

EFFECT OF ATORVASTATIN AND REMIFEMIN ON GLUCOCORTICOID INDUCED OSTEOPOROSIS IN RATS WITH EXPERIMENTAL PERIODONTITIS. A COMPARATIVE STUDY

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ABSTRACT

Periodontitis is a progressive destructive disease affecting the tooth supporting structures. Osteoporosis can enhance the rate and severity of periodontal destruction. In osteoporosis, lowering of the estrogen level leads to imbalance between bone formation and resorption. Different drugs were utilized to manage osteoporosis. Statins are cholesterol lowering drugs. Different studies have shown that statins have an anti-resorptive effect. Furthermore, Remifemin is a herbal drug that demonstrated certain favorable effects on bone integrity. The present study was performed to compare the impact of Atorvastatin and Remifemin in osteoporotic rats with induced periodontitis.

Materials and Methods: Fifty male albino rats weighing 300 ± 50 gm were enrolled in the current study. The rat population was divided into five groups, ten rats each: control group (Group I), Group II with ligature induced periodontitis, Group III with induced periodontitis and osteoporosis, the Atorvastatin-treated group (Group IV) and finally the Remifemin-treated group (Group V). Rats were sacrificed after five weeks. Histological and histomorphometric examinations were performed.

Results: Alveolar bone in Group V exhibited more bone formation with regular bone surface compared to Group IV. Histomorphometric results revealed that both treated groups showed substantial improvement in bone volume and in osteoblast count compared to Group II and III. However better results were noted in Group V.

Conclusion: Superior results were demonstrated with Remifemin concerning bone volume. The authors suggested utilization of Remifemin as a beneficial therapeutic drug.

KEY WORDS: Atorvastatin, histological, histomorphometry, osteoporosis, periodontitis, Remifemin.

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INTRODUCTION

Osteoporosis is a multifactorial disorder occurring due to discrepancy between bone mass reduction and replacement. It is manifested by decreased bone mass and impaired bone quality leading to bone fragility which increases the susceptibility to fracture. ⁽¹⁾ Primary osteoporosis is usually correlated to either postmenopausal estrogen loss or age related deterioration of the microarchitecture, both leading to uncoupling in bone remodeling process. ⁽²⁾ Secondary osteoporosis can result from long-term intake of glucocorticoids, diabetes mellitus, gastrointestinal disorders, hyperthyroidism, liver disease and hematologic disorders. ⁽³⁾ High levels of glucocorticoids have a major effect on bone gain and maintenance. Exposure to pharmacologic doses of glucocorticoids for a long period may lead to extensive bone loss and enhanced marrow adipogenesis. ⁽⁴⁾ Glucocorticoids intake can cause secondary hyperparathyroidism. The increase in parathormone secretion stimulates bone resorption and inhibits bone formation. Impaired vitamin D metabolism is a consequence of excessive glucocorticoids which in turn decreases calcium absorption and increases its loss in urine, all of which result in loss of bone. ⁽⁵⁾ In addition, elevated glucocorticoids levels suppress osteoblasts and increase its apoptosis which reduces bone formation. On the other hand, excessive glucocorticoid levels stimulate osteoclastogenesis. Thus, the net result will be bone loss. ⁽⁶⁾

Periodontitis is a progressive destructive disease of tooth supporting structures, initiated by specific bacteria from biofilms on tooth surfaces which elicit an immune-inflammatory response in the adjacent tissue. ⁽⁷⁾ Periodontitis is aggravated by different systemic disorders as diabetes mellitus, ⁽⁸⁾ pregnancy, ⁽⁹⁾ cardiovascular disease, ⁽¹⁰⁾ rheumatoid arthritis ⁽¹¹⁾ and osteoporosis. ⁽¹²⁾ Osteoporosis can modify the progression of periodontitis. In osteoporosis, low estrogen level leads to imbalance between bone resorption and formation. Likewise; in

periodontitis, bacteria cause direct bone destruction by means of its virulence factors, and also indirect bone destruction by stimulating the release of catabolic cytokines by host immune cells. Thus, the amount of bone resorption exceeds that of bone formation resulting in bone loss. ⁽¹³⁾

Different drugs were used to manage osteoporosis either by hindrance of bone resorption or by promoting bone formation. Examples of anti-resorptive drugs are calcitonin, estrogen replacement therapy, and bisphosphonates. ^(14,15) Statins are cholesterol lowering drugs which competitively inhibit HMG-CoA reductase enzyme at an early stage in the mevalonate pathway. ⁽¹⁶⁾ They are analogous to 3-hydroxy-3-methyl glutaryl-coenzyme A which is essential for biosynthesis of sterol. HMG-CoA reductase is a crucial enzyme in the pathways of cholesterol synthesis and osteoclasts activation. ⁽¹⁷⁾ Statins offered additional cholesterol-independent effects known as pleotropic effects. Statins anti-resorptive effects were proved by various studies. ^(18,19) Statins include Fluvastatin, Lovastatin, Cerivastatin, Pravastatin, Rosuvastatin, Simvastatin and Atorvastatin. However, statins have potential adverse effects. These adverse effects comprise muscle pain and weakness. ⁽²⁰⁾ Higher doses of statins were implicated in elevation of creatine kinase and liver function tests. ⁽²¹⁾ Therefore, it is suggested to find a potent drug for management of osteoporosis with minimal side effects.

Cimicifuga (Remifemin) is a herbal drug used to improve menopausal symptoms. ^(22,23) It was basically used to alleviate menopausal symptoms as hot flushes, anxiety and depressive moods. ⁽²⁴⁾ Remifemin is a safe non cytotoxic and non-carcinogenic drug. ⁽²⁵⁾ In addition, Remifemin demonstrated certain beneficial effects regarding bone integrity in rats with osteoporosis. ⁽²⁶⁾

Therefore, this study was performed to compare the therapeutic influence of Atorvastatin and Remifemin in osteoporotic rats with induced periodontitis.

MATERIALS AND METHODS

Fifty male albino rats of age ranging from 22-26 months were enrolled in this study. The rats' body weight was 300 ± 50 gm. They were kept in polypropylene cages in the animal house of Pharos University; ten rats each, with ad libitum access to water and normal diet. The rats were kept in a room temperature ranging from 22-24°C and were exposed to alternating light and dark cycles (12:12 hours). The study protocol was accepted by the Ethics Review Board of Faculty of Dentistry, Pharos University.

The rat population was distributed into five groups, ten rats each:

- Group I (control group): normal rats
- Group II (periodontitis group): rats were anesthetized using 90 mg/kg ketamine* and 15 mg/kg xylazine** intra-peritoneal injections. Ligatures with (4/0) sterile silk suture were knotted around the cervices of right and left mandibular molars. They were knotted on the vestibular side. These ligatures acted as a nidus for plaque accumulation leading to gingival irritation and to subsequent periodontal disease. Ligatures were removed after two weeks.⁽²⁷⁾
- Group III (periodontitis + osteoporosis group): Periodontitis was induced similar to Group II. Then, the rats received dexamethasone*** intramuscularly (7 mg/kg) once per week for 5 weeks.⁽²⁸⁾
- Group IV (periodontitis + osteoporosis + Atorvastatin group): Periodontitis and osteoporosis were induced similar to group III. The rats received 20 mg/kg Atorvastatin**** orally (once a day) for 5 weeks.⁽²⁹⁾

- Group V (periodontitis + osteoporosis + Remifemin group): Periodontitis and osteoporosis were induced similar to group III. Remifemin***** (100 mg/kg) was orally administered once a day for 5 weeks.⁽³⁰⁾

Histological Examination:

By the end of the study period, all rats were sacrificed under ether anesthesia. The regions of the mandible; where teeth were ligated, were dissected and fixed in 10% neutral buffered formalin for 48 hours. After eight weeks of decalcification in a 10% EDTA solution at pH 7.8, the specimens were gradient-dehydrated and embedded in paraffin. Then 4-5 μ m serial longitudinal sections were obtained and stained with Hematoxylin and Eosin (H & E) for histological evaluation of the alveolar bone and periodontal ligament (PDL). Inflammatory cells infiltration was scored from 0 to 3; no infiltrate (0), mild infiltrate (1), moderate infiltrate (2), and dense infiltrate (3).^(31,32)

Histomorphometric analysis

The quality of the alveolar bone was assessed in photomicrographs with a magnification of x400, using ImageJ 1.48v software program. The measured histomorphometric parameters included alveolar bone volume, osteoblast cell count and osteoclast cell count. In both histological and histomorphometric analysis, the histopathologist was blinded to the group distribution.^(33,34)

Statistical analysis of the data

Data were saved on the computer and were investigated using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov, Shapiro and D'agstino

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** Chanazine, Chanelle pharmaceutical manufacturing Ltd., Loughrea, Co. Galway, Ireland.

*** Sigma-Tec Pharmaceutical Industries, October 6, Egypt.

**** Ator (Atorvastatin), Egyptian Int. Pharmaceutical Industries Co. (EPICO), Egypt.

***** Remifemin, Enzymatic Therapy, Inc., USA.

tests were utilized to validate the normality of distribution of variables; **ANOVA** was utilized for the comparison between more than two groups for normally distributed quantitative variables, then **Post Hoc test (Tukey)** was performed for pairwise comparison. For comparison between different groups for not normally distributed quantitative variables, **Kruskal Wallis test** was advocated. While for pairwise comparison, **Post Hoc test (Dunn's for multiple comparisons test)** was used.

RESULTS

Histological analysis

In control group (Group I), the alveolar bone revealed normal histological architecture. The bone was dense compact showing viable osteocytes and normal Haversian system and lined with resting osteoblasts. The PDL bundles were running in their normal direction, with no inflammatory cells (**Fig. 1**).

Sections from periodontitis group (Group II) revealed moderate osteoclastic activity in the alveolar bone and mild inflammatory cells infiltrate between PDL fibers (**Fig. 2**). Whereas, marked bone

resorption, with thinning of alveolar bone trabeculae and increased osteoclastic activity were observed in the group with periodontitis and osteoporosis (Group III). The widened bone marrow spaces and the PDL fibers were heavily infiltrated with chronic inflammatory cells (**Fig. 3**).

Atorvastatin-treated group (Group IV) revealed newly formed bone, with alternating resting and reversal lines demarcating the fusion between old and new bone. There were also an increase in the number of lining plump shaped active osteoblasts and osteocytes. However, minimal osteoclasts were still evident within the Howship lacunae, with irregular outline of the alveolar bone. The PDL fibers showed mild inflammatory cells, with some dilated blood vessels (**Fig. 4**).

Alveolar bone in Remifemin-treated group (Group V) showed more bone formation with regular bone surface. The bony trabeculae were similar to that of normal intact bone lined by resting osteoblasts with no osteoclasts. The fibers of PDL were compact with increased vascularity. Neither osteoclasts nor inflammatory cells were detected (**Fig. 5**).

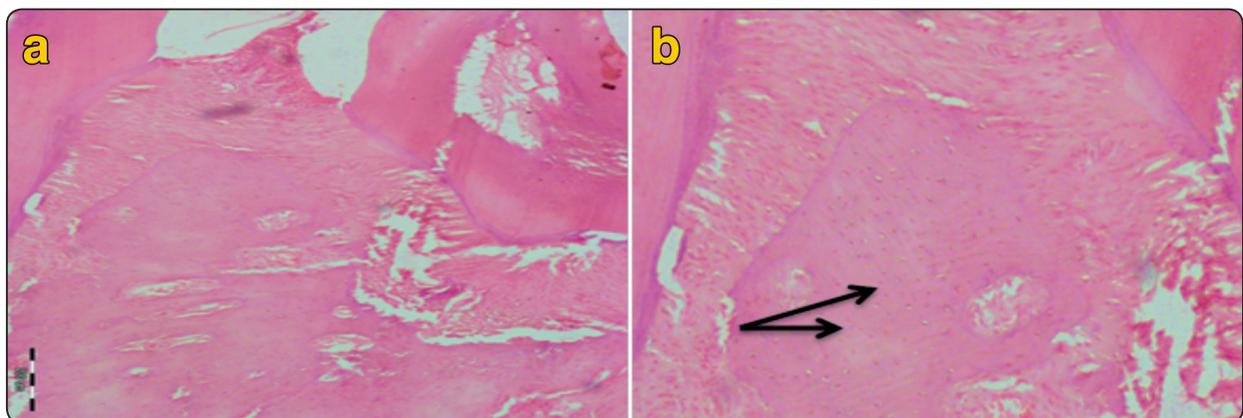


Fig. (1) A photomicrograph of control group (Group I) showing normal architecture of alveolar bone with multiple osteocytes (black arrows) and regular smooth outline. Dense PDL fibers with minimal interstitial spaces were noted. No inflammatory cells were detected. (a and b: H&E. aX100, b X400)

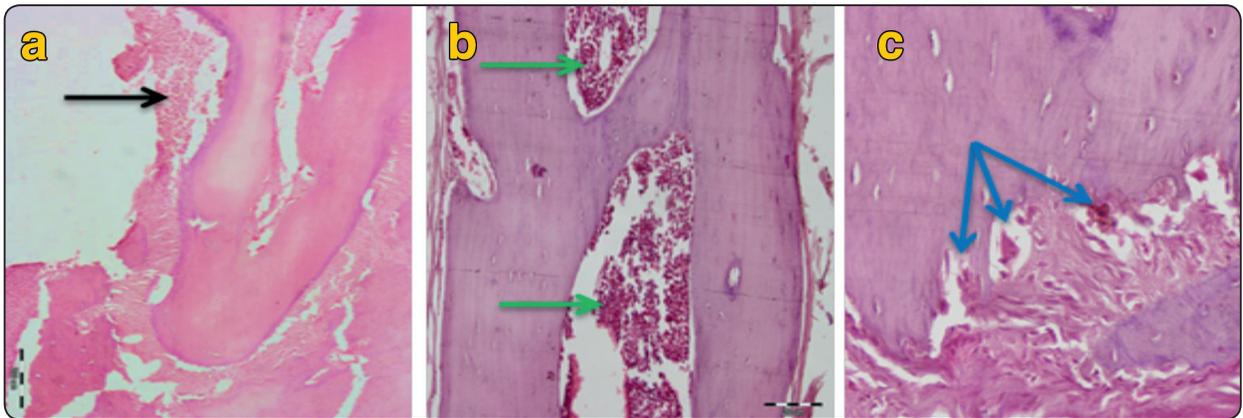


Fig. (2) A photomicrograph of periodontitis group (Group II) showing resorption of the alveolar bone with detached PDL fibers and moderate chronic inflammatory cell infiltrate in both PDL fibers (black arrow) and bone marrow (green arrows). Note osteoclasts within Howship lacunae (blue arrows). (a - c: H&E. aX100, bX200, cX400)

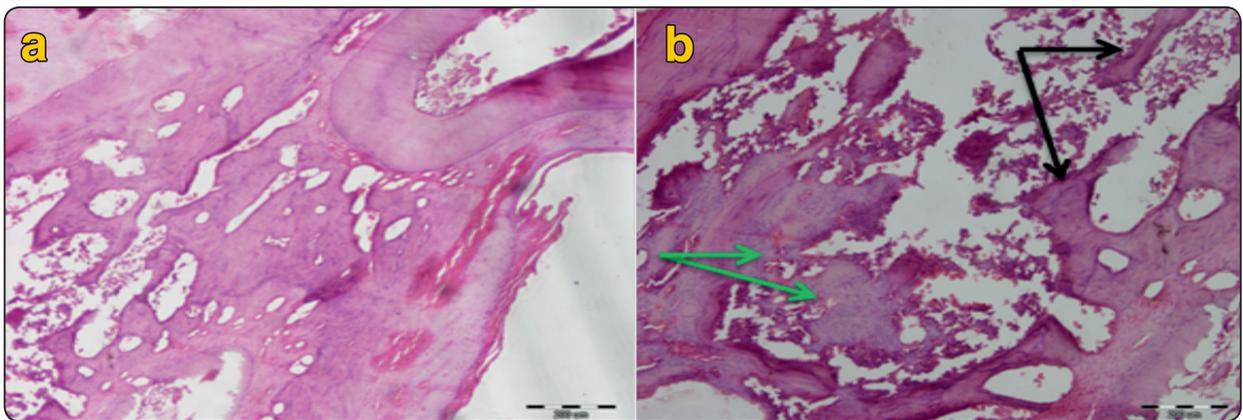


Fig. (3) A photomicrograph of periodontitis and osteoporosis group (Group III) showing massive resorption of alveolar bone with thinning of bony trabeculae (black arrows). Dense inflammatory cell infiltration (green arrows) with dilated blood vessels. (a and b: H&E. aX40, bX100)

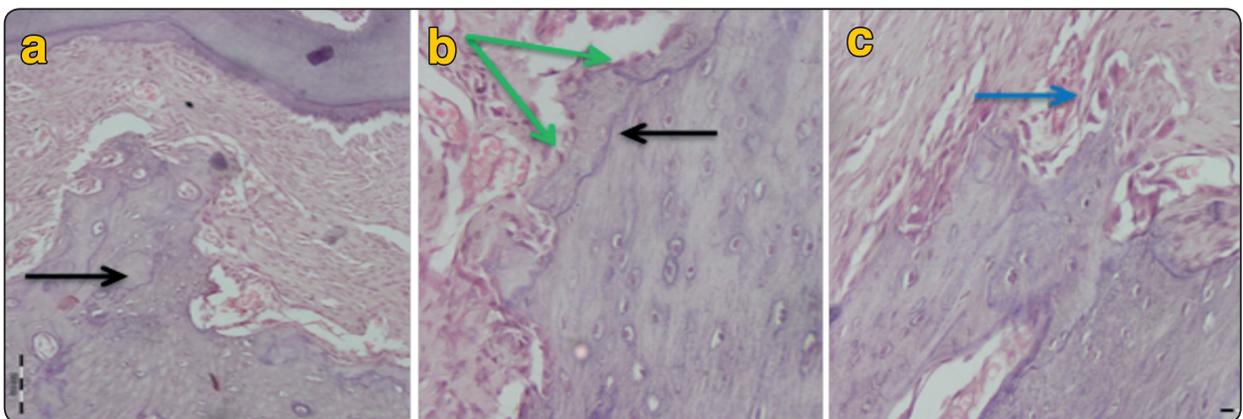


Fig. (4) A photomicrograph of Atorvastatin treated group (Group IV) showing newly formed bone with accentuated incremental lines (black arrows). Note the plump osteoblasts (green arrows) and few osteoclasts (blue arrow) within Howship lacunae. (a - c: H&E. aX200, b and c X400)

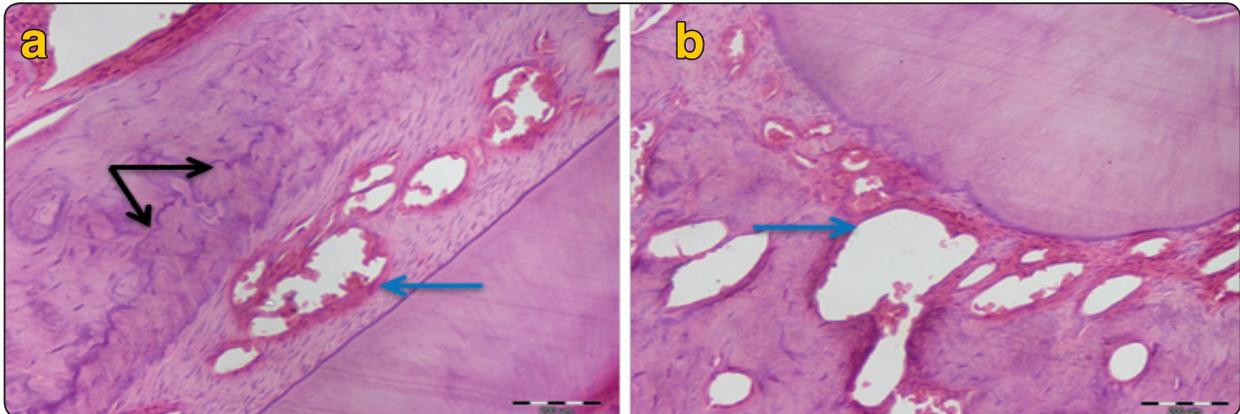


Fig. (5) A photomicrograph of Remifemin treated group (Group V) showing newly formed bone with regular outline and more accentuated incremental lines (black arrows). Dilated blood vessels (blue arrows) within the dense PDL fibers. No inflammatory cells were detected. (a and b: H&E, X400)

Histomorphometric results

The histomorphometric results supported those observed by the histological analysis. Control group (Group I) recorded the highest mean bone volume 81.4 ± 6.5 , with the highest mean osteoblast count 34.6 ± 3.2 , and lowest mean of osteoclast count 0 ± 1 .

Both the periodontitis group (Group II) and the periodontitis with osteoporosis group (Group III) showed significant reduction in the mean bone volume, 56.8 ± 10.1 and 46.5 ± 9.1 respectively ($p < 0.001$). The mean of osteoclast count recorded a significant elevation in both Group II by 1 ± 1 and in Group III by 3 ± 1 , ($p < 0.001$).

The treated groups (Group IV and V) showed a significant improvement in bone volume compared to Groups II and III. The Remifemin-treated

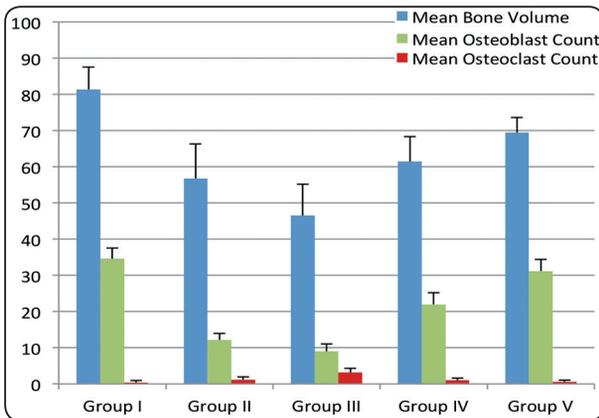
group (Group V) recorded higher rise in the mean bone volume (69.5 ± 4.4) than that recorded in Atorvastatin-treated group (Group IV) (61.5 ± 7.2). Though, no significant difference was identified between the treated groups, ($p > 0.05$). Likewise, both Groups IV and V displayed a significant rise in osteoblast count (22.0 ± 3.3 and 31.2 ± 3.4 respectively) compared to Group II and III. The osteoblast count in Group V was significantly greater than that of Group IV ($p < 0.001$). Moreover, the osteoblast count in Group V was near to that of Group I, with no significant difference, ($p > 0.05$). Additionally, the mean osteoclast count of Group IV was similar to that of Group V (1 ± 1) which was significantly less than that of Group III, ($p < 0.001$).

The results of histomorphometry were summarized and tabulated in **Table 1** and **Graph 1**.

TABLE (1): Comparison between the different studied groups according to histomorphometric results

	Group I (Control) (n = 10)	Group II (Periodontitis) (n = 10)	Group III (Periodontitis & osteoporosis) (n = 10)	Group IV (Atorvastatin- treated) (n = 10)	Group V (Remi- femin- treated) (n = 10)	Test of sig.	p
Bone volume							
Mean \pm SD.	81.4 ^a \pm 6.5	56.8 ^c \pm 10.1	46.5 ^d \pm 9.1	61.5 ^{bc} \pm 7.2	69.5 ^b \pm 4.4	F= 28.965*	<0.001*
Median (Min.–Max.)	78.3 (75.4–91.3)	55.7 (40.3–68.2)	49.6 (30.4–55.2)	65.1 (50.9–67.8)	67.1 (64.7–74.7)		
Osteoblast count							
Mean \pm SD.	34.6 ^a \pm 3.2	12.2 ^c \pm 1.8	9.0 ^c \pm 2.1	22.0 ^b \pm 3.3	31.2 ^a \pm 3.4	F= 158.12*	<0.001*
Median (Min.–Max.)	34.0 (31.0–40.0)	12.0 (10.0–15.0)	9.0 (6.0–12.0)	21.0 (18.0–27.0)	32.0 (27.0–35.0)		
Osteoclast count							
Mean \pm SD.	0 ^c \pm 1	1 ^b \pm 1	3 ^a \pm 1	1 ^{bc} \pm 1	1 ^{bc} \pm 1	H= 27.33*	<0.001*
Median (Min.–Max.)	0(0–1)	1(0–2)	2(2–4)	1(0–2)	0(0–1)		

F, *p*: *F* and *p* values for ANOVA test, Pairwise comparison between each 2 groups was done using Post Hoc Test (Tukey)
H, *p*: *H* and *p* values for Kruskal Wallis test, Pairwise comparison between each 2 groups was done using Post Hoc Test (Dunn's for multiple comparisons test) Common letters are not significant (i.e. Different letters are significant)
 *: Statistically significant at $p \leq 0.05$



Graph 1: Mean bone volume, mean osteoblast count and mean osteoclast count between different study groups

DISCUSSION

Periodontitis is an inflammatory disease which results in connective tissue damage and alveolar bone loss. Systemic disorders accelerate and aggravate the periodontal destruction. Osteoporosis potentiates the bone resorption by increasing the inflammatory factors that contribute in the progression of periodontitis.⁽³⁵⁾ In the current study,

periodontitis was induced by knotting ligatures around mandibular molars of rats in four study groups (Group II, III, IV and V). Results indicated that Group II (periodontitis group) revealed moderate osteoclastic activity. This is in accordance with Mizuno *et al.*⁽³⁶⁾ who advocated that alveolar bone loss occurs when ligature wire is placed around contact point between teeth. Osteoporosis was provoked by glucocorticoids in groups III, IV and V. In this study, Group III (periodontitis and osteoporosis) showed marked bone resorption, with thinning of alveolar bone trabeculae, increased osteoclastic activity and least osteoblast count (mean = 9 ± 2.1). This was in convenience with Hylley *et al.*⁽³⁷⁾ who demonstrated significant reduction in osteoblast count with limitation of new bone deposition in rats with Glucocorticoid-induced osteoporosis. Two different treatment modalities were examined in the present study Atorvastatin and Remifemin. The Atorvastatin-treated group (Group IV) showed newly formed bone, with alternating resting and reversal lines demarcating the fusion between old and new bone. Reversal lines indicate

the transition of bone resorption into bone formation phase.⁽³⁸⁾ Substantial elevation in bone volume was observed in Group IV (61.5 ± 7.2) compared to Groups II and III (56.8 ± 10.1 and 46.5 ± 8.6 respectively) which indicated the positive effect of Atorvastatin on bone formation. There was a significant rise in the osteoblast count (mean = 22 ± 3.3) compared to Groups II and III with a significant reduction in osteoclast count (1 ± 1) compared to that of Group III (3 ± 1) which again points to the advantageous influence of statins on bone integrity in cases with osteoporosis. These results confirm other studies that proved that Atorvastatin possesses dual action on bone, it can increase bone formation by osteoblasts and also decrease bone resorption by osteoclasts.^(39,40) Similarly, Chang *et al.*⁽⁴¹⁾ reported that Atorvastatin treatment impedes bone resorption and promotes bone formation. Esposito *et al.*⁽⁴²⁾ confirmed the valuable impact of statins in prevention of osteoporosis. This is not only attributed to the ability of statins to inhibit the mevalonic acid pathway,⁽⁴³⁾ but also due to its capability to upregulate the expression of osteoprotegerin which in turn antagonizes osteoclasts.⁽⁴⁴⁾ El-Nabarawy *et al.*⁽⁴⁵⁾ stated that lipophilic statins as Atorvastatin can significantly enhance the expression of vascular endothelial growth factor, a bone anabolic factor in osteoblasts. Furthermore, it was shown that lipophilic statins induce bone morphogenetic protein-2 (BMP-2) expression which in turn induce differentiation of undifferentiated mesenchymal cells to osteoblasts, thus it potentiates bone formation.^(46,47)

Marked bone formation was noticed in the Remifemin-treated group (Group V). The bony trabeculae were analogous to normal intact bone. The osteoblast count was the highest of all studied groups (31.2 ± 3.4) which was comparable to that of the control group (34.6 ± 3.2) showing no significant difference. Moreover, diminished osteoclast number was recognized (mean = 1 ± 1) which was significantly inferior to that of Group III. These results confirms those of Seidlova-Wuttke⁽⁴⁸⁾ who verified that Remifemin reduced bone marrow fat load and inhibited the release of pro-inflammatory cytokines, thus it can reduce osteoporosis

development. Furthermore, Cui *et al.*⁽⁴⁹⁾ concluded that Remifemin possesses preventive effect against bone-loss, improves the biomechanical features of bone and hinders bone resorption in ovariectomized rats. The ability of Remifemin to stimulate bone formation could be attributed to its ability to stimulate the osteoprotegerin production and to increase the serum osteocalcin and bone alkaline phosphatase.⁽⁵⁰⁾

Comparing both treated groups, Group V demonstrated higher level of bone volume than Group IV with significant increase in osteoblast count, which proved the superior effect of Remifemin compared to Atorvastatin concerning bone formation. Since randomized controlled trials proved the existence of statin-related side effects such as neuropathy, muscle pain and weakness, hepatic and pancreatic problems.⁽⁵¹⁾ Therefore, great interest is focused on using natural products as herbal Remifemin for the maintenance and improvement of bone health.⁽⁵²⁾

CONCLUSION

Within the highlights of the present study, it was concluded that both Atorvastatin and Remifemin enhanced bone formation and showed a protective role against osteoporosis. Superior results were demonstrated with Remifemin. Since it is a natural product, it does not possess the side effects encountered with statins. Thus, it is suggested to use Remifemin as a favorable osteoprotective drug.

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