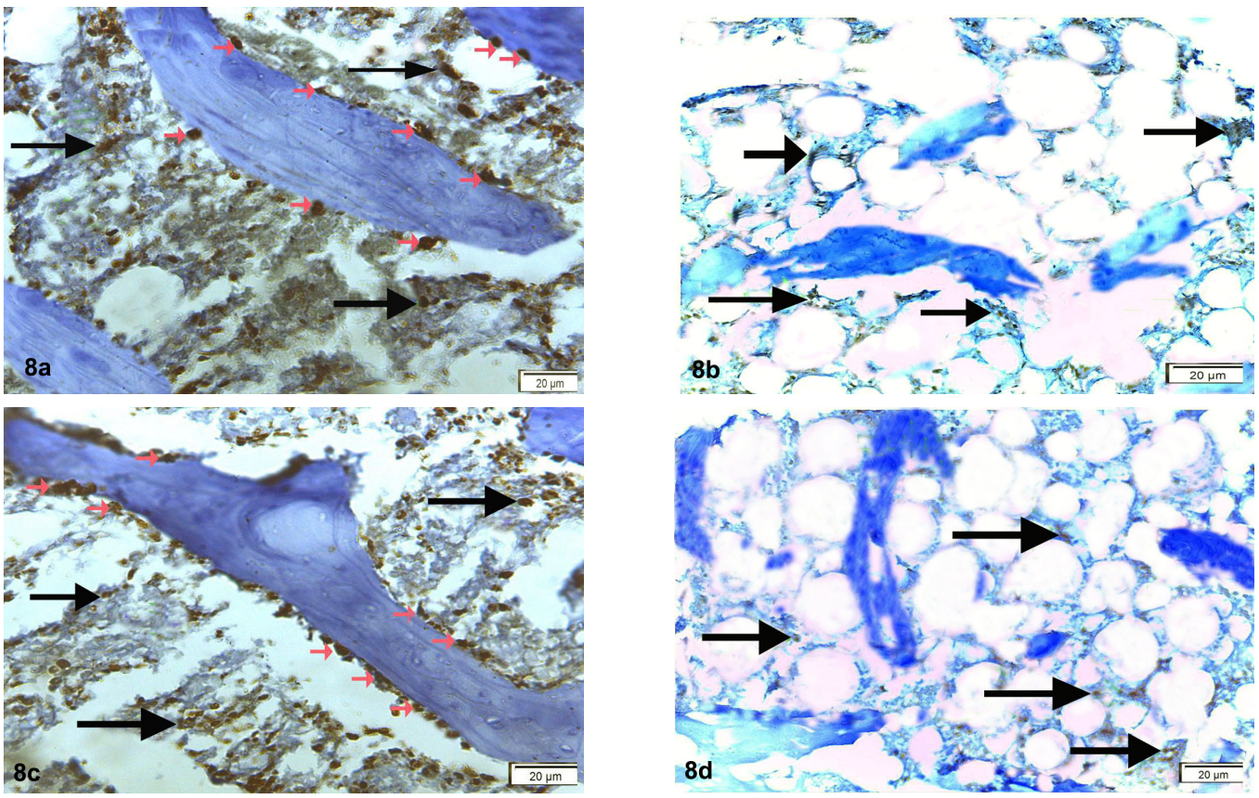

Fig. 8: Photomicrographs of transverse sections from rats' lumbar vertebrae immunostained with anti PCNA antibody (x400). 8a) Group I (control group) shows +ve PCNA immunoreactivity within the nuclei of osteoblasts (red arrows) and most of bone marrow cells (black arrows). 8b) Group II (OP group) displays -ve PCNA immunoreaction, yet, some bone marrow cells show +ve PCNA immunoreactivity (black arrows). 8c) Group III (CH group) presents numerous +ve PCNA osteoblasts (red arrow) and bone marrow cells (black arrows). 8d) Group IV (recovery group) shows -ve PCNA immunoreaction, however, some bone marrow cells are +ve PCNA immunoreactive (black arrows).

Table 1: Mean values (±SD) of chemical parameters in the studied groups

Parameters	Group I (Control)	Group II (OP group)	Group III (CH group)	Group IV (Recovery group)
Mean serum ALP (U/L)	122.76±1.65	307.33±6.49*	128.91±1.89#	301.39±8.16*$
Mean serum ca^{+2} (mg/dl)	10.71±0.12	6.96±0.96*	10.05±0.32#	6.52±0.88*$

* Significant compared to groups I and III.

Significant compared to groups II and IV with no significant difference compared to groups I.

$ Non significant compared to group II.

Table 2: Mean values (±SD) of morphometric parameters in the studied groups

Parameters	Group I (Control)	Group II (OP group)	Group III (CH group)	Group IV (Recovery group)
Thickness of compact bone (µm)	552.78±39.82	436.88 ±19.11*	548.22±32.14#	439.39±13.48*$
Area of trabecular bone (mm^2)	2727.8±194.3	1104.03±110.8*	2701.9±214.7#	1109.9±133.7*$
Area % of collagen fibers (compact bone)	94.62 ± 1.97	29.45 ± 3.12*	93.51 ± 2.76#	30.51 ± 3.05*$
Area % of collagen fibers (trabecular bone)	75.70± 4.02	13.93± 1.99*	73.95± 3.87#	14.69± 2.51*$
Area % of OPN in compact bone	23.57±2.02	0.71±0.18*	36.59±2.21α	0.79±0.16*$
Area % of OPN in trabecular bone	11.19±2.32	0.72±0.19*	20.09±2.11α	0.74±0.14*$
Number of PCNA +ve cells in compact bone	7.70±1.33	3.10±0.57*	17.50±2.46α	2.40±0.52*$
Number of PCNA +ve cells in trabecular bone	11.30±1.16	2.0±0.47*	20.10±1.85α	2.20±0.42*$

* Significant compared to groups I and III.

Significant compared to groups II and IV with no significant difference compared to groups I.

$ Non significant compared to group II.

α Significant compared to groups I, II and IV.

DISCUSSION

In the present study, male albino rats were used to examine the probable protective effect of CH against fluvoxamine maleate-induced OP; to avoid hormonal changes in female rats like estrogen deficiency that may cause OP[33]. Fluvoxamine maleate (FM) was given for 4 weeks in a non toxic dose of 2mg/kg/day; the total effective therapeutic human dose varies from 100 up to 300 mg[34] which is equivalent to about 1.8 to 5.4 mg in rats according to Paget[35], and these dose and duration were proved to be sufficient to induce OP in rats[26]. Both compact and trabecular bones were investigated in the current work and due to the multiple similarities between human and rat femur, at the macrostructural and microstructural levels, this site has gained vast importance in OP studies as an example for compact bone[36]. In addition, the trabecular bone of the lumbar vertebrae is at higher risk for OP changes and plays a key role in its monitoring[37]; therefore it was chosen for histological examination as an example for trabecular bone.

The current work revealed that FM when given alone in both groups II and IV (FM and recovery groups) produced a significant increase in serum alkaline phosphatase (ALP) and a significant decrease in serum calcium (Ca^{+2}) compared to the control; which indicates an increase in bone turnover, and this is consistent with several earlier OP studies[21,38,39]. This might be explained by that ALP is a cell membrane component of many tissues with the highest concentrations in the liver and bone osteoblasts. So, it is increased in skeletal system diseases as a compensatory reaction to bone destruction[17] due to increased bone turnover[38]. Whilst reduced serum Ca^{+2} in OP group could be attributed to increased bone resorption causing hypercalcemia, which triggers a compensatory mechanism to reduce its intestinal absorption and increase its renal excretion[40]. In group IV (recovery group), these findings was enlightened by previous researchers who stated that FM exerted negative effect on bone metabolism that persisted for a prolonged time after its discontinuation[8].

In the present study, bone sections from both groups II and IV presented the same histological alterations. There were areas of pale stained matrix, indistinct cement lines, decreased bone thickness, thinning and interruption of bone trabeculae, multiple resorption cavities housing many osteoclasts. These findings go on line with former studies[26,41] and were confirmed by morphometric measurements; significant decrease in the mean thickness of compact bone and mean area of trabecular bone compared to control, which is in agreement with other OP studies[42,43,44,45]. This might be explained by the disturbance between bone formation and resorption, resulting in remodeling dysregulation with subsequent loss of bone matrix[46]. Another explanation could be the increased rate of bone resorption over that of bone formation[47]; as regular cement lines mark the edge between old and newly deposited bone and are defined as matrix layers laid down at any time resorption is followed by bone deposition[48]. Also, bone sections displayed wide marrow spaces with numerous fat cells and this might be attributed to decreased trabecular bone thickness that leaves more space for the bone marrow[49]. Also the shift in differentiation of mesenchymal cells toward adipocytes instead of osteoblastic lineage in bone marrow, results in increased number of fat cells and inactive bone marrow[50].

Moreover, there was irregular eroded endosteal surface with loss of osteoblasts which was reported by previous researchers following the use of SSRIs and they attributed these changes to the decline in osteogenic proliferation and differentiation[48,51]. This finding was established immunohistochemically by the significant decrease in OPN and PCNA expression compared to the control, which reflects the activity of osteoblasts, their maturation and the beginning of bone mineralization activity[52,53,54]. Low expression of OPN as a bone mineralization marker was formerly reported and it was associated with increased bone fragility and matrix porosity[22,23]. Additionally, a decrease in the mean number of PCNA immunostained positive osteoblasts was previously reported with the long term use of SSRIs and FM; as they directly inhibit osteoblasts formation and function and induce their apoptosis[8,51,54,55].

Demineralization of bone following FM was evident in Mallory's trichrome stained sections where bone was formed mostly of immature unmineralized matrix with small areas of mineralization that might be attributed to the decreased number of osteoblasts responsible for bone mineralization[54]. This was confirmed morphometrically by the significant decrease in the mean area percent of collagen fibers compared to the control, which is in agreement with previous OP studies[27,54,56].

Moreover, bone sections revealed apoptotic osteocytes and this is consistent with Rochefort[16] who reported osteocytes loss or their presence in few numbers within indistinct lacunae and in irregular orientation as a key feature in the pathogenesis of OP. Besides, whatever the cause of OP, osteocyte cell death occurs resulting in a condition called osteonecrosis or dead bone[57]. Moreover, there was apparent widening of osteocytes lacunae in this work that might be explained by the occurrence of osteocytic osteolysis which has a Ca^{+2} homeostatic effects[58]. Consistent with this, previous scientists have found widened osteocytic lacunae in OP rats and other conditions of bone loss[27,59,60]. Osteocytic osteolysis is the removal of perilacunar matrix by osteocytes, where they are able to acidify their lacunar-canalicular space through the production of protons via carbonic anhydrase-2 and the release of protons via proton pumping ATPases. This demineralizes the bone matrix and free calcium, while MMP-13, tartrate resistance acid phosphatase and cathepsin K remove the organic components of the perilacunar matrix[61].

With long term use of SSRIs, the negative effect of peripheral serotonin outweighs the positive central effect[62]; as they inhibit osteoblasts differentiation, proliferation and mineralization, decrease matrix proteins formation and lower bone marrow density (BMD), inhibiting bone development[8,54]. The effect of SSRIs on bone is governed by activation of 5-HT receptors on osteoblasts[9]; binding

of peripheral serotonin to these receptors triggers 5-HT-R1b/PKA/CREB/cyclins signaling cascade, inhibiting cAMP production and phosphorylation mediated by protein kinase A (PKA), thus leads to decreased expression of cyclin genes and decreased osteoblast proliferation[63]. Another explanation was given by Gustafsson *et al*.[64] who reported that SSRIs have increased osteoclastic activity by stimulating receptor activator of nuclear factor kappa-B ligand (RANKL) and inhibiting osteoprotegrin (OPG). RANKL, also known as osteoclast differentiation factor, is produced by osteoblasts and marrow stromal cells. When binds to its RANK receptor on osteoclasts and preosteoclasts, it promotes their proliferation and differentiation[65]. OPG is a circulating protein receptor also expressed by osteoblasts and marrow stromal cells. It inhibits osteoclast formation by binding RANKL, thus prevent the stimulatory cell to cell interaction with preosteoclasts and inhibit RANKL/RANK interactions[66]. Decreasing osteoblasts activity while maintaining osteoclasts function leads to a shift in the bone remodeling balance towards bone resorption and loss[47]. Besides, an in-vitro study provided evidence that SSRIs effect on bone cells is direct in nature rather than serotonin-dependent[8].

On the other hand, concomitant administration of CH with FM in group III (CH group) resulted in significant decrease in serum ALP and significant increase in serum Ca^{+2} compared to both groups II and IV, yet, there was no significant change when compared to the control. This might be explained by that collagen peptides (CP) could reduce bone turnover and promote calcium uptake, thus increase bone formation as was proved previously[21,67,68].

Bone sections from group III (CH group) revealed noticeable preservation in the bone microstructure. Osteocytes inside their lacunae were seen in between regular lamellae and the bone matrix appeared deeply eosinophilic with multiple distinct cement lines. The bone trabeculae of lumbar vertebrae appeared thicker and more continuous, with smaller highly cellular marrow cavities compared to OP group. This is in harmony with numerous scientists who documented the protective effect of CH on bone histology in ovariectomized OP rats[21,37,69]. Also CH intake was found to increase bone mass in growing rats following treadmill training[70] and increase BMD in postmenopausal women with primary age-related OP[71]. Furthermore, in a study done on OP in aged mice, CH was found to improve the bone architecture in terms of more branching and anastomosing trabeculae and normalizing their integrity[72]. Morphometric measurements were supportive to the histological findings; there was significant increase in the mean thickness of compact bone and in the mean area of trabecular bone when compared to FM groups (II and IV). This is in accordance with an earlier study, which investigated the effect of CH in magnesium deficient OP rats and proved that it could attenuate bone brittleness by increasing cortical thickness[2]. Similar finding was reported by Liu *et al*.[21] who stated that CP supplementation could inhibit bone loss with significant increase in the trabecular bone area in ovariectomized OP rats. Adding up, no visible osteoclasts were detected in the present work and this might be accredited to the role of CH in inhibiting osteoclasts formation and activity[73]. Others stated that CH intake has exerted a significant effect on reducing tartrate-resistant acid phosphatase-5b which is a specific and sensitive biomarker of osteoclasts and its concentration is recognized as a specific index of bone resorption, so CH plays an important role in reducing bone absorption served by osteoclasts[74].

Moreover, the endosteum was lined with numerous osteoblasts and this was confirmed by significant increase in the mean area percent of OPN and in the mean number of PCNA immunoreactivity in CH group compared to both osteoporotic groups. In agreement with this, previous researchers have reported that CP could improve osteoblasts proliferation and differentiation and accelerate matrix mineralization with increased OPN expression[75]. They found that when cultured human osteoblasts were treated with CP for seven days, their number increased significantly compared to untreated cells[76]. Furthermore, the effect of CH on the proliferation of osteoblasts was determined by a colorimetric immunoassay based on quantitating bromodeoxyuridine incorporation into the newly synthesized DNA, and it was found to increase the osteoblastic proliferation[37]. The outcome of CH on bone osteoblasts number, activity and mineralization could be elucidated by the ability of CH to increase the expression of bone matrix genes that are responsible for bone formation such as ALP, type 1 collagen and OPN. Osteoblasts express OPN and osteocalcin during the mineralization phase. OPN appears prior to osteocalcin and is expressed during the stage of active proliferation. The expression of ALP and OPN accelerates mineralization and leads to increased calcium deposition[77]. The increased expression of OPN and osteocalcin was proved by different studies following CH treatment associated with prevention of apoptosis[72,78,79].

Adding up, Mallory's trichrome stained sections showed that bone was formed mostly of mineralized matrix with significant increase in the mean area percent of collagen fibers compared to FM groups. This is consistent with former studies that compared CH treated rats to ovariectomized OP rats[27], and could be elucidated by the increased number of osteoblasts and enhancement in their activity that results in increased bone mineralization and synthesis of organic bone matrix components[68,78,80]. Also, a CH-enriched diet was found to improve bone collagen metabolism and BMD[20]. Adding up, Kim *et al*.[37] supported this point by demonstrating the effects of CH in-vitro and in-vivo. In the in-vitro tests; it was observed that CH has increased osteoblasts proliferation and ALP activity, increased bone matrix proteoglycans and improved bone collagen metabolism, collagen synthesis and collagen type1 alpha1 gene expression. In the-vivo tests; they found significant increase in BMD in the lumbar vertebrae. Increased osteoblasts activity is due to interaction between type I collagen and alpha 2, beta 1 integrin receptors on osteoblasts cell membranes which induces their differentiation leading to increased ALP

activity and expression of bone matrix proteins such as type I procollagen, OPN and matrix Gla protein. Moreover, CH intake was found to significantly increase the level of alpha 2, beta 1 integrin receptors[72]. Increasing BMD could be assigned to the composition of bone; as it is made of approximately 70% inorganic salts (minerals) and 30% organic matrix, with collagen accounting for over 90% in this organic part. Minerals play a significant role in bone mass but without enough collagen, taking large amount of minerals is wasteful, as there is inadequate framework for minerals to attach to. Hence, collagen provides a framework where calcium adheres on, creating a hardened soft tissue framework[71,81].

CONCLUSION

Data from this study provide evidence that Fluvoxamine maleate (a common SSRI) leads to osteoporosis affecting both compact and trabecular bones. Also this work proved that concomitant administration of collagen hydrolysate could protect the bone tissue by enhancing osteoblasts proliferation and mineralization. Therefore, it is advisable to use CH for patients assigned to SSRIs therapy, during the course of treatment; to prevent bone loss and reduce the incidence of osteoporosis.

CONFLICTS OF INTEREST

There are no conflics of interest.

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الملخص العربى

هيدروليسات الكولاجين ضد ترقق العظام المحدث بماليات الفلوفوكسامين في الجرذان البيضاء: دراسة هستولوجية وهستوكيميائية مناعية

سهام عبد الحميد محمد القلعاوى[١]، مها محمد أبو جازيه[٢]، سالى أحمد عبد العزيز الإختيار[٢]، داليا ابراهيم اسماعيل[١]

[١]قسم الهيستولوجيا، كلية الطب، جامعة القاهرة
[٢]قسم الهيستولوجيا، كلية الطب، جامعة كفرالشيخ

الخلفية: ترقق العظام هو مرض عظام تنكسى سائد بين المرضى الذين يتلقون مثبطات امتصاص السيروتونين الانتقائية مثل ماليات الفلوفوكسامين. وهيدروليسات الكولاجين مكون غذائي له تأثير مضاد للارتشاف.

الهدف من العمل: تقييم التأثير الوقائي المحتمل لهيدروليسات الكولاجين ضد ترقق العظام المحدث بماليات الفلوفوكسامين فى ذكور الجرذان البيضاء البالغين.

المواد والطرق: تم تقسيم ست وثلاثون جرذاً إلى أربعة مجموعات ، المجموعة الأولى (المجموعة الضابطة) ، المجموعة الثانية (مجموعه ترقق العظام): وتم حقنها بماليات الفولوفكسامين يومياً لمدة أربعه أسابيع ، المجموعة الثالثة (مجموعة هيدروليسات الكولاجين): تلقت ماليات الفلوفوكسامين بالإضافة الي هيدروليسات الكولاجين بالفم لمدة أربعة أسابيع ، المجموعة الرابعة (مجموعة التعافى): تلقت ماليات الفولوفوكسامين فقط لمدة أربعة أسابيع ثم تركت بدون إعطاء أى أدويه لمدة أربعه أسابيع أخرى. تم قياس نسبة الفوسفاتيز القلوي والكالسيوم فى مصل الدم. وتم تحضير عينات العظم من عظمة الفخذ اليمنى وعظمة الفقرة القطنية الأولى وصباغتها بصبغة الهيماتوكسيلين و الإيوسين ، صبغة مالوري ثلاثي الألوان ، صبغات هستوكيميائية مناعية ضد البروتين العظمى الجسرى وضد مولد المضاد النووى للخلايا المتكاثرة. - وتلا ذلك تحليل قياسى وإحصائي.

النتائج: أظهرت المجموعتان الثانية و الرابعة ارتفاعًا ذا دلالة إحصائية في الفوسفاتيز القلوي وانخفاضًا ذا دلالة إحصائية في الكالسيوم مقارنةً بالمجموعة الضابطة. أظهر فحص قطاعات العظم وجود تغيرات هستولوجية واضحة ؛ ظهرت الخلايا العظمية بنوايا صغيرة وداكنة فى داخل ثغرات واسعة مع وجود اتساع فى قنوات هافرسى. كما أظهر قالب العظم أماكن ضعيفة الصبغة ، تجاويف عديدة ، كما وجد تجاويف متآكلة بداخلها خلاياعظمية آكلة. كما وجد إنخفاضاً ذو دلالة إحصائية فى متوسط سمك العظم المحكم ، متوسط مساحة العظم الحويجزي ، متوسط النسبة المئوية للتفاعل المناعي بالبروتين العظمى الجسرى ، ومتوسط عدد الخلايا المتفاعلة مناعياً مع مولد المضاد النووى للخلايا مقارنةً بالمجموعة الضابطة. وأظهرت المجموعة الثالثة متوسط إنخفاضاً ذا دلالة دلالة إحصائية في الفوسفاتيز القلوي

وارتفاعًا ذا دلالة إحصائية في الكالسيوم. أظهر العظم حفاظاً أكثر على البنية الهستولوجية. كما وجد إرتفاعاً ذا دلالة إحصائية فى متوسط سمك العظم المحكم ، متوسط مساحة سطح العظم الحويجزى ، متوسط النسبة المئوية للتفاعل المناعي بالبروتين العظمى الجسرى ، ومتوسط عدد الخلايا المتفاعلة مناعياً مع مولد المضاد النووى للخلايا مقارنةً بالمجموعتين الثانية و الرابعة.

الإستنتاج: هيدروليسات الكولاجين له تأثير قوى فى حماية العظم من ترقق العظام المحدث بماليات الفلوفوكسامين.