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Evaluating the therapeutic effect of Diallyl disulfide compared to that of Alderonate on Glucocorticoids induced osteoporosis in rats: Biochemical and histomorphometric analysis.

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Keywords

- Osteoporosis;
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- RANKL

Abstract

Introduction: The prevalence of Glucocorticoids (GC) use made Glucocorticoid induced osteoporosis (GIO) an important form of secondary osteoporosis. Bisphosphonates (Alderonate) are considered as pharmacological agents in prevention and treatment of GIO. Garlic containing Diallyl disulfide (DAS) has received special attention for its beneficial effects as an antioxidant. The present study attempted to evaluate the Alendronate, DAS, and the combination of Alderonate and DAS effect on bone gene expression of RANKL and OPG, serum biochemical parameters [Ca, P, alkaline phosphatase (ALP)] and histological assessment of tibia in animal model of GIO. **Material and Method**: Fifty pathogen albino male rats were allocated in five groups:Control group(C), Methyl prednisolone group (M), methyl prednisolone Aldenronate group (A) Methyl prednisolone Diallyl disulfide (D), Diallyl disulfide Alendronate Methyl prednisolone (AD) group.Gene expression studies, biochemical parameters, histological and morphometric studies were assessed for all animals. **Results:** Among the study groups, Methyl prednisolone exposure provoked decrease in OPG (0.26±0.16) increase in RANKL (3.57±.39) gene expression, decrease in serum ALP(85.38±6.3),Ca(6.41±0.89), P(1.9 ±0.35) levels and decrease in trabecular thickness (57.01±23.22).Alderonate and Dially disulfide concomitant administration was shown to increase OPG(2.84 ±0.53)and decrease in RANKL (0.63 ± 0.27) gene expression, increase serum ALP(187.75±24.93),Ca(10.330 ±.69)and P(3.16 ±0.43) levels,increase trabecular thickness(154.7±31.7)($p<0.001$). **Conclusion:** Diallyl disulfide can add advantage to Alderonate in treatment of glucocorticoid induced osteoporosis.

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INTRODUCTION

Bone is composed of extracellular matrix which is formed of organic components and inorganic components .The organic components include collagen type I and non-collagenous proteins, on the other hand, the inorganic component includes the apatite – crystallic form Ca_3 $(PO_4)_2$. Also there are different cell types .Osteoblasts originating from mesenchymal cells in the bone marrow. They have high proteosynthetic activity and are rich in alkaline phosphatase. Bone matter formation and mineralization are their main function. Also they are responsible for maturation and activity of osteoclasts. Osteoclasts are formed from hematopoietic cells (monocyte macrophage lineage). They contain lysosomes filled proteolytic enzymes for example: collagenase, gelatinase, cathepsins and acid phosphatase [1].

Bone remodeling means formation of organized structure of bone to allow the adaptation to biomechanical forces to keep bone integrity. It helps also maintaining calcium and phosphate homeostasis [2]. Peak bone mass is reached at the age 25. Mostly, five years remodeling balance occurs which means that bone resorption is balanced by bone formation. After this period continuous loss of bone mass begins, where bone resorption predominates over bone formation, this loss is about 0,5 % per year [3].

Osteoporosis is reduction of bone mass and decrease of bone strength resulting in bone fragility and fracture. It is diagnosed by bone mineral density (BMD) at the hip and/or the spine at least 2.5 standard deviations below in comparison with the bone mass of young healthy adults as determined by dual-energy X-ray absorptiometry (DXA) .Osteoporosis does not have symptoms and evidence till fracture occurs. Early diagnosis of osteoporosis is important for effective therapy and for diagnosing patients with fracture risk [3].

Glucocorticoids (GC) are therapeutic agents used for their potent anti-inflammatory and immunosuppressive activities [4]. The prevalence of GC use made Glucocorticoid induced osteoporosis (GIO) an important form of secondary osteoporosis [5]. Rapid loss of bone integrity is present in the early phase of glucocorticoid treatment due to decreased bone formation leading to the fracture risk. However, resorption occurs later in the course of treatment resulting in a condition characterized by chronic decrease in bone turnover [6].

Calcuim, phosphates and hormones influencing bone metabolism (parathormone, calcitonin, and vitamin D) can be laboratory markers of bone metabolism. There are also specific markers of bone metabolism. Bone-specific alkaline phosphatase (ALP) which is an important marker for differentiation of osteoblast; its serum level of activity reflects the extent of calcification [7]. Added to bone specific marker, several molecules have a role in matrix formation, calcium deposition and mesenchymal to osteoblastic differentiation. These molecules include Osteoprotegerin (OPG), receptor activator of nuclear factor kappa B ligand (RANKL), osteopontin, osteocalcin, collagen type I and bone morphogenic protein-2 (BMP-2) [8].

Osteoprotegerin is a soluble cytokine belonging to Tumor Necrosis Factor receptor superfamily. It functions as inhibitor of RANKL. Osteoblast produces RANKL which causes osteoclast differentiation in bone marrow through its receptor RANK. OPG acts as a decoy receptor blocking RANKL, thus preventing RANK receptor activation, osteoclast differentiation and bone resorption. RANKL and OPG ratio is considered to be an index in bone formation/resorption. In high RANKL/OPG ratio, osteoclastosis is favored, while low ratio indicates osteoblastogenesis [5, 8].

Bisphosphonates and teriparatide are considered as pharmacological agents in prevention and treatment of GIO. However, biphosphonates are more popular as antiosteoporotic drugs. They can prevent osteoporosis at the hip and spine in patients initiating GCs, and increase BMD in patients on long-term GCs [9].

Chemically, bisphosphonates are analogues of inorganic pyrophosphate. Pyrophosphate is produced in the body by many anabolic processes. It is hydrolyzed into two constituent phosphate groups. Bisphonates as analogues are stable and resistant to hydrolysis. They bind to the hydroxyapatite crystals of bone and prevent its dissolution. By altering the structure of side chains on the carbon atom, the biological activity of the bisphosphonate can be modified. For example, bisphosphonates having nitrogen in their side chain have the most potent antiresorptive effect (such as alendronate, risedronate, ibandronate, and zoledronic acid)[9].

Beside pharmacological treatment in GIO, attention to nutrition is also essential to prevent protein and calcium intake deficiencies. Calcium and vitamin D have been used for years in GIO, but there are controversies about their effectiveness. However, any deficiency in calcium and vitamin D could be harmful to patients receiving GCs [5].Intake of diets rich in plant foods can reduce severity of many common chronic diseases such as osteoporosis. Among plant food, garlic has received special attention for its beneficial effects [10].

Garlic cutting releases allicin, the main volatile organosulfur compound. Enzymatic reactions on allicin produces allyl sulfur compounds including Diallyl disulfide (DAS) [11]. DAS is an unsaturated oil present in garlic having a strong ability to lower lipid in plasma. The antioxidant ability of garlic has been attributed to DAS. Additionally, garlic has been reported to contain phytoestrogen which also have chemopreventive actions [12].

The present study aims to compare the effect of Alendronate on osteoporosis with that of Diallyl disulfide and to study the combined effect of Alderonate and Diallyl disulfide on GIO in an animal model .This will be done by analyzing markers of bone remodeling (OPG and RANKL gene expression in tibia, serum alkaline phosphatse, serum Ca and P, histological and morphometric assessment of tibias.

Material and Methods:

Chemicals:

Methyl prednisolone Solumedrol :(methylprednisolone sodium succinate for injection)40 mg Act-O-Vial System (Single-Use Vial)—Each mL (when mixed) contains methylprednisolone sodium succinate equivalent to 40 mg methylprednisolone; also 1.6 mg monobasic sodium phosphate anhydrous; 17.46

mg dibasic sodium phosphate dried; and 25 mg lactose hydrous (Pfizer)

Diallyl disulfide: (purity 80%, the remaining 20% being diallyltrisulfide and diallyl sulfide) and allylmercaptan (AM) (purity 80%, remaining 20% composed of sulfides) (Sigma-Aldrich).

Alderonate: Alendex tablet contain 40mg of alendronic acid equivlant to contain 52.2 mg alendronate sodium trihydrate product of unipharm co, al obour city cairo Egypt diluted in saline solution.

Methods:

Fifty pathogen free adult male wistar albino rats weighing 200-230 g were included in the present study. The animals were housed and acclimatized for five days prior to the experiment and were allowed free access to standard rat water and nutrition The animals were given nutrition in the from a standard rat chow containing 0.75 %calcium, 0.6 % phosphorus, 500 IU/kg vitamin D3 (pars animals food) to minimize nutritional variability among rats and to compensate for nutritional deficiency of calcium, phosphorus, and vitamin D3 that may increase bone turnover and bone mass loss and decrease bone strength. They were kept on this diet through the study. The duration of the experiment was five weeks. All procedures followed the guidelines for care and handling of animals of the international ethics committee on animal welfare. The ethical guidelines of Alexandria University on laboratory animals and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 80-23, revised 1978) were adopted. Further, the Alexandria Faculty of Medicine Ethical Committee approval was obtained.

Fifty rats were allocated in five groups: Control group(C) ,Methyl prednisolone group (M) in which animals in this group received intramuscular injection of methyl prednisolone (0.2 mg/kg 3 times / week for the 5-week period) [13], Methyl prednisolone and Aldenronate group(A) animals received subcutaneous Alendronate 1 mg/kg / day [14] concomitantly with methyl prednisolone, Methyl prednisolone and Diallyl disulfide (D) animals received oral Diallyldisulfide 80 mg/kg body weight [15] concomitantly with methyl prednisolone and lastly animals received oral Diallyl disulfide with subcutaneous Alendronate concomitantly with methyl prednisolone were allocated in (AD) group. At the end of the study rats were anaethesized by ether and sacrificed. Blood was collected from the aorta for serum alkaline phosphatase, total calcium and phosphate. The tibias were removed and dissected from each animal, one tibia from each rat was stored in 5 ml of 10% EDTA in RNA*later* (Ambion)at -20°c for gene expression of RANKL and OPG by real-time polymerase chain reaction RT-PCR)[16]and the other tibia was fixed in formaldehyde for histological assessment.

Gene expression studies:

Total RNA was extracted from 20 mg bone tissue after lysis and homogenization, using a silicate gel technique provided by the Pure Link RNA Mini Kit (Life Technologies).The amount of extracted RNA was measured by extinction at 260 nm; the contamination with proteins was determined with the 260/280 ratio. A260/A280 greater than 1.9 was included in the study. Total

RNA was reverse-transcribed into cDNA using high capacity reverse transcriptase kit(Applied Biosystem) .To detect the mRNA of RANKL and OPG in bone samples , primers had been matched to the mRNA sequences of the target genes (NCBI Blast software).As housekeeping gene, beta actin was used [17, 18].

The PCR amplification was performed in a 25µl reaction volume including SYBR green PCR Master Mix (Applied Biosystems) using ABI 7900 sequence detector (Applied Biosystems). The reaction was performed with 10 min of initial stage to activate the DNA polymerase, followed by 40 cycles at 95°C for 15 s and 60°Cfor 1 min. Single product formation was confirmed by melting point analysis and comparative CT method was used to calculate relative gene expression with beta actin as an endogenous control. For statistical analysis of the CT values, $2^{-\Delta\Delta CT}$ method was applied for each specific primer and real-time PCR [17].

Biochemical studies:

After blood withdrawal, each sample was centrifuged at4°C for 15 min at 3000 rpm, serum was collected and storedat-20°C.Serum alkaline phosphatase(ALP) was measured using p-nitrophenylphosphate as the substrate [19].Total calcium and acid phosphate concentration in serum were determined by spectrophotometery using commercially available test kit ((BioMerieux) [20].

Histological and morphometric studies:

Tibias were fixed in 10% formaldehyde, decalcified in 10% EDTA, dehydrated, embedded in paraffin, sectioned at 3-5 µm, and stained with hematoxylin and eosin (H&E) [21].Digital images from H&E stained sections were obtained using a digital camera (Olympus DP20) connected to microscope (Olympus BX41). Images were taken at magnification of (100 X) and (400 X). The bone trabecular thickness was determined using images at magnification of (100 X). Measurements were expressed in the form of length in (µm) using NIH ImageJ (v1.49) software.

Statistical analysis:

Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed with IBM SPSS statistics, version 23.0 (IBM Inc.). The results were analyzed by one-way analysis of variance (ANOVA) followed by a post-test for multiple comparisons and *p*-value ≤ 0.05 was defined to be statistically significant.

Results

Gene expression of OPG

The present study showed that methyl prednisone decreased significantly gene expression of OPG (0.26±0.16) as compared to control rats (3.57±.39) where ($p<0.001$).Alderonate and Diallyl disulfide administration were shown to increase gene expression of OPG versus Methyl prednisone group (1.76±0.42) and (1.12 ±0.38), respectively. Rats received both Alderonate and Diallyl disulfide revealed significant difference of expression OPG (2.84±0.53) compared to controls and to other treated groups ($p <0.001$)(Fig. 1A).

Gene expression of RANKL

Administration of methyl prednisone resulted in significant increase of RANKL gene expression (3.98±0.68) when compared with control rats (0.25±0.32) where ($p <0.001$).On the contrary, Alderonate and Diallyl disulfide intake decreased RANKL gene expression in both groups (1.27± 0.34) and (2.84± 0.49) respectively as compared to Methyl prednisone rats. No significant difference was detected on comparing

group received combination therapy to control rats but significant decrease was observed when compared to M and D groups (Fig. 1B)

Serum ALP

Injection of methyl prednisolone caused significant decrease of ALP (85.38±6.3) compared to control group (208.25±12.06). Treatment with Alendronate (116.12±14.9) and Diallyl disulfide (107.75±5.75) caused significant increase of serum ALP compared to Methyl prednisolone group. Rats receiving combination therapy showed significant increase of serum ALP(187.75±24.93) as compared to other treated groups ($p < 0.001$) (Fig. 2A).

Serum Ca^{2+}

It was revealed that the group receiving methyl prednisolone showed significant decrease of serum Ca (6.41± 0.89) as compared to control group (10.23±0.67). There was also a significant increase of serum Ca in Alendronate group (9.18±.95131) and Diallyl disulfide group (8.92±1.03) compared to methyl prednisolone group. In addition, there was significant increase of serum P in Alendronate group (2.88±0.38) and Diallyl disulfide group (2.79±0.36) as compared to Methyl prednisolone group (1.9±0.35).Serum Ca. and P in animals receiving Alendronate and Diallyl disulfide(10.33±0.69) (3.16±0.43) respectively didn't show significant difference when compared to control animals.(Fig. 2B).

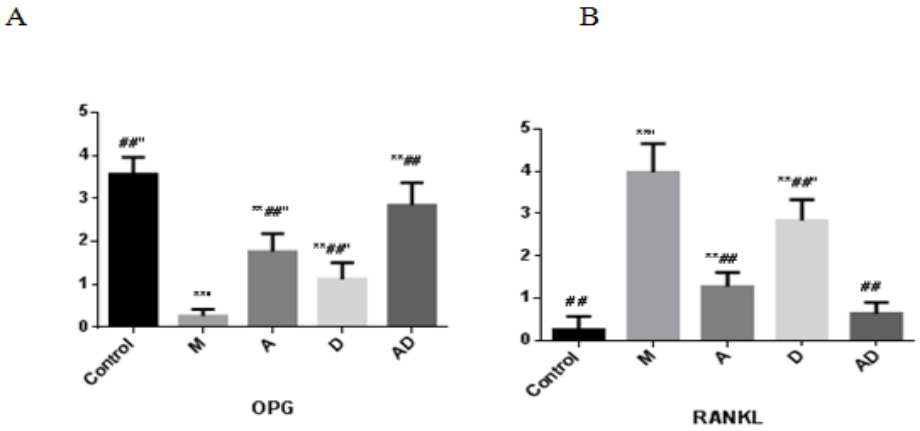

Fig 1: Gene expression studies A) Gene expression of ,OPG B) Gene expression of RANKLE. Data are presented as means±SD using one-way ANOVA test (n=8). ** P<0.001 versus control group. # P<0.05 versus M group.## P<0.001 versus M group.'P<0.05 versus AD group. ''P<0.001 versus AD group **.Abbreviations**:**M;** methyl prednisolone.A; Alendronate.D; Diallyl disulfide**.AD;** alendronate plus methyl prednisolone.OPG; Osteoprotegerin. RANKL; receptor activator of nuclear factor kappa B ligand.

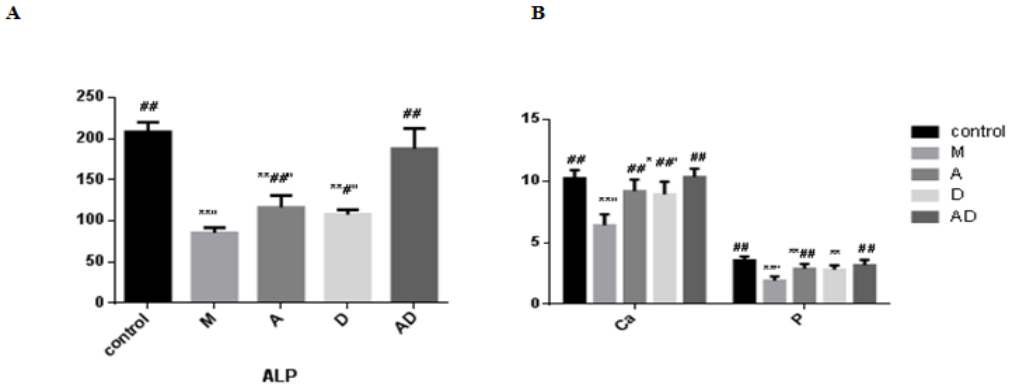

Fig 2. Serum measurements, A) Serum level of ALP, B) Serum levela of Ca and P. Data are presented as means±SD using one-way ANOVA test (n=8). ** P<0.001 versus control group. # P<0.05 versus M group.## P<0.001 versus M group.'P<0.05 versus AD group. ''P<0.001 versus AD group **.Abbreviations**:**M;** methyl prednisolone.A; Alendronate.D; Diallyl disulfide**.AD;** alendronate plus methyl prednisolone.OPG; Osteoprotegerin. RANKL; receptor activator of nuclear factor kappa B ligand.ALP; alkaline phosphatase.

Table 1 Changes in of OPG, RANKL gene expression, serum ALP, Ca, P and Trabecular thickness among the studied groups

	Control	M	A	D	AD
OPG	3.57±0.39$^{\#\#\,\prime\prime}$	0.26 ± 0.16$^{**\,\prime\prime}$	1.76 ± 0.42$^{**\#\#\,\prime\prime}$	1.12 ± 0.38$^{**\#\#\,\prime\prime}$	2.84 ± 0.53$^{**\#\#}$
RANKL	0.25±0.32$^{\#\#}$	3.98±0.68$^{**\,\prime\prime}$	1.27±0.34$^{**\#\#}$	2.84±0.49$^{**\#\#\,\prime\prime}$	0.63±0.27$^{\#\#}$
ALP	208.25±12.06$^{\#\#}$	85.38±6.3$^{**\,\prime\prime}$	116.13±14.9$^{**\#\#\,\prime\prime}$	107.75±5.75$^{**\#\,\prime\prime}$	187.75±24.93$^{\#\#}$
Ca	10.22±0.67$^{\#\#}$	6.41±0.89$^{**\,\prime\prime}$	9.18±0.95$^{\#\#}$	8.93±1.03$^{*\#\#\,\prime}$	10.33±0.69$^{\#\#}$
P	3.54±0.33$^{\#\#}$	1.9±0.35$^{**\,\prime\prime}$	2.88±0.38$^{**\#\#}$	2.79±0.36**	3.16±0.43$^{\#\#}$
Trabecular thickness	184.36±68.75$^{\#\#}$	57.01±23.22$^{*\,\prime\prime}$	122.65±11.76$^{*\#\#}$	141.74±20.66$^{\#\#}$	154.7±31.7$^{\#\#}$

Values are presented as mean ± SD. * P<0.05 versus control group**.** ** P<0.001 versus control group. $^{\#}$ P<0.05 versus M group.$^{\#\#}$ P<0.001 versus M group.**'**P<0.05 versus AD group. ''P<0.001 versus AD group **.Abbreviations**:**M;** methyl prednisolone.A; Alendronate.D; Diallyl disulfide**.AD;** alendronate plus methyl prednisolone.OPG; Osteoprotegerin. RANKL; receptor activator of nuclear factor kappa B ligand.ALP; alkaline phosphatase.

Histological and morphometric assessment of the tibias:

Light microscopic examination of the tibias of the methyl prednisolone treated group, revealed a decrease in the bone trabecular thickness and increased the intertrabecular distance when compared to the control group. In consistency morphometric analysis recorded that bone trabecular thickness in methyl prednisolone treated group (57.01±23.22) was significantly decreased when compared to the control group (184.36±68.75). In addition, decreased number of osteocytes inside lacunae and increased number of empty lacunae were noticed in bone tissue of methyl prednisolone group. (Figure 3) (Table 1).

On the other hand, light microscopic examination of tibias of the groups treated by Alendronate or Diallyl disulfide, showed increased bone trabecular thickness and decreased intertrabecular distance, when compared to methyl prednisolone treated group. Morphometric analysis showed that the increase in the bone trabecular thickness was of a significant value, when comparing group received Alendronate (122.65±11.76 um) or Diallyl disulfide (141.74±20.66) to methyl prednisolone treated group (57.01±23.22). Decreased number of empty lacunae and increased osteocytes inside lacunae were also noticed by light microscopic examination of bone tissues of these two groups compared to the methyl prednisolone group. (Figure 3,4) (Table 1).

Tibias of animals treated with both Alendronate and Diallyl disulfide showed nearly normal bone tissue, as osteocytes were revealed inside lacunae and almost no empty lacunae were recorded in most fields. Furthermore, light microscopic examination revealed that the bone trabecular thickness and intertrabecular distance became nearly normal. Morphometric analysis revealed a significant increase in the bone trabecular thickness of group received both Alendronate and Diallyl disulfide (154.7±31.7) when compared to prednisolone treated group (57.01±23.22). (Figure 3,4) (Table 1).

Comparing the bone trabecular thickness of the three treated groups; Groups A, D and AD,

to the control group, revealed that their measurements were less than that of the control group but only group A showed a significant difference.(Table 1).

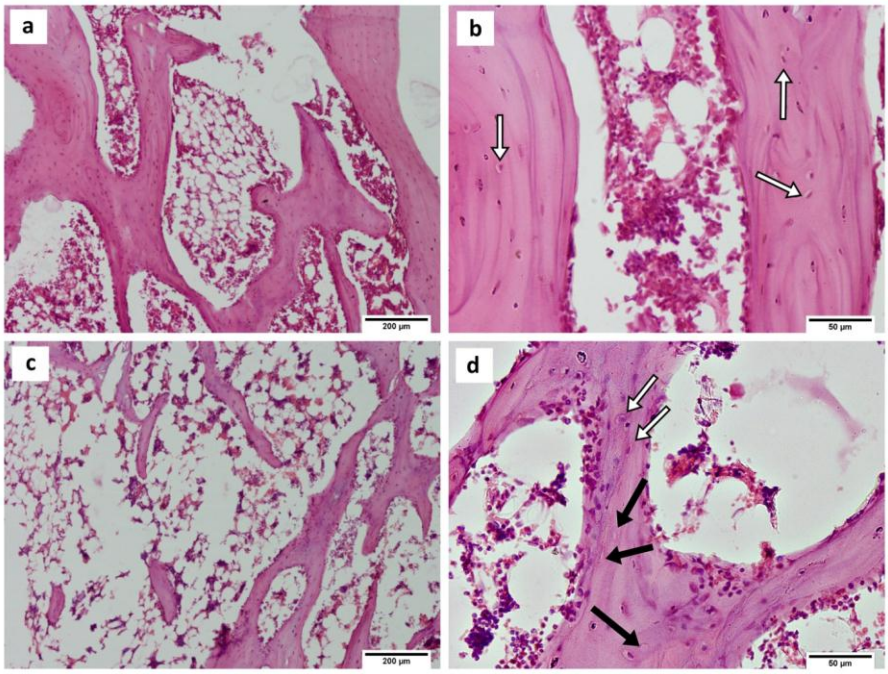

Fig 3. (a) Photomicrograph of the tibia of the control group X100 magnification, showing normal bone tissue with normal trabecular thickness and normal intertrabecular distance. (b) Photomicrograph of the tibia of the control group X400 magnification, showing osteocytes inside lacunae (white arrow). (c) Photomicrograph of the tibia of M group X100 magnification, showing thin bone trabeculae and increase intertrabecular distance. (d) Photomicrograph of the tibia of M group X400 magnification, showing few osteocytes inside lacunae (white arrow) and empty lacunae (black arrow). (H&E stain).

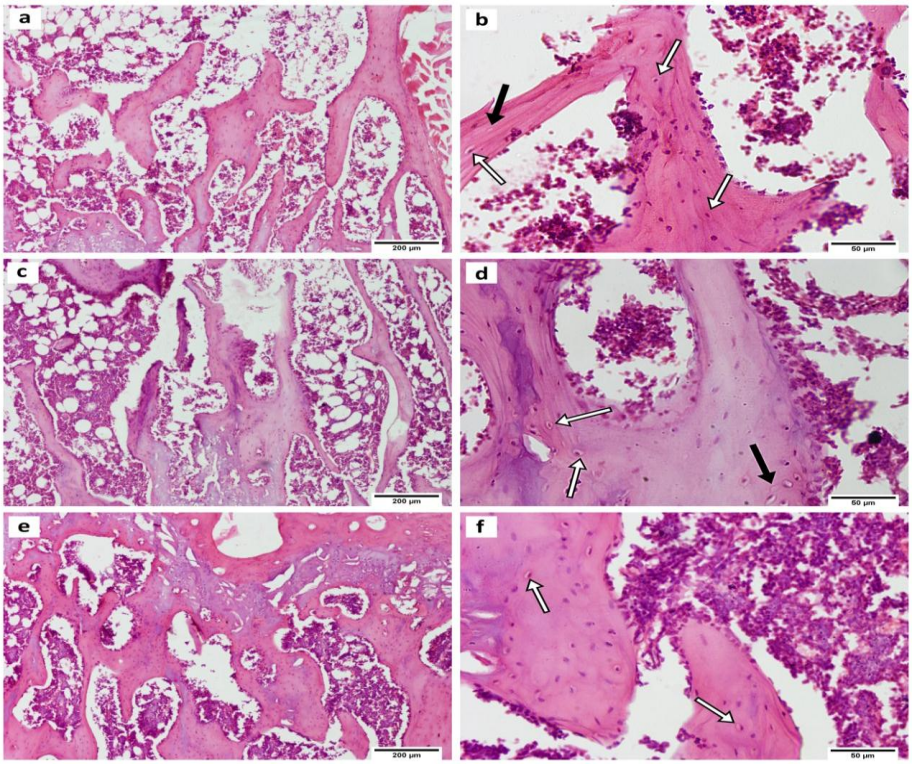

Fig 4. (a,c) Photomicrographs of the tibia of (A & D) groups X100 magnification showing increased thickness of the bone trabeculae and decreased intertrabecular distance. (b,d) Photomicrographs of the tibia of (A & D) groups X400 magnification, showing few empty lacunae (black arrow) and osteocytes inside lacunae (white arrow). (e) Photomicrograph of the tibia of AD group X100 magnification, showing mostly normal bone tissue with normal trabecular thickness and normal intertrabecular distance. (d) Photomicrograph of the tibia of AD group X400 magnification, showing osteocytes inside lacunae (white arrow) and no empty lacunae. (H&E stain).

Discussion

The bone is under constant remodelling, with repeated cycles of bone resorption by osteoclasts followed by deposition of new bone by osteoblasts. GCs induced-osteoporosis (GIO) occurs as a result of an immediate and persistent decrease in bone formation and a rapid and transient increase of bone resorption [22].

The present study compared the efficacy of Alendronate in treating osteoporosis with that of Diallyl disulfide in an animal model of GIO by analysing markers of bone remodelling (gene expression of RANKL and osteoprotegerin in tibia, serum alkaline phosphatse, serum Ca and P, histological and morphometric assessment of tibias.

Our results showed that glucocorticoid administration reduced gene expression of osteoprotegerin (OPG) and increased receptor activator of NF-κB ligand (RANKL). The main action of GC is on osteoblasts, decreasing their replication and impairing differentiation and maturation, leading to decreased bone formation [23]. Also GC decrease osteocytes function and increase their apoptosis resulting in impairment of their ability to detect and repair bone microdamage, the increase of osteoblast and osteocyte apoptosis is associated with caspase3 activation [24].

Glucocorticoids stimulate osteoclasts proliferation by suppressing synthesis of OPG, an inhibitor of osteoclast differentiation and by stimulating production of RANKL, in cells of the osteoblast lineage, including osteoblasts and osteocyte. They act as a key regulator of osteoclast recruitment, activation and survival in the presence of colony-stimulating factor-1 (CSF-1) [25].

These findings are in agreement with Chmielnicka et al. who found stimulation of osteoclast proliferation in patients treated with corticosteroids [26]. Others observed that serum OPG concentrations are significantly decreased in patients treated with systemic GCs. This decrease in OPG is more marked than the GCs-induced increase in RANKL, leading to an increased RANKL/OPG ratio that may favour GIO [27, 28].

Glucocorticoids administrated group showed significant decrease of serum ALP as GCs inhibit alkaline phosphatase activity and synthesis of type I collagen and bone proteins, such as osteocalcin, which are biomarkers of osteoblast activity [29]

Glucocorticoid induced osteoporosis group demonstrated significantly decreased of serum Ca and P as compared to control group. This can be explained as, GCs inhibit calcium resorption at the renal tubule and calcium absorption in the bowel through a vitamin D-independent mechanism, they decrease synthesis of calcium binding protein and deplete mitochondrial ATP (inhibiting calcium release by mitochondria) [30]. GCs acting directly on the kidney and indirectly, via induction of secondary hyperparathyroidism, lower tubular reabsorption of phosphate and lead to phosphaturia. In line with this finding, a previous study showed that GCs increase the amiloride-sensitive Na+/H+ exchange activity in the renal proximal tubule brush border vesicles and decrease the Na+ gradient-dependent phosphate uptake;

increased acid secretion and phosphaturia follow [31].

In Glucocorticoid induced osteoporosis group, tibias showed thin bone trabeculae and increase intratrabecular distance. Decreased number of osteocytes inside lacunae and increased number of empty lacunae were also noticed in bone tissues, indicating osteocytes death. Osteocytes are present inside lacunae, when they die, the lacunar space is maintained leaving a "shallow grave" [32]. This is supported by bone loss induced by GCs. In line with Bouvard et al, it was shown that GCs decreased bone formation of tibias with a reduced mineral deposition rate, bone formation rate and a significant decrease in trabecular bone volume and trabecular thickness [33].

Bisphosphonates such as Alendronate are effective in the treatment and prevention of GIO. In our study, levels of bone markers suggested stimulation of bone formation in the Alendronate treated group as there is increased level of OPG and decreased RANKL gene expression as compared to control group. Alendronate group also showed increased serum level of alkaline phosphatase, Ca and P as compared to GIO group.

Bisphosphonates (including Alendronate), work by inhibiting the osteoclast enzyme farnesyl pyrophosphate synthase (FPPS) which is important for osteoclast function[34].The benefits of bisphosphonates in GIO have been ascribed primarily to their antiresorptive effect and secondarily to possible inhibition of osteoblasts apoptosis [35].

Many studies using a variety of experimental models of osteoprosis demonstrated that bisphosphonates are able to inhibit osteoclast-mediated bone resorption and they stimulate bone formation [36, 37]. Bitto *et al*. showed that Alendronate treatment improved GIO in rats as it increased bone strength, bone mineral density and ameliorated histological damage of both cortical and trabecular bone matrix in femur head [38]. In the present study, histological and morphometric assessment of the tibias of Alendronate group showed, increased bone trabeculae thickness and decreased intratrabecular distance, with a decrease in the number of empty lacunae and increased osteocytes inside lacunae, denoting increase bone formation and decreased osteocytes death. These histological findings were in line with Ali et al [39]. Although, this protection didn't reach the aimed expectations, as morphometric analysis revealed that the increase in bone trabecular thickness is still less than that of the control with a significance difference.

Our result suggested also, that Diallyl disulfide (DAS) may have protective effects against GIO. The use of DAS in treatment of osteoporosis showed improvement of bone markers as increased of OPG and decreased RANKL bone gene expression, increased serum level of ALP as compared to GIO group indicated increase bone formation and decrease absorption by inhibiting osteoclats activity by DAS. These findings are supported by significant increase of serum level of Ca and P as compared to GIO group. Histological and morphometric assessment of tibias showed increased bone formation and decreased osteocytes death when compared to GIO group. Comparing the bone trabecular thickness of

this group to the control group showed that Diallyl disulfide group is near to the normal measurements, as no significant difference was detected. This is in accordance with previous experimental findings that demonstrated positive effects of garlic on bone, e.g. increased BMD in ovariectomized rats either by improved calcium uptake, estrogenic functions, inhibiting osteoclasts [40-42] or by reducing oxidative stress [43].

Diallyl disulfide blocks the formation of free radicals with significant upregulation in activity and transcription of several antioxidant enzymes including Glutathione-S-transferase (GST) activity thereby restoring the redox homeostasis. So the garlic components are not only suitable for acute therapy but also for long term prophylaxis. In addition, Ehnert et al, found that DAS can protect osteoblasts from oxidative damage induced by free radicles and might improve bone matter and stability [44].

It is interesting to note that significant increase of bone gene expression level of OPG, decrease RANKL and increase serum level of ALP concentrations were seen in rats treated with combined therapy DAS with Alendronate in comparison with that treated with Alendronate only. These results indicate that adding DAS to Alendronate in GIO-rats may work in a synergistic manner with Alendronate in reducing bone loss in GIO-rats. It is also indicated by augmented serum calcium and phosphate along with enhanced histological structure of the tibias, as the bone tissues appeared almost normal and bone trabecular thickness is more or less equivalent to the control group. These findings may be explained by working on two different mechanisms namely, antiresorptive and antioxidant mechanisms. The first directed towards osteoblasts and the latter towards osteoclasts. Two key cell types which are responsible for the whole process of bone remodeling [35, 44].

Conclusion:

We have demonstrated that co-treatment of Alendronate and DAS through duration of 5 weeks have significantly improved the biochemical bone indices and restored microarchitecture of tibias of the GIO rats as compared to Alendronate and DAS single treatment groups. The combined use of Alendronate and DAS may be a new treatment strategy for preventing bone loss and reversing bone.

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