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Full Length Research Paper

Ameliorating effect of combination of simvastatin and residronate on glucocorticoid induced osteoporosis model in rats

Ahmed Shata[1]*, Alaa El-Din L. Firgany[2] and Ahmed A.M. Abdel-Hamid[2]

[1]Department of Clinical Pharmacology, Mansoura University, P. O. Box 35516, Mansoura, Egypt.
[2]Department of Histology and Cell Biology, Mansoura University, P. O. Box 35516, Mansoura, Egypt.

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Osteoporosis is one of the major problems facing older people of both sexes. This study was undertaken to investigate the synergistic effects of combination of statins and bisphosphonates in a glucocorticoid-induced osteoporosis (GIO) model. Thirty rats were divided into five groups; control group (I), GIO group (II) (given oral prednisolone, pred 30 mg/kg, per 2 days for 6 weeks), group III (treated by pred + risedronate, risedr 1mg/kg), group IV (treated by pred + simvastatin, sim 10 mg/kg) and group V (treated by pred +sim + risedr). Histological study of the femoral neck bone as well as biochemical assessments of serum alkaline phosphatase (ALP), carboxy-terminal collagen crosslinks (CTX), osteoprotegrin (OPG), leptin, Ca and phosphate were performed. The significant decreases in the trabecular bone thickness, area ($p < 0.001$), and the significant increase in bone marrow fat cells area ($p < 0.001$) detected in pred group, were corrected to be nearly similar to control values in groups III, IV and V. Groups that received sim showed better remodeled smoother surfaces bone trabeculae compared with other groups. Combination of sim + risedr shows a significant decrease of bone specific ALP in comparison to pred group ($p < 0.05$). Also, there was significant decrease of CTX in all treated groups in comparison to pred group ($p < 0.05$). On other hand, significant improvements of osteoprotegrin levels were noted in all treated groups ($p < 0.05$), however, there were insignificant changes in leptin levels among groups. In conclusion, addition of statins to bisphosphonate has qualitative rather than quantitative positive effect in experimental osteoporosis which might shorten the duration of therapy and increase efficacy when combined together.

Key words: Statins, bisphosphonates, glucocorticoids, osteoporosis.

INTRODUCTION

Osteoporosis is a common, chronic disease, defined by decreased bone mass and altered microarchitecture,

*Corresponding author. E-mail: ahmedmhes@mans.edu.eg, Fax: +20 (50) 2223613-124.

resulting in increased bone fragility and susceptibility to fracture (Johnell and Kanis, 2006). It is a common condition, and an economic burden associated with multiple deleterious consequences, including deformity, pain, loss of ambulation, and death. Early prevention and treatment of osteoporosis is very important to avoid these complications. Current drugs for the prevention and treatment of osteoporosis include estrogen, selective estrogen receptor modulators, calcitonin, and bisphosphonates (Cramer et al., 2007), and some of these drugs produce multiple adverse side effects on different tissues (Plotkin et al., 1999).

Despite large problem in the aging society, the discovery of antiresoptive agent is very slow. Also, it was found that women who took statin drugs were less likely to experience a fracture than women who did not take statin drugs (Alexandra et al., 2007).

Despite the development of a number of guidelines for treatment of osteoporosis, management of the condition is not straightforward (Mazziotti et al., 2011; Bultink et al., 2014; Yeh et al., 2014). Several anabolic agents have been investigated in animal models of osteoporosis but it is associated with multiple drawbacks and increase in the risk of fracture may be due to abnormal bone quality (John and Seeman, 2007).

All approved osteoporosis treatments are of the antiresorptive class and it induced a significant decrease in fracture risk, but they do not decrease the risk completely (Alexandra et al., 2007). An increase in bone mineral density did not seem to account for this reduction in fracture risk, so the mechanism by which statins seems to protect against fractures remains unclear (Jadhav and Jain, 2006). So in the present study we try to evaluate role of simvastatin in conjunction of bisphosphonate in osteoporosis model induced by methylprednisolone.

MATERIALS AND METHODS

Drugs

(a). Risedronate (Actonel 5 mg) was dissolved in pathogen free normal saline to make a concentration of 10 mg/ml
(b). Simvastatin (Zocor 40 mg) was dissolved in 4 ml methylcellulose (0.5%) to make a concentration of 10 mg/ml.
(c). Prednisolone

Animals

All procedures were evaluated and approved by the Ethics committee of the University of Mansoura and the study procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals. A total of 30 female Sprague–Dawley rats weighing 250 to 275 g were used in this study. During the experiment, animals were housed under controlled environmental conditions and were maintained in plastic cages with free access to food and water and were kept at a constant temperature of 22 ± 1°C with 12 h light/dark cycle for at least 1 week before the experiment.

Animal groups

Rats that survive throughout the study were divided into five groups (each group contained 6 rats):

Group I: Received saline (0.5 ml) served as negative control group.
Group II (pred): Was given oral prednisolone (pred) at 30 mg/kg per 2 days for 6 weeks to produce GIO and serve as positive control (Fujita et al., 2011).
Group III (pred + risedr): Was given daily with pred by above mentioned dose plus receiving risedronate (risedr) orally through NG tube (1mg/kg) (Fujita et al., 2011)
Group IV (pred + sim): Was given daily with pred by above mentioned dose plus receiving simvastatin (sim) orally through NG tube (10 mg/kg) (Wang et al., 2013).
Group V (pred + sim + risedr): Was given daily with pred by above mentioned dose plus receiving sim and risedr as doses mentioned before.

All protocols were approved by our local committee of Animal Care and Use Committee.

Biochemical analysis

At the end of the study, animals were killed under general anesthesia with chloral hydrate (400 mg/kg i.p.) after blood collection by cardiac puncture. Blood was centrifuged and serum stored immediately at -20°C for analysis. Commercially available enzyme linked immunosorbent assay (ELISA) kits for b-ALP (IDS Plc), CTX (Nordic Bioscience Diagnostics, Beijing, China), OPG (IDS Ltd), leptin (Abcam's Leptin Rat ELISA (Enzyme-Linked Immunosorbent Assay) were used to evaluate sera for each animal according to the manufacturer instructions.

Determination of Ca and phosphate (Source: Barnett et al., 1973; Daily and Ertingshausen, 1972)

Histological and electron microscopic study

The hind limbs were disarticulated at the hip. The femurs were carefully dissected and cleared from adjacent muscles. The right femoral neck was processed for preparation of decalcified specimens. First, fixation was done immediately in neutral buffered formaldehyde for 2 days. After fixation, the proximal parts of the right femurs were processed for preparation of decalcified specimens using the chelating agent ethylenediaminetetraacetic acid in the form of its disodium salt (5.5 g ethylenediaminetetraacetic acid in 90 ml distilled water and 10 ml formaldehyde 37 to 40%). Decalcification was carried out for 4 weeks, during which time the decalcifying solution was changed every day (Bancroft and Gamble, 2002). The decalcified specimens were dehydrated and processed to form paraffin blocks. Serial longitudinal sections in the femoral neck, 5 µm thick, parallel to the long axis of the bones, and were prepared. Then, the sections were stained with hematoxylin and eosin stain (H&E) and periodic acid Schiff's stain (PAS) (Bancroft and cook, 1994). The left femoral neck was sectioned with a saw. Specimens were then kept in 2.5%

Table 1. Effect of treatment on Ca, phosphorous and alkaline phosphatase.

Parameter	Ca (mg/dl)	Phosphate (mg/dl)	Alkaline phosphatase (u/ml)
Control	9.63±0.24	8.16±0.22	123.67±2.11
Pred	8.78±0.2	8.55±0.19	130.17±1.4
Pred+risedr	9.42±0.25	7.85±0.2	125.17±2.29[c]
Pred+sim	9.47±0.19	7.88±0.23	127.17±1.47[c]
Pred+sim+risedr	9.73±0.15[b]	7.63±0.19[b]	119.33±1.31[b]

aP < 0.05; when compared to control, bP < 0.05 when compared to positive control; cP < 0.05; when compared to pred + sim + risedr group.

glutaraldehyde after a thorough washing in a buffer solution for a few minutes to remove any debris (Lu et al., 2014). After dehydration and the critical point dryer procedures, tissue samples were mounted on the scanning electron microscope (SEM) stubs with carbon tape and were gold-coated. The bone was examined and photographed with a JEOL JSM 6510 lv SEM (Japan) .

Morphometric study

Digital quantitative assessment of the thickness (in um), the percentage area of the trabecular bone, fat cells and heamopoetic tissue in bone marrow were measured in each histological slice by using an image analyzing software (Image-Pro Plus, version 6.0; Media Cybernetics, Bethesda, Md) (Griffith et al., 2010). The mean of all fields measured from each animal was used for statistical analysis. Histological analysis was carried out by 2 experts (each with more than 10 years experience in histological slides examination) (Griffith et al., 2010).

Statistical analysis

All data are expressed as means ± standard deviation (SD). The significance of differences were assessed by a two-way repeated-measures analysis of variance (ANOVA) followed by Tukey's multiple comparison test. For all other data, comparisons between different treatments were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. In all cases, a probability error of 0.05 was selected as the criterion for statistical significance. Graphs were drawn using GraphPad Prism (version 4.0 for Windows).

RESULTS

Effect of tested drugs on Ca, phosphate and alkaline phosphatase

Combination of sim + risedr increases significantly calcium and decrease significantly phosphate in relation to positive control group ($p < 0.05$) but not exceed physiological limits (Table 1). Also, combination of therapy exerted a significant decrease of bone specific alkaline phosphatse in comparison to positive control group ($p < 0.05$).

Effect of tested drugs on CTX, osteoprotegrin and leptin

There was a significant increase of bone resorption marker (CTX) and significant derangement of osteoprotegrin in positive control group (Table 2). There was significant decrease of CTX in all treated groups in comparison to positive control ($p < 0.05$). On other hand, significant improvements of osteoprotegrin levels were noted in all treated groups ($p < 0.05$). There were insignificant changes in leptin levels among groups (Table 2).

Light microscopic results

In control group, by H&E stain the rat femoral neck was made of cancellous bone made of thick dense bone trabeculae that separated the irregular bone marrow spaces containing hemopoetic cells rather than fatty tissue (Figure 1A). In sections stained with PAS of the control group, intense PAS positive reaction with occasional appearance of cement lines around the osteocytes lacunae were the salient features seen in the bone matrix of the trabeculae (Figure 2A and B).

In the second (pred) group, sections of femoral neck were stained with H&E severe bone resorption in the form of marked thinning and irregularity of the bone trabeculae. The bone marrow spaces were wider and showed marked increase in the fat area compared with the control group (Figure 1B and C). Moreover, the bone matrix exhibited a diffuse decrease in the intensity of PAS stain both/specially in the core of trabeculae and at the resorption bay of the endosteal surface, with complete absence of cement lines. Furthermore there was disarray

Table 2. Effect of treatment on CTX, osteoprotegrin and leptin.

Parameter	CTX (pmol/L)	Osteoprotegrin (pmol/L)	Leptin (ng/ml)
Control	80.17±1.94	45.83±1.87	1.1617± 0.039
Pred	92±2.74[a]	34.67±2.22[a]	1.1967±0 .039
Pred+risedr	79.67±1.9[b]	56±2.52[b,c]	1.1550± 0.022
Pred+sim	79.17±2.32[b]	61±2.35[b]	1.1283± 0.018
Pred+sim+risedr	77.83±2.01[b]	66.67±2.06[b]	1.1267±0 .016

aP < 0.05; when compared to control, bP < 0.05 when compared to positive control; cP < 0.05; when compared to pred + sim + risedr group.

of the endosteal surface of the bone in the form of appearance of resportion bars and irregular spicules (Figure 2C and D).

In the third (pred + risedr) group, by H&E stain, the rat femoral neck showed thicker bone trabeculae with increased bone matrix density compared with the second group. The fat areas in bone marrow spaces are relatively decreased compared with the second group (Figure 1D). The endosteal surface showed a row of activated osteoblasts surrounded by condensed bone matrix. Moreover, there were numerous dark basophilic cement lines around the lacunae of osteocytes in the thickened bone trabeculae (Figure 1E). The one matrix of these trabeculae showed a strong PAS positive reaction (Figure 2E).

In the fourth (pred + sim) group, by H&E stain the rat femoral neck there was homogenously increased bone density observed in most bone trabeculae which appeared thick and dense while few others were thinner and displayed irregular non-homogenous bone density (Figure 1F and G). The bone marrow spaces showed a significant decrease in the fatty tissue area compared with the second group (Figure 1F). Furthermore, the matrix of bone trabeculae exhibited an intense positive PAS reaction (Figure 2F).

In the fifth (pred + sim + risedr) group, by H&E stain the rat femoral neck showed the normal intact bone architecture with almost complete restoration of the bone density and appearance. The bone trabeculae appeared thick and dense together with a considerable decrease of fatty tissue area in bone marrow spaces compared to the second group (Figure 1H). The bone matrix showed a diffuse increase in intensity of PAS stain reaction in the matrix of bone trabeculae compared with the second group (Figure 2G).

Electron microscopic results

By scanning electron microscope (SEM) of the control group, bone trabeculae of the rat femoral neck appeared as thick dense bars with smooth intact surface (Figure 3A). On the other hand, by SEM of the pred per se treated group, there was marked resorption of bone trabeculae of the rat femoral neck which appeared thin, short and atrophied together with multiple erosions and pores of the bone matrix surrounding bone marrow spaces. The bone substance had a decreased density of the bone and marked irregularity of the surface of bone trabeculae (Figure 3B and C) which acquired a smoother surface in the better remodeled bone on accompanying sim either + pred or + risedr in the fourth and fifth, respectively (Figure 3E and F). In addition, by SEM of the third, fourth and fifth groups, there was a relative increase in the density of the bone substance together with a relatively thicker bone trabeculae compared with the second group (Figure 3D to F). On the other hand, the bone marrow spaces appeared relatively narrower in the third group compared with the second group (Figure 3D) and smooth intact surfaces of these trabeculae are seen extending to marrow spaces in the fifth group (Figure 3F).

Statistical analysis of the second group data showed significant decreases in the trabecular bone thickness, area ($p < 0.001$) and hematopoietic tissue area ($p < 0.05$), while it showed a significant increase in bone marrow fat cells area ($p < 0.001$) compared with control group. On the other hand, the other three groups showed significant increases in trabecular thickness area ($p < 0.001$) and hematopoietic tissue area ($p < 0.05$) as well as significant decrease in the bone marrow fat cells area ($p < 0.001$) compared with second group (Figure 4 and Table 3).

DISCUSSION

Osteoporosis is a major public health problem leading to morbidity and mortality in many individuals. Treatment for osteoporosis has generally relied on mechanisms that decrease osteoclastic bone resorption. In the study we evaluated the role statins in treatment of methylprednisolone induced osteoporosis. Effect of statin

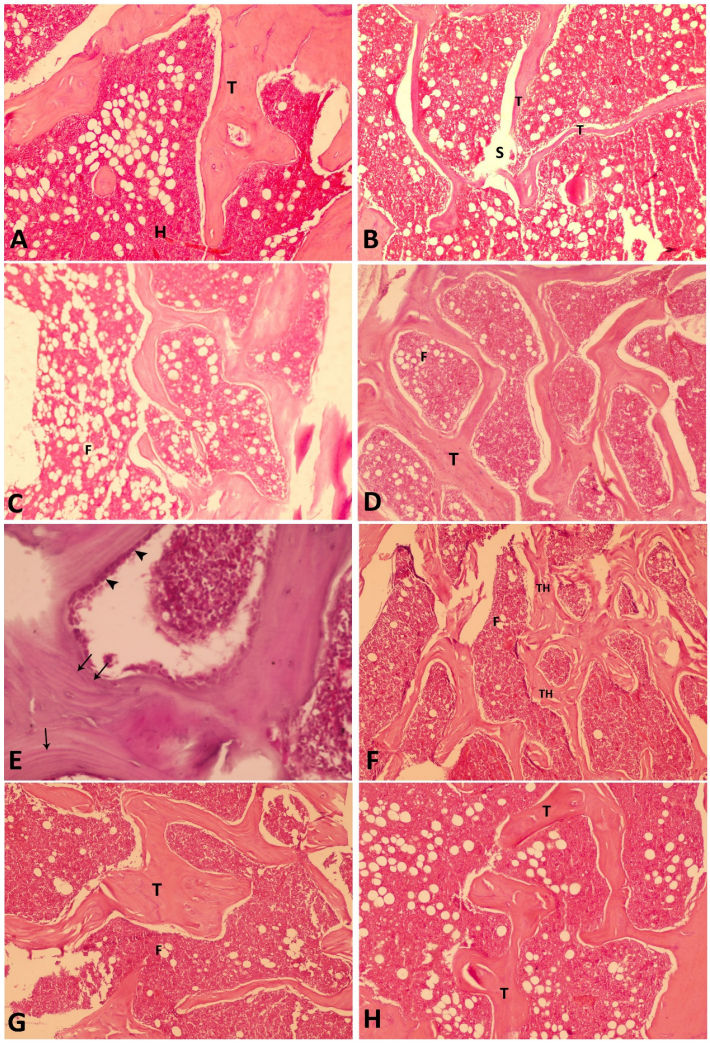

Figure 1. Photomicrographs of sections in the rat femoral neck in all groups stained with H&E. Normal cancellous bone architecture made of thick dense bone trabeculae (T) separated by bone marrow spaces distended with hemopoetic cells (H) and some fatty tissue (arrows) are seen in control group (Figure A). Severe bone resorption in the form of marked thinning of the bone trabeculae (T) with widening of the bone marrow spaces (S) containing a noticeable increased fatty tissue (F) (Figure B and C). In pred + risedr group (Figure D), thicker bone trabeculae (T) with increased bone matrix density compared with the second group is observed with a relatively decreased fatty tissue (F) in bone marrow spaces. Row of activated osteoblasts (arrow heads) at the endosteal surface surrounded by condensed bone matrix containing numerous dark basophilic cement lines (arrows) are also seen in the pred + risedr group (Figure E). Although most of bone trabeculae (T) are thick and dense homogenous in the pred + sim group, some are thinner, irregular and non-homogenous (TH) enclosing bone marrow spaces having minimal fatty tissue (arrow heads) (Figure F and G). Well-formed bone architecture with almost complete restoration of the bone density and appearance are observed in the pred + sim + risedr treated group (Figure H) which exhibits thick dense bone trabeculae (T) surrounding bone marrow spaces distended with hemopoetic cells nearly similar to that of the control group (H&E, Figure A to D, F to H ×100; Figure E ×400).

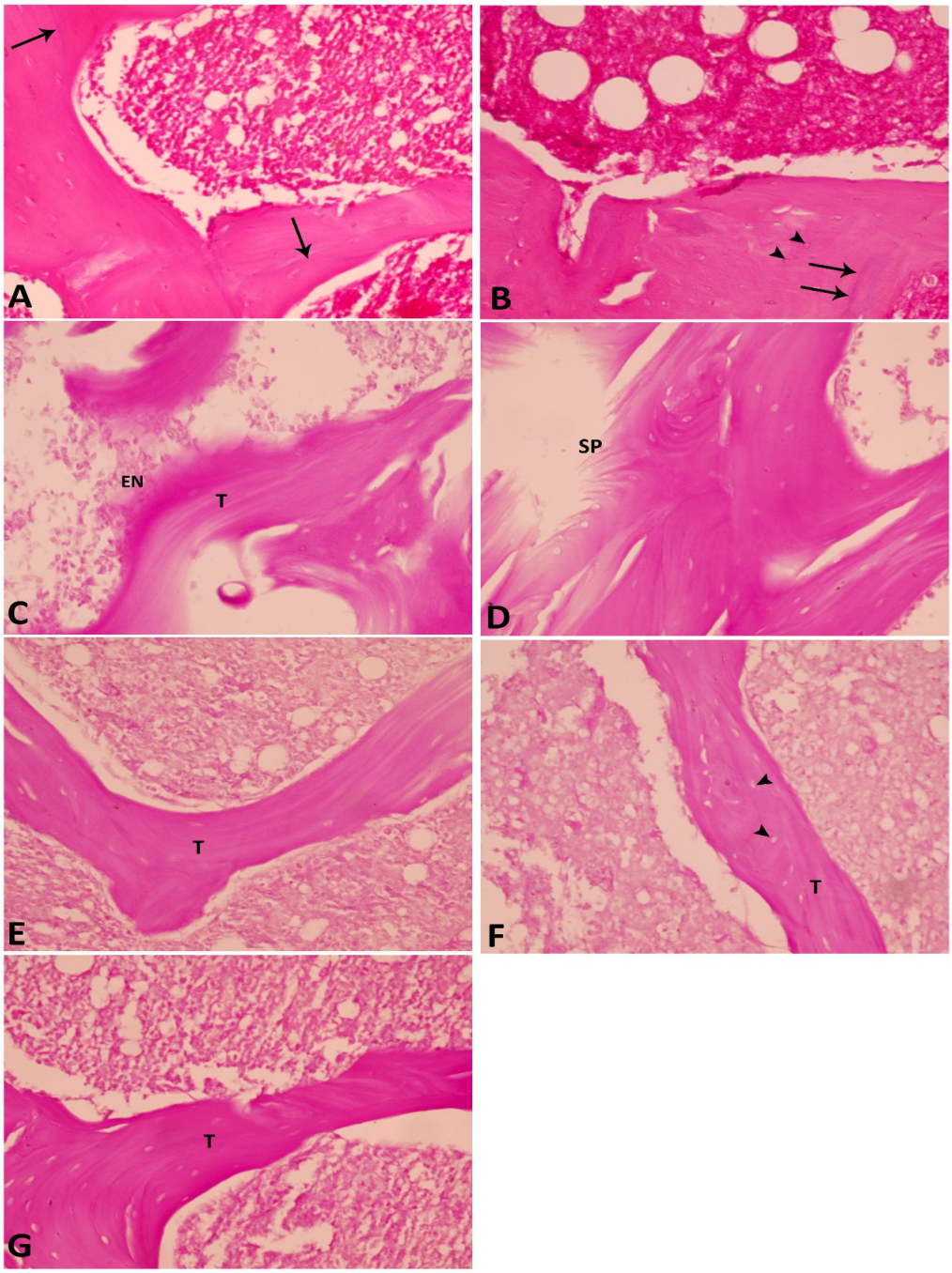

Figure 2. Photomicrographs of sections in the rat femoral neck in all groups stained with PAS. The bone matrix in the trabeculae of the control group (Figure A and B) shows an intense PAS positive reaction with occasional appearance of dark cement lines (arrows) and dispersed bone lacunae (arrow heads). On the other hand, the pred per se treated group (Figure C and D) shows a diffuse decrease in the intensity of PAS stain particularly in the core of trabeculae (T) and at the resorption bay of the endosteal surface (EN) along with appearance of resorbed bars and spicules (SP) and complete absence of cement lines. On the other hand, in pred + risedr treated group (Figure E), strong PAS positive bone matrix in the thickened bone trabeculae (T) is seen. Similarly, in the pred + sim (Figure F) and in the pred + sim + risedr (Figure G) -treated groups, the matrix of bone trabeculae (T) exhibits intense positive PAS reaction surrounding the lacunae of osteocytes (arrow heads) (PAS Figure A to G ×400)

Table 3. Mean and standard deviation of various morphometrical parameters in different groups.

Parameter	Control	Pred	Pred+risedr	Pred+sim	Pred+sim+risedr
Trabecular bone area (%)	28.9±2.8	13.6±4.3**	27.7±3.9$^{\#\#}$	25.8±4.6$^{\#\#}$	30.8±3.5$^{\#\#}$
Bone marrow fat cells area (%)	20.6±3.1	54.9±3.8**	23.6±4.1$^{\#\#}$	26.6±2.9$^{\#\#}$	21.5±2.9$^{\#\#}$
Hematopoietic tissue area (%)	50.5±4.2	31.5±4.5*	48.7±3.4$^{\#}$	47.6±3.7$^{\#}$	47.8±5.1$^{\#}$

*P<0.05 and **P<0.001 compared with control; $^{\#}$P<0.05 and $^{\#\#}$P<0.001 compared with pred per se group.

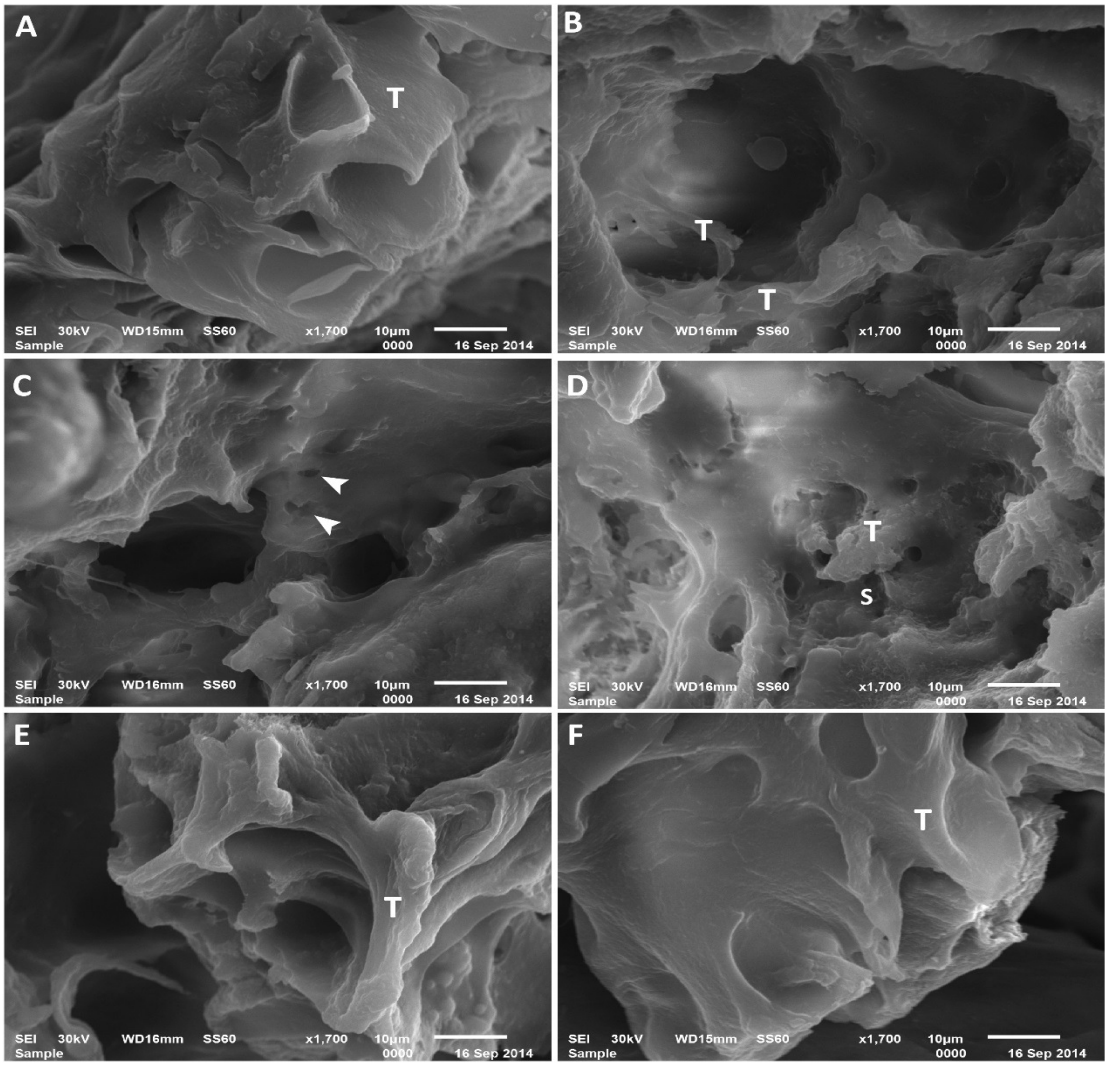

Figure 3. Electron micrographs of sections in rat femoral neck of all groups. The control group displays thick bars of bone trabeculae (T) which have a smooth and intact surface (Figure A). On the other hand, in pred per se treated group (Figure B and C), an obviously decreased density of the bone along with atrophy of its trabeculae (arrows) which appear thin, short and have irregularity in their surface and multiple erosions and pores (arrow heads) in their matrix. However, in the pred + risedr treated group (Figure D), increased density of the bone substance, relatively thicker bone trabeculae (T), but with irregular surface, and relatively narrower bone marrow spaces (S) are observed compared with the second group. Similarly, both pred + sim treated (Figure E) and pred + sim + risedr (Figure F) treated groups exhibit relatively thicker bone trabeculae (arrows), however with a smoother surface and better remodeled, and their bone substance shows a distinct increased in the density compared to the second group (SEM, A to F ×1700)

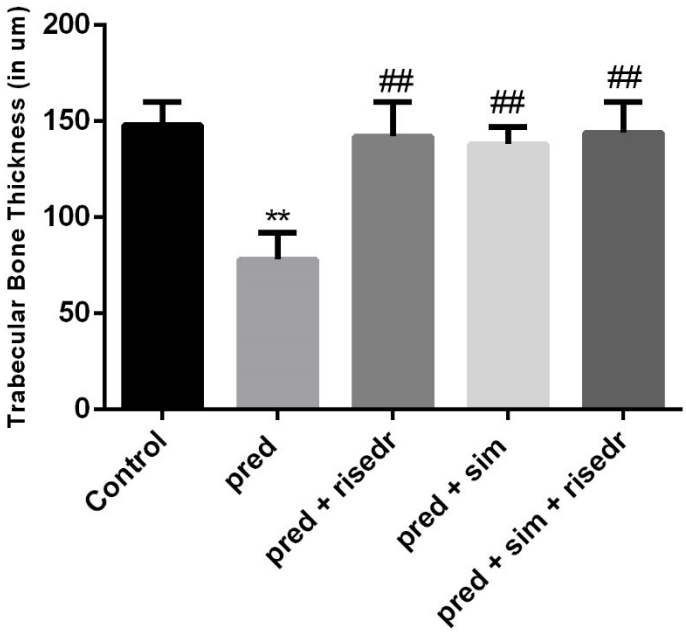

Figure 4. Histogram showing mean ± SD of the trabecular bone thickness (in um) in different groups. **p< 0.001 compared with control
##p<0.001 compared with pred per se treated group.

appear to be mediated through inhibition of bone resorption and stimulation of bone growth, this effect is synergistic when used conjointly with bisphosphonate.

Osteoporosis is a common disease in the elderly population. The progress of this disease results in the reduction of bone mass and can increase the incidence of fractures. Drugs presently used clinically can block the aggravation of this disease. However, these drugs cannot increase the bone mass and may result in certain side effects. Most of the current therapies available for its treatment are limited to the prevention or slowing down of bone loss rather than enhancing bone formation. Recent discovery of statins (HMG-CoA reductase inhibitors) as bone anabolic agents has spurred a great deal of interest among both basic and clinical bone researchers. *In vitro* and some animal studies suggest that statins increase the bone mass by enhancing bone morphogenetic protein-2 (BMP-2)-mediated osteoblast expression.

The ability of bisphosphonates, calcitonin, estrogen and related compounds, vitamin D analogues to increase bone mass is relatively small, certainly not more than 2% per year. It is desirable, therefore, to have a satisfactory and universally acceptable drug that would stimulate new bone formation and correct the disturbance of trabecular microarchitecture, which is a characteristic of established osteoporosis. Although patients using weekly bisphosphonate medication follow their prescribed dosing regimens better than those using daily therapy, overall compliance and persistence rates were suboptimal (McCombs et al., 2004). So the need to add another drug that enhance effect of bisphophosnate and shorten duration of therapy is a good idea and should be evaluated in those categories of patients.

Ruan et al. (2012) said that statins, also known as HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors, have been widely prescribed for cardiovascular disease (CVD) for decades. Nonetheless, several studies have demonstrated that statins exert bone anabolic effect and may be helpful for the treatment of osteoporosis. Several experiments have analysed the mechanisms of bone anabolism regulated by statins.

Tsartsalis et al. (2012) show statins, osteoporosis and adipogenesis share the same pathway, RANKL/OPG. It would appear that an imbalance in this pathway could be responsible for the manifestation of some metabolic disorders such as diabetes mellitus, atherogenesis, multiple myeloma, osteoporosis. Possibly in the future, drugs which can intervene in this biochemical and pathophysiological cascade, like statins, in a variety of doses, could be used for the management of ectopic ossification syndromes and other bone disorders, even as an additive treatment. Until then, further large longitudinal randomized controlled studies for each statin separately are required to confirm this hypothesis.

Although a limited number of case-control studies suggest that statins may have the potential to reduce the risk of fractures by increasing bone formation, other studies have failed to show a benefit in fracture reduction (Jain, 2006). Randomized, controlled clinical trials are needed to resolve this conflict. One possible reason for the discrepancy in the results of preclinical, as well as clinical studies is the liver-specific nature of statins. Considering their high liver specificity and low oral bioavailability, distribution of statins to the bone microenvironment in optimum concentration is questionable. To unravel their exact mechanism and confirm beneficial action on bone, statins should reach the bone microenvironment in optimum concentration. Dose optimization and use of novel controlled drug delivery systems may help in increasing the bioavailability and distribution of statins to the bone microenvironment. Discovery of bone-specific statins or their bone-targeted delivery offers great potential in the treatment of osteoporosis.

On the other hand, Rejnmark et al. (2004) discussed that sim caused no changes in BMD at the lumbar spine, total hip, femoral neck, or whole body at week 52 or 78. However, a significant increase in BMD was found in response to sim at the forearm. Within the sim group, changes in cholesterol levels did not correlate to BMD changes at any site and conclude that their results do not support a general beneficial effect of sim on bone. Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-COA) reductase. Usually, it has been known that they have efficacy on coronary artery diseases and hyperlipidemia (La Rosa and Vupputuri, 1999). Cholesterol synthetic pathway may be important in bone metabolism and that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins may increase bone formation. An experimental observation reported that statins increase bone formation in rodents and that statins have an important role for the cholesterol synthetic pathway in bone formation. This may be via potent bone-forming growth factors, the bone morphogenetic proteins (BMPs). However, other published studies have challenged the effect on fracture risk.

It was found that adipose tissue leptin and OPG expressions are related to osteoporosis in patients with COPD (Pobeha et al., 2011) but in our study no significant changes of leptin was recorded. Some epidemiological studies have suggested that statin use may be associated with increased bone mineral density (BMD) and decreased fracture risk in humans.

Alam et al. (2009) hold that statin/ACS implants show BMP-2 expression and osteoinductive activity that is similar to those of rhBMP-2/ACS implants. Mundy et al. (1999) state that lovastatin and sim increased bone formation when injected subcutaneously over the calvaria of mice and increased cancellous bone volume when orally administered to rats. Thus, in appropriate doses, statins may have therapeutic applications for the treatment of osteoporosis. Another study by Meier et al. (2000) showed that current exposure to statins is associated with a decreased risk of bone fractures in individuals' age 50 years and older. Wang et al. (2000) mentioned the association between statin use by elderly patients and reduction in the risk of hip fracture. Sugiyama et al. (2000) suggest that statins, if they are selectively targeted to bone, have beneficial effects in the treatment of osteoporosis or bone fracture. Garrett et al. (2000) stated that statins increased bone formation and bone mass in rodents, suggesting a potential new action for these compounds, which may be beneficial in patients with established osteoporosis where marked bone loss has occurred. It was found that sim abated oxidative stress, increased NO production, subsequently attenuating osteoporosis. In the *in vitro* studies, the protective effects against H_2O_2-induced cell injury were examined in the MG-63 human osteoblastic cells. It was found that sim ameliorated H_2O_2-induced cell loss and cell apoptosis and increased alkaline phosphatase (ALP) activity in osteoblastic cells (Yin et al., 2012).

In conclusion, the effect of statins on bone mineral density and fracture risk in retrospective studies suggests an exciting new direction for research in bone formation that may lead to advances in the therapy of osteoporosis. Implementation of statins with bisphosphonate may shorten duration of therapy and increase efficacy of drugs.

Conflict of Interest

The authors have not declared any conflict of interest.

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