The Possible Protective Effect of MethylSulphonylMethane on Experimentally-Induced Osteoporosis in Adult Male Albino Rat

Original Article

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ABSTRACT

Background: Osteoporosis (OP) is the most common metabolic bone disorder which can increase risk of fracture. Glucocorticoid (GC) is the most common cause of secondary osteoporosis. Methyl sulfonyl methane (MSM) is an organosulfur molecule that is used as a precursor for synthesis of many amino acids and maintaining normal connective tissues.

Aim of the Work: The current work aimed to explore the possible protective potency of MSM on glucocorticoid induced osteoporosis in adult male albino rat model.

Materials and Methods: Thirty adult male albino rats were divided into three groups; control, glucocorticoid treated (7 mg/kg, once/week dexamethasone I.M injection) and MSM treated groups (400 mg/kg/day, orally). The blood samples were subjected to biochemical tests (calcium, phosphorus and alkaline phosphatase levels). Sections from rat tibia were examined histologically, immunohistochemically for the expression of osteopontin (OPN), Receptor activator of nuclear factor- kappa B ligand (RANKL), and d-Transferase-Biotin-dUTP nick end labeling (TUNEL) and by scanning electron microscope .

Results: Glucocorticoid caused significant reduction in both serum calcium of and phosphorus levels with significant elevation in the level of alkaline phosphatase, compared to the control group. In addition, it produced marked increase in thickness of periosteum, and marked thinning of compact bone of the diaphysis with many fissures, degenerated osteoblasts and osteoporotic cavities. Significant upregulation of (RANKL), (TUNEL),and (OPN), markers for bone resorption, apoptosis and bone formation respectively were noticed. Co-administration of MSM improved osteoporosis in glucocorticoid induced rat model.

Conclusion: Glucocorticoid induced osteoporosis in adult male rats. Marked improvement was achieved by co-administration of MSM.

Key Words: Apoptosis, glucocorticoid, MSM, osteoporosis.

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INTRODUCTION

Osteoporosis (OP) is a progressive bone disease characterized by a decline in bone mass and density with consequent increase in fracture risk. The bone mineral density (BMD) is declined accompanied by deterioration of bone microarchitecture and bone proteins are altered in amount and variety[1]. Early stages of bone loss are asymptomatic. But once bones have been weakened by osteoporosis, Kyphosis is common, loss of height over time and bone fracture occur much more easily than expected[2]. Worldwide, more than 200 million individuals have osteoporosis with risk of fracture as high as 40% in osteoporotic patients[3]. According to the recent International Osteoporosis Foundation (IOF) report, 53.9% of postmenopausal women have osteopaenia and 28.4% have osteoporosis. On the other side, 26% of men have osteopaenia and 21.9% have osteoporosis[4].

Secondary osteoporosis occurs when an underlying disease or drug causes osteoporosis. Glucocorticoid (GC) is the most common cause of both secondary and iatrogenic osteoporosis. Previous and current exposure to glucocorticoids inhances the risk of bone loss and fracture[5]. Glucocorticoid is widely used in inflammatory rheumatic disorders (as polymyalgia rheumatica & rheumatoid arthritis) and lung disorders (as chronic obstructive lung diseases asthma &)[6]. High concentrations GCs of

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dramatically decline the rate of bone formation, number, activity of osteocyte and osteoblast[7]. Moreover, GCs are also associated with a decline in osteocyte viability and osteoblast function, including changes in matrix around the osteocyte lacunae[8].

Methylsulfonylmethane (MSM) is an organosulfur molecule naturally occurring in human body and many foods. Eating these foods unprocessed is the best way to consume optimal amounts of this natural compound. MSM is classified by FDA as GRAS (generally recognized as safe) that is not toxic to humans[9]. It is used as a precursor for the synthesis of many amino acids. It maintains normal connective tissues in the body because of its sulfur content;. MSM has been shown to exert anti-inflammatory, antioxidant, chemo preventive, anti-atherosclerotic and free radical scavenging effects[10]. Several reports presume in vitro the evidence of the antiapoptotic and antimicrobial activities of MSM[11]. MSM encourages mesenchymal stem cells differentiation into osteoblasts. Also, the effect of MSM on osteogenesis was identified in vivo. MSM promotes osteogenic differentiation and bone formation of alveolar bone[12]. Therefore, the current study was conducted to study the effect of methylsulphonylmethane on osteoporosis that was induced experimentally by glucocorticoids in adult male albino rat model.

MATERIALS AND METHODS

Drugs and chemicals

Dexamethasone Sodium Phosphate, a product of Amriya pharmaceutical company, was obtained from El-Ezaby pharmacy, Shebin El-Kom, Menoufia, Egypt, in the form of 8 mg/2ml ampoule Solution for injection.

Methyl Sulphonyl Methane, a product of Eva pharmaceutical company, was obtained from El-Ezaby pharmacy, Shebin El-Kom, Menoufia, Egypt, in the form of tablets, each contains 1000 mg Methyl Sulphonyl Methane.

Animals:

This study was carried out on thirty adult male albino rats, of Sprague Dawley strain. They were purchased from El helw animal house, Tanta, Egypt, with average weight of 180-200 g. Animals were housed in in clean properly ventilated metallic cages in the animal house of the Faculty of Medicine, Menoufia University, standard laboratory temperature (25±2ºC), illumination (12 h light/dark) and humidity of approximately 50%, all over the experiment. The animals were supplied with free access to food and water.

ETHICAL CONSIDERATION

This experiment was done according to the Animal Care and Ethical Committee Guidelines of the Faculty of Medicine, Menoufia University, Egypt, 82021 ANAT3and according to the international regulations for use of laboratory animals.

Experimental design:

After one week adaptation period, rats were randomly divided into3 groups each one 10 rats

Group I: 10 adult male albino rats were kept without any treatment all over the experimental periods as control group for all experimental groups.

Group II (Experimentally-induced Osteoporosis group) (GC group): This group included 10 rats. Each rat received intramuscular injection of dexamethasone (7 mg/kg, once/week; about 1.4 mg/ rat or about 0.35 ml of dexamethasone ampoule) for 4 weeks to induce Osteoporosis[13] then kept without treatment for another four weeks.

Group III (Glucocorticoid group treated with MSM): This group included 10 rats. Each rat received intramuscular injection of dexamethasone (7 mg/kg, once/ week) and at the same time methylsulphonylmethane (400 mg/kg/day) dissolved in 0.2 ml normal saline for 4 weeks orally by gavage[14] then continued MSM only for another 4 weeks.

Tissue preparation:

By the end of the experiment (8 weeks), the body weight of the rats were registered, and the blood samples were assembled in tubes from the retro-orbital venous plexus for the biochemical evaluation. Rats were anaesthetized by diethyl ether inhalation and then sacrificed. One of the tibiae of each rat was collected for the histological and immunohistochemical study and the other tibia was fixed in 1% glutaraldehyde in phosphate buffer and processed for scanning electron microscope (SEM).

Evaluation methods:

Body weight measurement:

Body weight measurements were recorded for all rats every week over the experimental period.

Biochemical analysis:

The blood samples were gathered from each rat in non-heparinized capillary tubes and serum was separated by centrifugation at 4000 rpm for 15 minutes for the biochemical assay. The serum was stored at −20°C before analysis using a Synchron cx5 autoanalyzer (Beckman, CA, USA). Concentrations of serum calcium[15], phosphorus[16] and alkaline phosphatase[17] were measured spectrophotometrically using specific diagnostic reagent kits from Olympus Diagnostica (Clare, Ireland) and an Olympus AU 2700 analyzer (Mishima, Japan) at menoufia clinical pathology laboratory[18].

Histological examination:

From each rat in each group, the collected tibiae were fixed for 4 days in 10% buffered formal saline, decalcified for 10 days by (Ethyline- Diamline Tetra-acetic acid EDTA) (***Shriram et al.***, 2010)[19], double embedded in methyl benzoate celliodine to prepare paraffin blocks. Longitudinal sections of 5 um thickness from the upper end and shaft of the tibiae were done and processed for histological, immunohistochemical studies, morphometrical assessment and statistical analysis. Sections were stained by hematoxylin and eosin (H&E) for histological examination[20] and Alzarine red stain for detection of calcium in bone[21].

Immunohistochemical examination:

The 5-µm tibial paraffin sections were deparaffinized then rehydrated in descending grades of alcohol. Endogenous peroxidase was blocked by inserting the sections in 3% hydrogen peroxide (H2O2). A protein blocker was used to block nonspecific binding sites then the primary antibody anti-OPN (rabbit polyclonal, 1:100, Sigma-Aldrich, Cairo, Egypt), anti-RANKL (Rabbit polyclonal, 1:200, Sigma-Aldrich, Cairo, Egypt) and Monoclonal mouse Transferase-Biotin-dUTP nick end labeling (TUNEL) antibody (Rabbit polyclonal, 1:200, Sigma-Aldrich, Cairo, Egypt) were added with overnight incubation. After that, biotinylated goat-polyvalent secondary antibody at a concentration of 2% (Vector, Peterborough, UK) was applied for 10 minutes (37°C) then the avidin-biotin-peroxidase complex (Vector) was added[22].

Scanning Electron Microscopic (SEM) examination:

The other tibia from each rat was immediately dissected longitudinally, irrigated with saline to remove the bone marrow then dipped in 1% Triton-X-100 at room temperature for 20 min in an ultrasonic cleaner (Bandelin Sonorex RK156, Berlin, Germany) to bring out all soft tissues. Specimens were fixed by 2.5% glutaraldehyde solution, washed with 0.1 M Sorensen's phosphate buffer for two hours at 4°C, and post-fixed with 1% osmium tetraoxide. the specimens were dehydrated by using ascending alcohol gradients, dried and coated with gold using sputter-coated SCD/005, Germany (***Shah et al.,*** 2019)[23]. The tissue was put on a copper stub and was examined by a scanning electron microscope (SEM) philips XL30, (Holland) in the EM unit, college of Medicine, Tanta University.

Morphometric analysis:

The thickness of the outer fibrous layer of the periosteum in H&E stained sections, the compact bone thickness in H&E stained sections was estimated by drawing a line from endosteum to just beneath the periosteum, the number of osteogenic cells, osteoblasts and osteoclasts, the area percent and color intensity of Ca staining in Alzarine red stained sections, the number of Osteopontin, RANKL and TUNEL positive cells in immunostained sections and the trabecular thickness in scanning electron microscopic photographs in different groups were measured using Image J software version K 1.45.

For each parameter, five non-overlapping fields (400x) were selected from each specimen randomly using Leica DML B2/11888111 microscope contained Leica DFC450 camera. The data was subjected to statistical analyses. This was done in Anatomy and Embryology Department, Faculty of Medicine, Menoufia University.

Statistical analysis:

The data were expressed as the mean ± SD. The data was analyzed using SPSS (Statistical Package for Social Science) (Inc., Chicago, IL, USA) version 23. One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used to evaluate the significance of

differences between groups. The significance of the results was expressed by the *P value* (probability of chance): The results were considered statistically significant and nonsignificant when the *p-values* were <0.05 and <0.05 respectively.

RESULTS

Body weight results:

The body weight of the GC group was significantly decreased ($p < 0.001$) (47.42±1.94 vs. 109.24±0.52) compared to the control one. On the contrary, the weight of GC group treated with MSM revealed a significant increase ($p < 0.001$) in its (93.62±1.79 vs. 47.42±1.94) when compared to the GC group (Figure 1a).

Biochemical results

Serum calcium level:

The serum calcium level of the GC group decreased significantly ($p < 0.001$) (8.22±0.53 vs. 10.63±0.43) Compared to the control group. However, The serum calcium level was significantly increased ($p < 0.001$) in the GC group treated with MSM Compared to the GC group, (9.92±0.39 vs. 8.22±0.53) (Figure 1 b).

Serum phosphorus level:

A significant decrease in the serum phosphorus level ($p < 0.001$) was observed in the GC group (2.82±0.43 vs. 4.9±0.63) compared to the control group, while the GC group treated with MSM showed a significant increase ($p < 0.001$) in the level of serum phosphorus (4.23±0.52 vs. 2.82±0.43) compared to the untreated GC group (Figure 1 c).

Serum alkaline phosphatase level:

The level of serum alkaline phosphatase exhibited a significant increase ($p < 0.001$) in the GC group compared to the control one (207.67±14.61 vs. 128.33±13.50), however, the GC group treated with MSM showed a significant decline ($p < 0.001$) in the level of serum alkaline phosphatase (145.00±14.99 vs. 207.67±14.61) compared with the untreated GC group (Figure 1d).

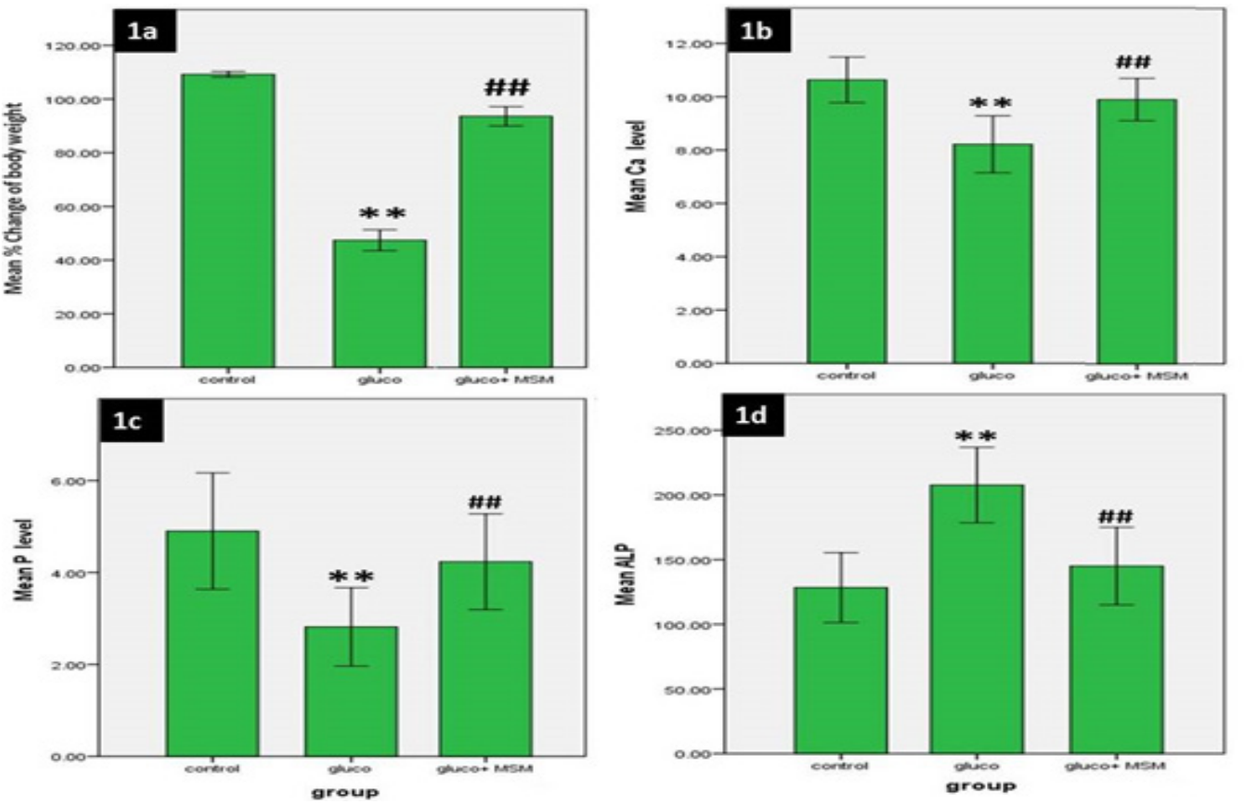

Fig. 1: (a) Mean percentage change of body weight (g), (b) Mean of calcium level (mg/dl), (c) Mean of serum phosphorus level (mg/dl), (d) Mean of serum alkaline phosphatase level (mg/dl). ** *P*<0.001 as compared to control group, ## *P*<0.001 as compared with glucocorticoid group.

Histopathological results:

Longitudinal sections of the tibial shaft of the control group stained with H&E exhibited normal histological architecture formed of uniform compact bone with regular periosteum and endosteum lining the external and internal aspects recepectively. The periosteum had outer fibrous layer (OFL) formed of dense collagen fibers and inner osteogenic layer (IOL) formed of osteoblasts (ob) and many osteogenic cells (og). Compact bone of tibial diaphysis showed many numerous Haversian systems with regular walls of their canals, surrounded by osteocytes present inside their lacunae and arranged in lamellae. The bone marrow was highly cellular with nearly no fat cells. Longitudinal sections through the upper end of the tibia consisted of uniform bony trabeculae surrounding bone marrow inbetween. It contained osteocytes within their lacunae and osteogenic cells on the trabecular surfaces (Figure 2).

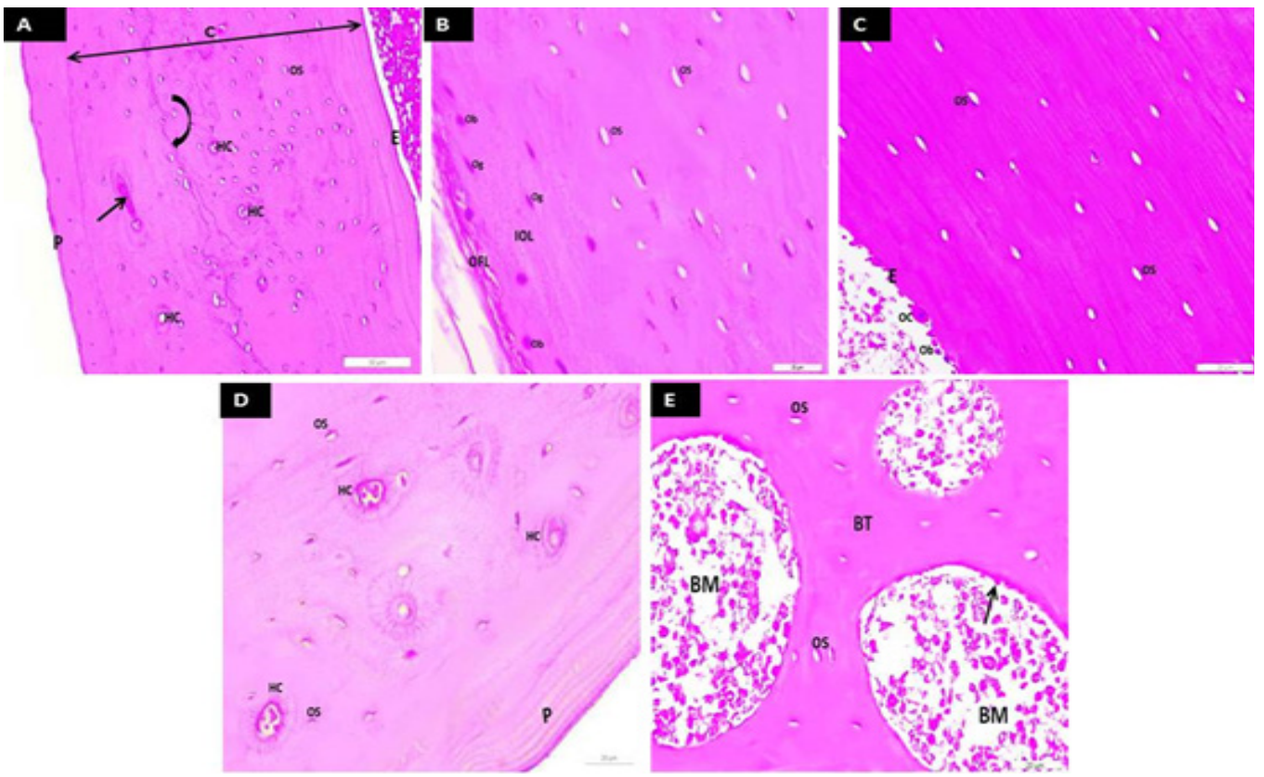

Fig. 2: (A,B,C,D,E) H&E staining of tibial sections of the control group showing: (A) Regular periosteum (P) and endosteum (E). Haversian canals (HC) within the compact bone. Volkmann's connection (straight arrow) connects some Haversian canals together. Normal thickness (double-headed arrow) of the compact layer of the cortex (C). Normal osteocytes (OS). Cement line (curved arrow) between new and old. (B) The two layers of the periosteum, outer fibrous layer (OFL) and inner osteogenic layer (IOL) contain cubical osteoblasts (ob) with rounded nuclei and many osteogenic cells (og) with elongated nuclei. Osteocytes (os) inside their lacunae in the compact bone. (C) The endosteum (E) formed of osteoblasts (Ob) with rounded nuclei and osteoclasts (OC) which is a multinucleated cell with acidophilic cytoplasm. (D) Regularly arranged Haversian canals (HC) within the compact bone. (E) The upper end of the tibia, the epiphysis, has bony trabeculae (BT) with bone marrow spaces (BM) inbetween. Osteocytes (OS) within their lacunae appear in the trabeculae. Osteogenic cells line the trabeculae (black arrow). Scale bars = 50 µm (A), 20 µm (B,C,D and E).

Compared to control group, GC group revealed marked increase in thickness of the outer fibrous layer of the periosteum with irregularity of both periosteum and endosteum in the form of resorption cavities, erosions, fissures or even subperiosteal tunnel. The osteoblasts in the inner osteogenic layer of the periosteum were degenerated and the osteoblasts on the endosteal surface were few in number. Compact bone of the diaphysis showed marked thinning with many fissures and cracks. Multiple osteoporotic cavities of variable sizes were also present in the compact bone. Haversian canals became wide and few in numbers with irregular walls and irregular distribution. The osteocytes were present inside widened lacunae and showed irregular distribution with loss of the normal pattern of lamellae around the Haversian canals. Some osteocytes were degenerated with pyknotic nuclei while some lacunae were empty and wide. Disruption of bone matrix with irregular basophilic areas within acidophilic matrix was detected and many congested blood vessels within the cortex. The upper end showed that the trabeculae were thin, irregular and discontinuous with many fissures and cracks. The bone marrow spaces between these trabeculae were widely separated and showed many fat cells (Figure. 3).

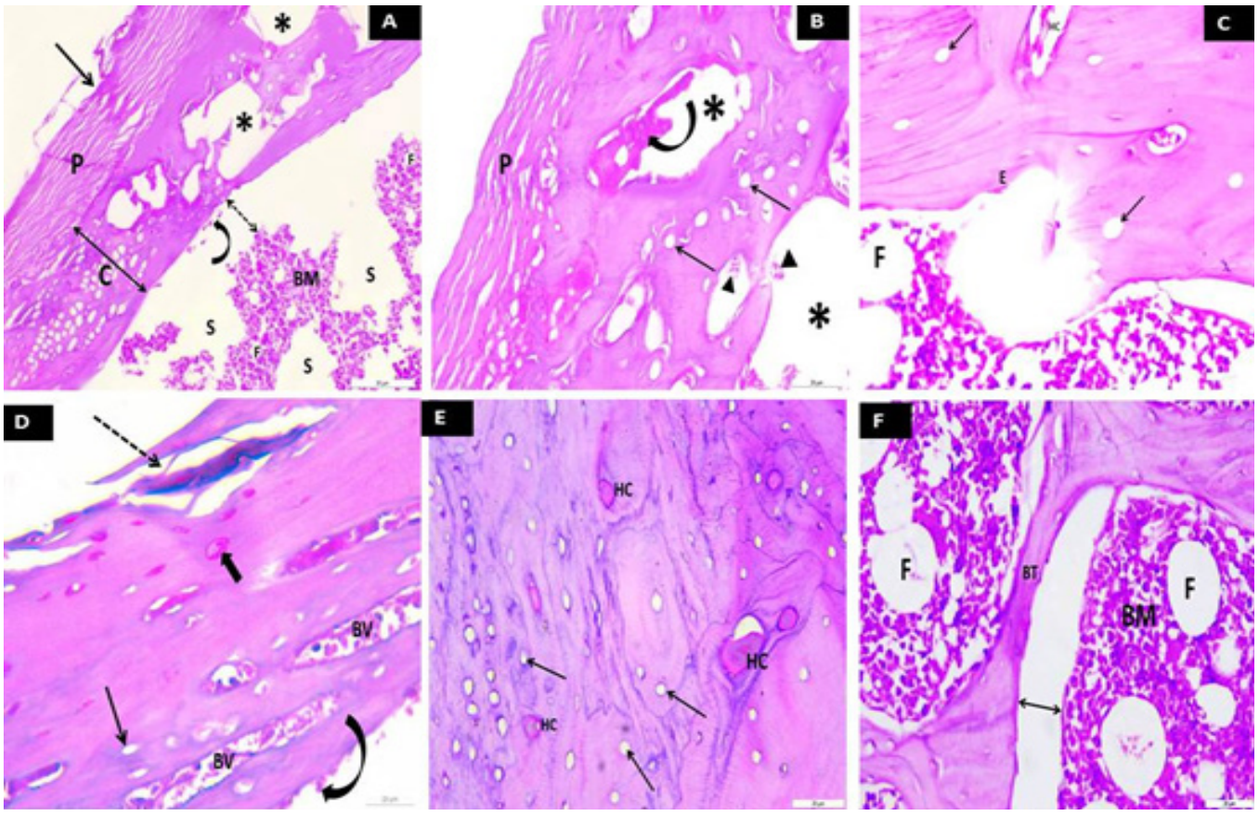

Fig. 3: (A,B,C,D,E,F) H&E staining of tibial sections of the GC group showing: (A) Apparently thickened fibrous periosteum (P) with irregular surface (black arrow).The endosteum is lined by few number of osteoblasts (curved arrow). Marked thinning (double-headed arrow) of the compact layer of the cortex (C) with appearance of many osteoporotic cavities (*) within it. The bone marrow (BM) shows many fat cells (F) which may fuse together forming large space (S) with widening of the gap between bone marrow and compact bone (dashed double headed arrow). (B) Markedly thickened fibrous periosteum (P). Lacunae (straight arrow) are widened and empty. Many resorption cavities (*) appear, some of these cavities contain osteoclasts (arrow head) or granulation tissue (curved arrow). (C) Irregular, eroded endosteum (E). Some empty lacunae (black arrow) and widened irregular Haversian canal (HC) in the bone matrix. Bone marrow contains fat cells (F). (D) Subperiosteal tunnel (dashed arrow) within the fibrous periosteum. Markedly dilated congested blood vessels (BV), empty lacuna (thin arrow) and degenerated osteoblasts (thick arrow) within the cortex. Few numbers of osteoblasts on the endosteum (curved arrow). (E) The epiphysis with very thin bony trabeculae (BT). The bone marrow (BM) shows many fat cells (F) with widening of the gap between bone marrow and compact bone (double head arrow). Scale bars = 50 μm (A), 20 μm (B,C,D,E and F).

Most of histopathological findings found in the GC group was improved by MSM treatment as thickness of the outer fibrous layer and inner osteogenic layer of periosteum became normal with abundant osteogenic cells and few degenerated osteoblasts. Periosteum and endosteum were nearly regular. Many osteoblasts were present on the endosteal surface. Compact bone of diaphysis nearly restored its normal thickness and architecture with few and small fissures, cracks. Few, small resorption cavities were still present. Haversian canals restored its normal shape, number and size with few congested blood vessels within the cortex. Osteocytes appeared nearly normal inside their lacunae and restored their regularity in lamellae around the Haversian canals. Some lacunae were wide, empty and few multinucleated osteoclasts were seen in the resorptive cavities. The upper end showed thick, continuous trabeculae with normal osteocytes and separated by normal cellular bone marrow (Figure. 4).

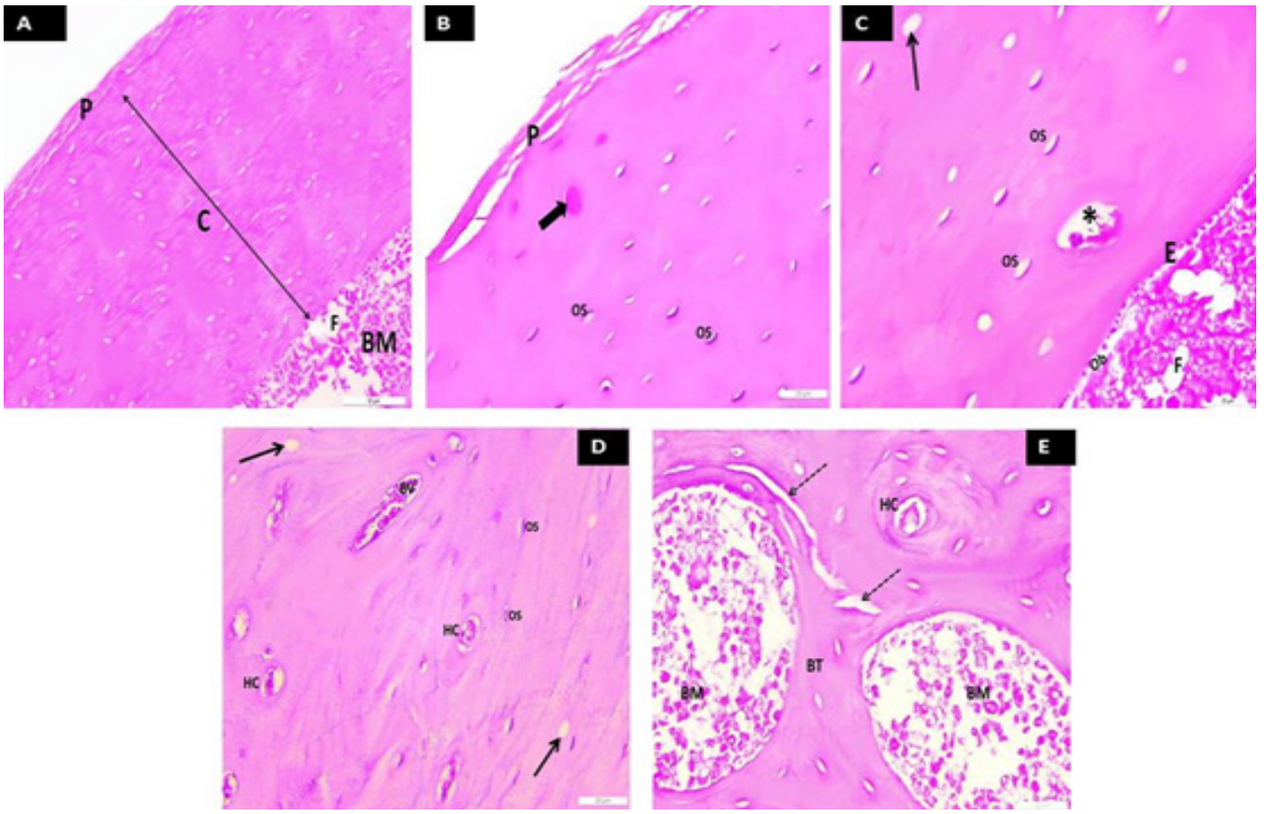

Fig. 4: (A,B,C,D,E) H&E staining of tibial sections of the GC group treated with MSM showing: (A) Apparent decrease in the thickness of the fibrous periosteum with increase the cortical bone thickness (C) compared to GC treated group. Bone marrow (BM) appears cellular with few fat cells (F). (B) Restoration of the thickness of the periosteum (P). Degenerated osteoblasts (thick arrow) are seen while many normal osteocytes within their lacunae (OS) are observed. (C) Regular endosteum (E) with normally distributed osteoblasts (Ob) on the endosteal surface. Normal osteocytes within their lacunae (OS) are observed while some empty lacunae (black arrow). An osteoporotic cavity (*) near the endosteal surface. Normal cellular bone marrow (BM) with few fat cells (F). (D) Regular, normal sized Haversian canals (HC) surrounded by normal osteocytes (OS) within their lacunae while few lacunae are empty (black arrow). Dilated congested blood vessel (BV) can be observed. (E) bony trabeculae (BT) which are regular and thick but have some fissures (dashed arrow) and normal bone marrow spaces (BM) between them. Normal Haversian canals (HC) which have regular wall with normal osteocytes regularly arranged around them in lamellae also appear. Scale bars = 50 µm (A), 20 µm (B,C,D and E).

Statistically, the outer fibrous layer thickness and osteoclast number in GC group were increased significantly (P <0.001) in the GC group as compared to the control group (181.41±2.357 vs. 101.15±1.856 and 2.182±.0952 vs. 0.141±.0424 respectively). These results were declined significantly (P <0.001) in the GC group treated with MSM as compared to the GC group without treatment (112.35±1.763 vs. 181.41±2.357 and 0.603±.0163 vs. 2.182±.0952 respectively) (Figure. 5).

The Compact bone thickness, osteoblast number and osteogenic number in GC group were significantly declined (P <0.001) in the GC group as compared to the control group (205.10±1.009 vs. 333.06±0.989, 10.087±0.441 vs. 18.017± 0.234 and 4.86±1.138 vs. 12.65±0.187 respectively). These results were increased significantly (P <0.001) in the GC group treated with MSM as compared to GC group without treatment (310.50±0.750 vs. 205.10±1.009, 17.097±0.291 vs, 10.087±0.441 and 9.65±0.369 vs. 4.86±1.138 respectively) (Figure. 5).

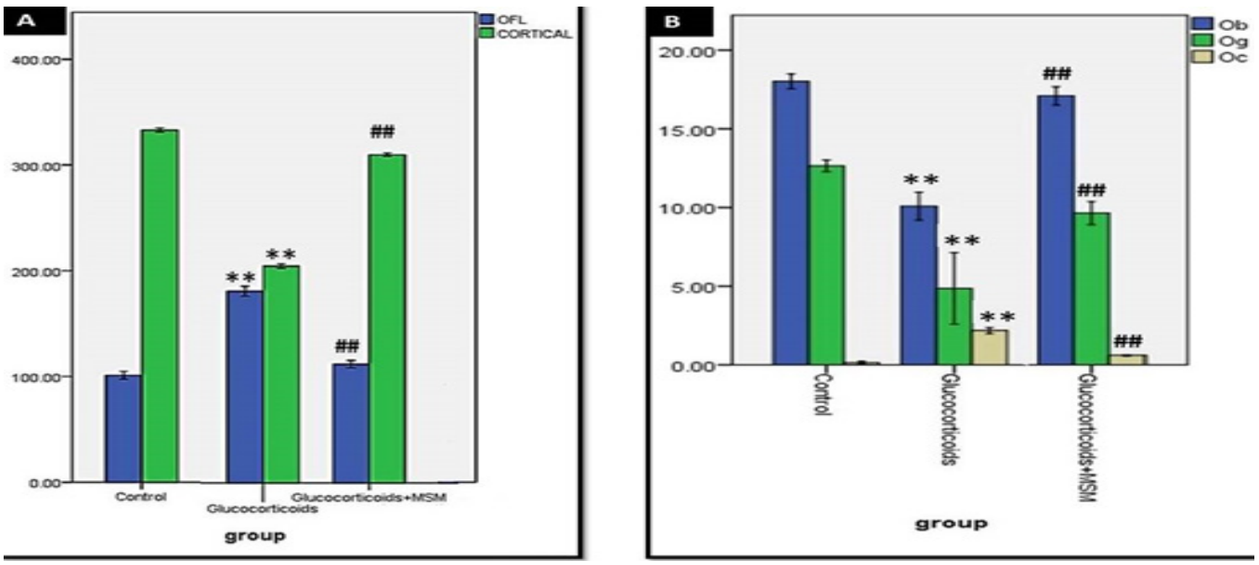

Fig 5: (A,B): A histogram of the different studied groups showing : (A) The mean thickness of outer fibrous layer and compact bone (um). (B) The mean cortical bone osteoblast, osteogenic and osteoclast number. ** *P*<0.001 as compared to the control group, ## *P*<0.001 as compared to glucocorticoid group.

Alzarine red stained longitudinal sections through the tibial shaft showed significant decline (*P* <0.001) in the area percentage and color intensity of Ca staining in GC group as compared to control one (1.767±0.277 vs. 59.458±0.859 and 34.558±2.249 vs. 179.476±3.372 respectively). These results were increased significantly (*P* <0.001) in the GC group treated with MSM as compared to the untreated GC group (26.226±1.105vs. 1.767±0.277 and 126.670±1.278 vs. 34.558±2.249 respectively). (Figure. 6 A,B,C,D).

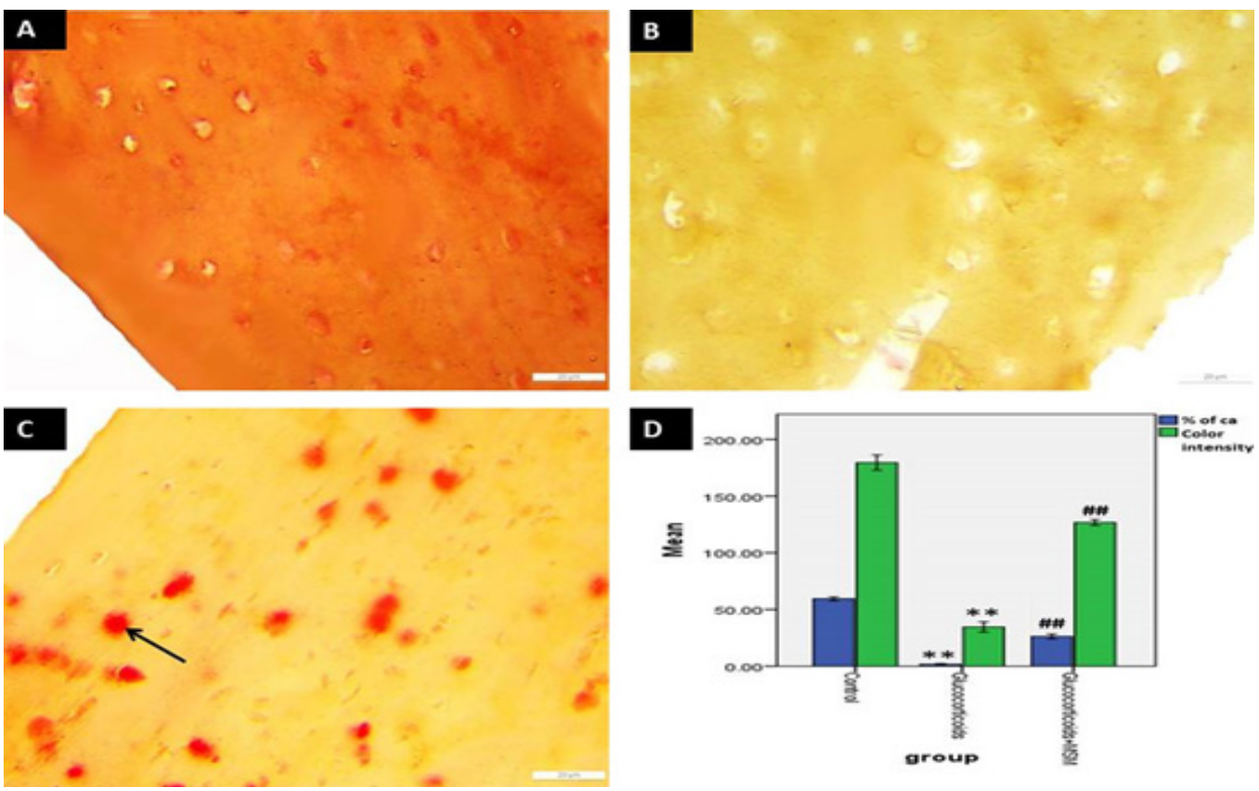

Fig. 6: (A,B,C,D) Alzarine red staining of tibial sections showing: (A) Intense staining throughout the cortical bone matrix the tibial shaft of the control group (Scale bar = 20 µm). (B) Severe reduction in the intensity of staining of the cortical bone matrix of the GC group. (C) Appearance of many orange-red stained calcified nodules (arrow) within the cortex of the GC group treated with MSM. (D) A histogram showing the mean area percentage (%) and color intensity (pixel) of Ca staining. Scale bar = 20 µm (A,B and C). ** *P*<0.001 as compared to the control group, ## *P*<0.001 as compared to glucocorticoid group. Scale bars = 20 µm.

Immunohistochemical results

Osteopontin (OPN) immunoreactions in tibial sections revealed a significant decrease (P<0.001) in the GC group compared to control group (1.843±0.336 vs. 9.310±0.3761). In addition, the OPN immunoreactions in the GC group treated with MSM was also significantly increased compared with the untreated GC group (7.745±0.406 vs. 1.843±0.336) (P<0.001) (Figure 7 A,B,C and J).

Further, RANKL and TUNEL immunoreactions in tibial sections exhibited a significant increase (P<0.001) in the GC group compared to control group (10.857±0.218 vs. 4.105±0.436 and 41.517±0.861 vs. 2.478±0.275 respectively). In addition, the RANKL and TUNEL immunoreactions in the GC group given MSM revealed also a significant decline (P<0.001) compared to the untreated GC group (5.840±0.293 vs. 10.857±0.218 and 7.847±0.236 vs. 41.517±0.861 respectively) (Figure 7 D,E,F,G,H,I and J).

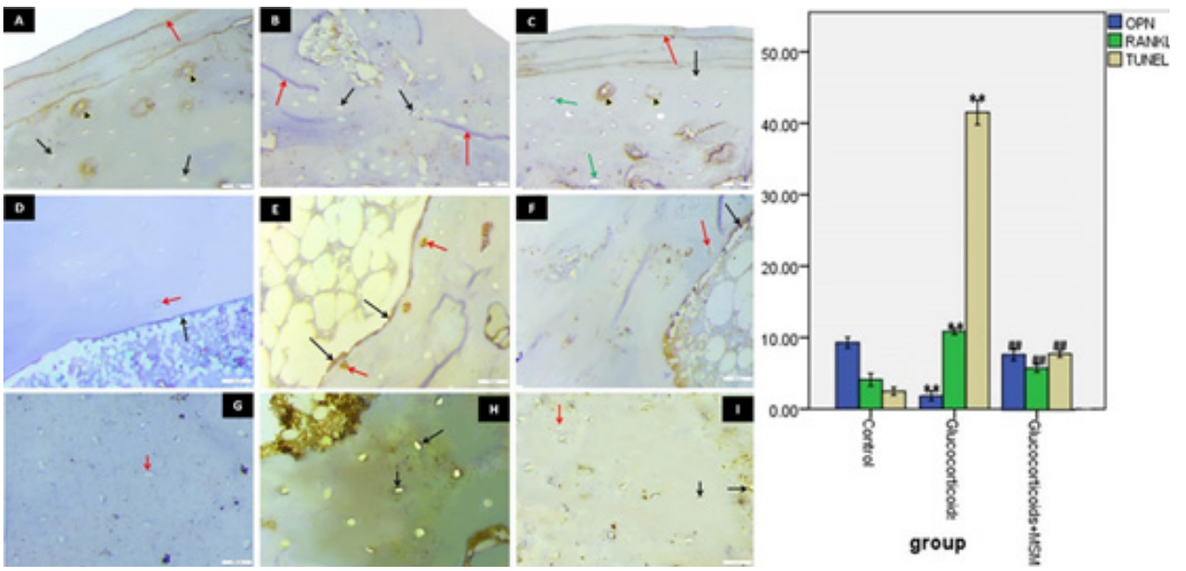

Fig. 7: (A-J) Representative photomicrographs of immunostained tibial sections from the different studied groups showing: (A) positive reaction to osteopontin of the control group with brownish coloration of the cement lines (red arrow), osteocytes (black arrow) and around the Haversian canals (arrow head). (B) Negative reaction to osteopontin of the GC group with no brownish coloration of the cement lines (red arrow), osteocytes (black arrow). (C) Positive reaction to osteopontin of the GC group treated with MSM as brownish coloration of the cement lines (red arrow), some osteocytes (black arrow) and around the Haversian canals (arrow head) as a reaction to osteopontin immune staining while some osteocytes show negative reaction (green arrow). (D) control group showing negative reaction to RANKL with nearly no brownish coloration of the osteoblast (black arrows) or osteoclast (red arrows). (E) GC group showing brownish coloration of many osteoblasts (black arrow) and osteoclasts (red arrow) indicating positive reaction to RANKL. (F) GC group treated with MSM showing few brown stained osteoblasts (black arrow) and osteoclasts (red arrow) as a reaction to RANKL. (G) control group showing negative reaction to TUNEL with nearly no brownish coloration of the osteocytes (red arrow). (H) GC group showing brownish coloration of many osteocytes (black arrow) indicating positive reaction to TUNEL. (I) GC group treated with MSM showing few brown stained osteocytes (black arrow) as a reaction to TUNEL and some osteocytes with negative reaction (red arrow). (J) A histogram showing a significant decline in OPN positive immunoreactions in the GC group compared with the control one. GC group treated with MSM exhibits significant increase in OPN immunoreactions compared to the untreated GC group. RANKL and TUNEL immunoreactions exhibited a significant increase in the GC group compared to control. In addition, the RANKL and TUNEL immunoreactions in the GC group given MSM revealed a significant decline compared to the untreated GC group. ** P<0.001 as compared to the control group, ## P<0.001 as compared to glucocorticoid group. Scale bars = 20 µm

Electron microscopic results:

Scanning electron microscopic examination of tibial diaphysis of control rats showed normal thickness of the compact bone with uniform periosteum and endosteum surrounding the bone marrow (Figure 8 A). Cancellous bone in tibial epiphysis showed normal thickness and continuity of the trabeculae and normal bone marrow spaces (Figure 8 B). Haversian system appeared with regular and uniform Haversian canals (Figure 8 C). Collagen fibers showed normal thickness and arrangement (Figure 8 D).

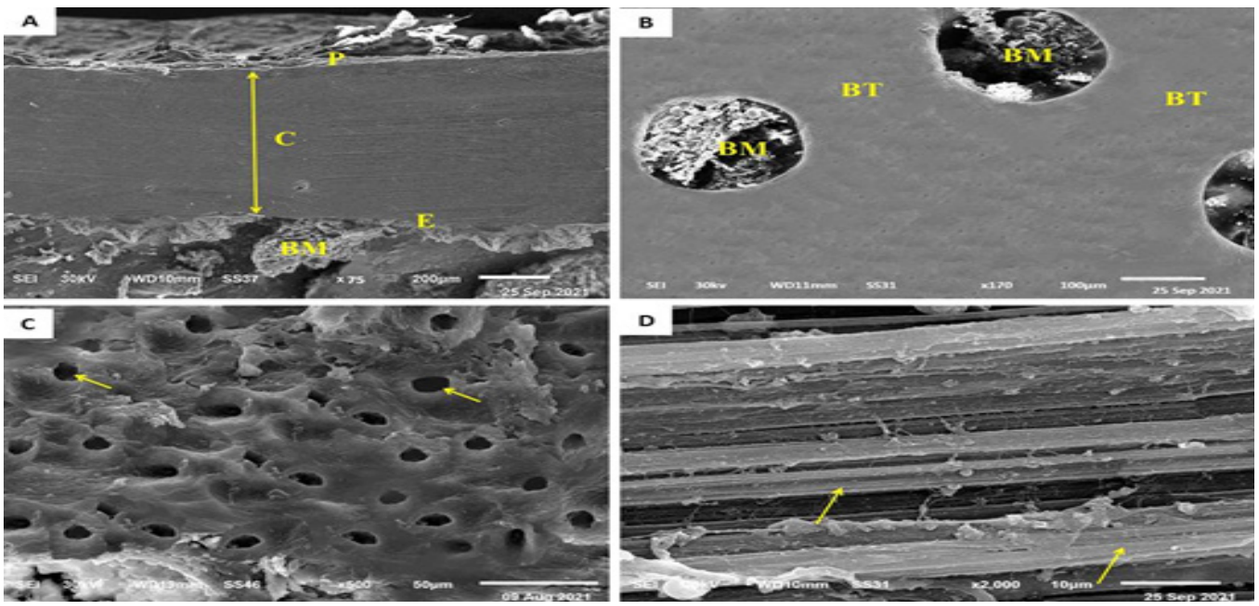

Fig. 8: (A,B,C,D) Scanning electron micrographs of tibial sections in the control group showing: (A) the cortical bone (C) with normal thickness (double headed arrow) enveloped with uniform outer fibrous periosteum (*P*) and lined by inner endosteum (E). Bone marrow (BM) is seen on the inner side of the bone (Scale bar = 200 µm). (B) Normal thickness and continuity of the bone trabecula (BT) with normal bone marrow spaces (BM) (Scale bar = 100 µm). (C) Many Haversian canals which are uniform in size (arrow) (Scale bar = 50 µm). (D) Normal thickness and arrangement of collagen fibers in the cortical bone (arrow) (Scale bar = 10 µm).

Scanning electron microscopic examination of tibial diaphysis of GC rats showed thinning and fissures in the cortical bone with visible osteoporotic cavities. The outer cortical surface showed considerable irregularity and disturbed periosteum was observed (Figure 9 A). Cancellous bone in tibial epiphysis showed irregular bone trabeculae which may be fractured and widened bone marrow spaces (Figure 9 B). Haversian system appeared irregular and widened and were not regularly arranged (Figure 9 C). Collagen fibers showed marked dissociation (Figure 9 D).

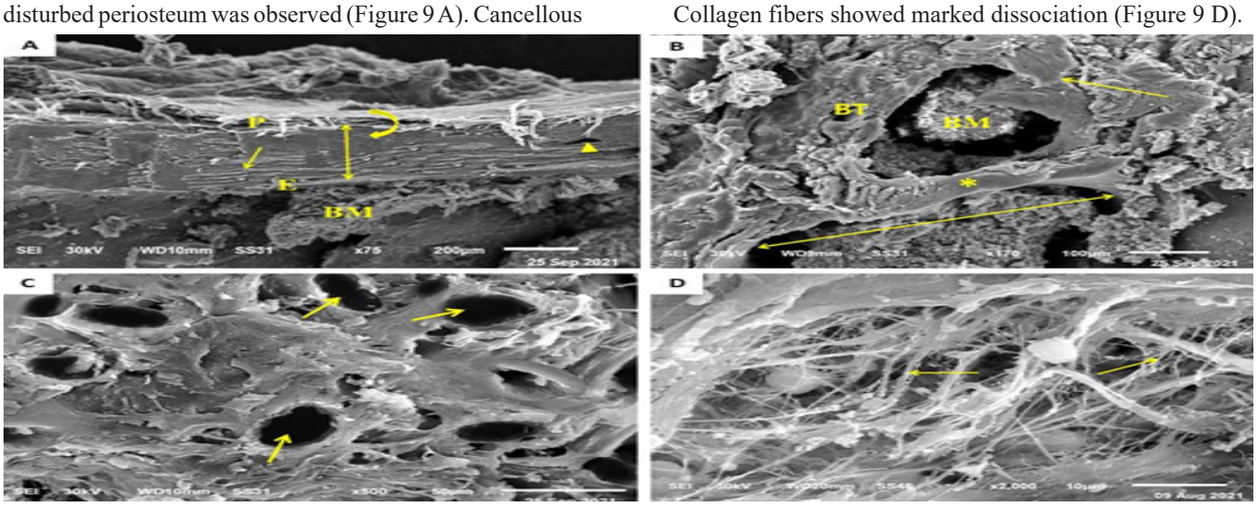

Fig. 9: (A,B,C,D) Scanning electron micrographs of tibial sections in the GC group showing: (A) Restoration of the normal thickening of the compact bone (double head arrow) compared to control group, with regular periosteum (*P*) and endosteum (E). There is some irregularity of the outer cortical surface (arrow) and a fissure (white thick arrow) is also seen (Scale bar = 200 µm). (B) More or less regular continuous bone trabeculae (BT). However, some of them are apparently thin (arrow head). Bone marrow cavities (BM) are nearly regular (Scale bar = 100 µm). (C) Many Haversian canals which are more or less uniform in size (arrow) compared to control one (Scale bar = 50 µm). (D) some dissociation of collagen fibers (arrow) (Scale bar = 10 µm).

Scanning electron microscopic examination of tibial diaphysis of GC rats treated with MSM showed restoration of the normal thickening of the compact bone with nearly normal periosteum, endosteum and bone marrow spaces (Figure 10 A). The cancellous bone of tibial epiphysis showed restoration of normal thickness and continuity of the trabeculae and normal bone marrow spaces (Figure 10 B). Mostly the Haversian systems had uniform sized Haversian canals (Figure 10 C). Collagen fibers showed less dissociation (Figure 10 D).

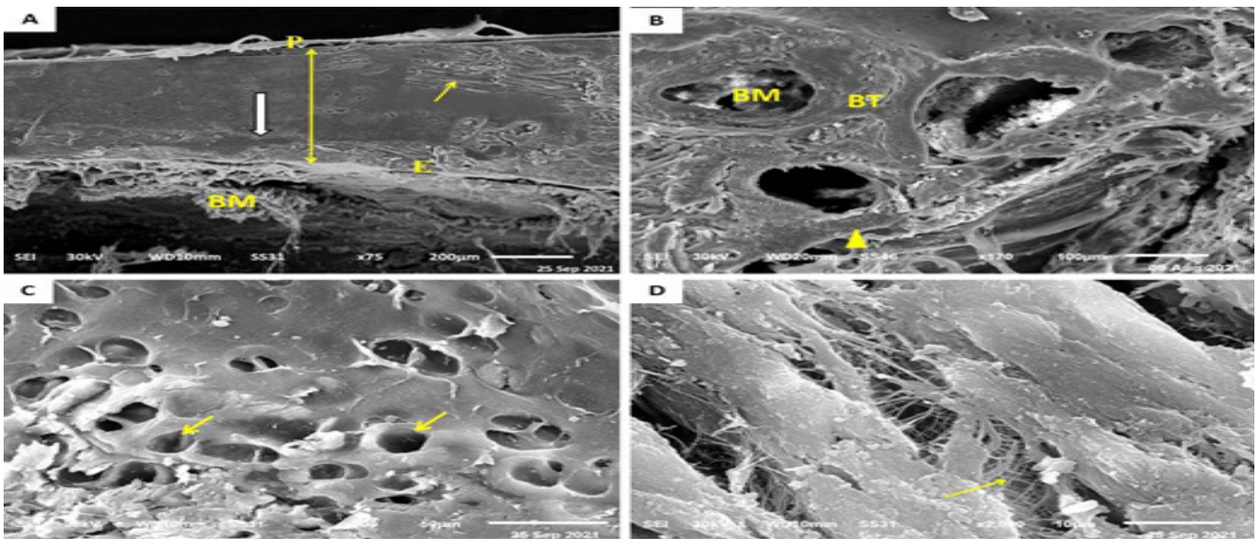

Fig. 10: (A,B,C,D) Scanning electron micrographs of tibial sections in the GC group showing: (A) Thin cortical bone in relation to control group (double headed arrow) with appearance of an osteoporotic cavity (arrow head), the outer cortical surface (arrow)with marked irregularity and disturbed periosteum (*P*). A fissure in the compact bone (curved arrow) can also be seen. Bone marrow (BM) is seen on the inner side of the bone. (Scale bar = 200 µm). (B) Apparently thin (*), irregular bone trabeculae (BT) with fractured one (arrow). Widened (double headed arrow) bone marrow spaces (BM) are seen. (Scale bar = 100 µm). (C) Irregular and widened Haversian canals (arrow) which are not regularly arranged (Scale bar = 50 µm). (D) Marked dissociation of collagen fibers (arrow) (Scale bar = 10 µm).

Statistically, scanning electron microscopic examination of tibial diaphysis of GC group revealed that the trabecular thickness was decreased significantly ($P < 0.001$) as compared with control group (32.5517±0.799 vs. 53.6783±1.471). In addition, there was also a significant increase ($P<0.001$) in trabecular thickness in the GC group given MSM compared with the GC group (45.8217±0.511 vs. 32.5517±0.799) (Figure 11).

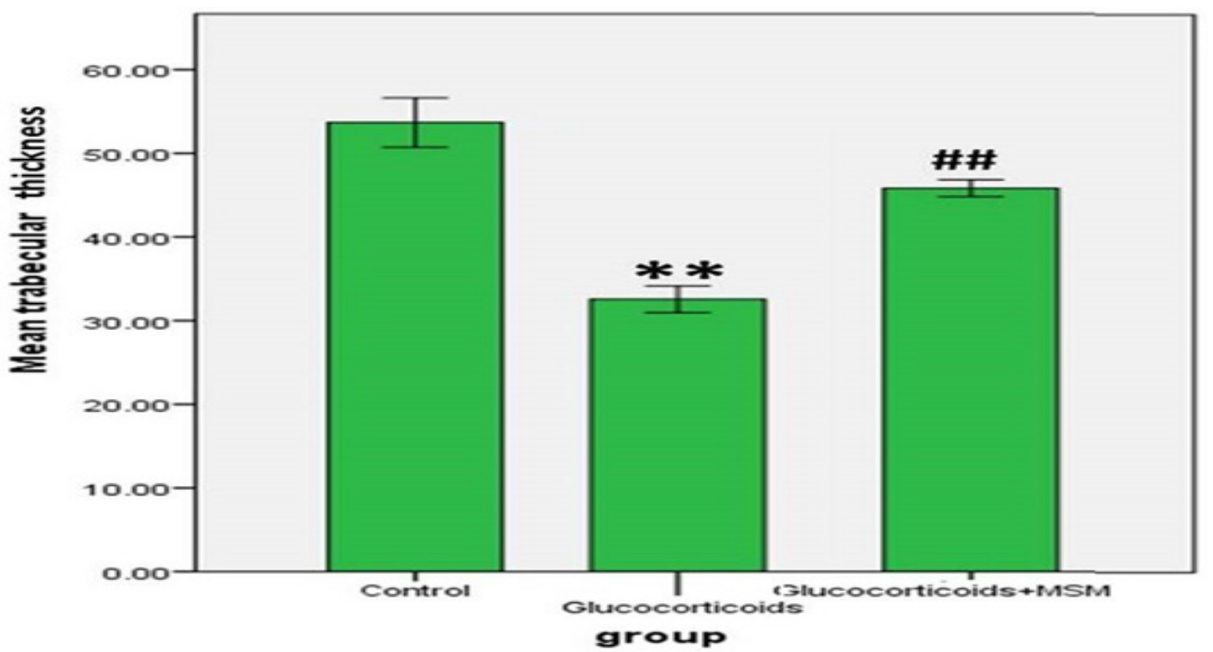

Fig. 11: A histogram showing a significant decrease in trabecular thickness in the GC group as compared to control group (**: $p < 0.001$). GC group given MSM exhibits a significant increase in trabecular thickness as compared to GC group (##: $p < 0.001$).

DISCUSSION

Osteoporosis is most common chronic metabolic bone disease worldwide[24]. It results from dysregulation of bone remodeling, as the rate of bone resorption is exceeding bone formation, resulting in bone loss[25]. It is one of the most destructive side effects of glucocorticoid treatment used for the management of auto-immune and inflammatory diseases[13].

Compared to synthetic medications natural products are often safe for use with few side effect. MSM has been used widely as a dietary supplement as it is natural organosulfur compound. Owing to its antioxidant and anti-inflammatory properties MSM has protective effects against various disorders. Several clinical studies have documented the beneficial effect of MSM for treating osteoarthritis of knee joint, repetitive stress injuries, allergies, stomach ulcers, certain bladder disorders like interstitial cystitis, and wounds[26, 27].

The present work was performed to investigate the biochemical, histological, immunohistochemical and scanning electron microscopic bone changes of GC induced rat model and to assess the role of MethylSulphonyl Methane on it.

The laboratory rat is the commonest used model for glucocorticoid induced osteoporosis (GIOP) as it is convenient, cost-effective, easy and safe to handle[28]. Moreover, its biomechanics, histomorphometry, and imaging methodologies have been well established[29, 30]. Similar to the human disease, in rat model, it was found that after beginning the administration of GC, an early phase characterized by excessive bone resorption mediated by osteoclast occurs followed by the chronic phase of reduced osteoblastogenesis and bone formation resulting in early and highest rate of bone loss. So, it was found that adult rats were appropriate animal models of GIOP models[31]. Many similarities at the macrostructural and microstructural levels are present between the human and rat tibiae[32]. Among other corticosteroids dexamethasone was reported as the most powerful inductor of osteoporosis, in animal models. Furthermore, it has long-term action thus no need for daily injections. So, it was selected to induce osteoporosis in our study[28].

Our study revealed a significant decrease of body weight in GC treated rats. This was in accordance with ***Lin et al.***[30]. who found that the mean body weight of the rats was decreased after treatment with prednisone and that the linear relationship between mean body weights and the dosages of prednisone revealed a dose-dependent manner.

The decrease in body weight was explained by ***Liu et al.***[33] who stated that GC treatment suppressed appetite, produced apparent glucolipid metabolic disturbances and hyperinsulinemia. Moreover, GC decreased levels of mRNA expression of the orexigenic neuropeptides; AgRP, NPY, and anorexigenic neuropeptide; CART, in rats hypothalamus. When NPY/AgRP is released, food intake is increased.

In this experiment, a significant decline was noticed in the serum calcium and phosphorus levels in glucocorticoid treated rats. This was in accordance with ***Gabr et al.***[34] who declared that administration of glucocorticoids resulted in a significant reduction in in the serum Ca level of male and female osteoporotic rats compared with their normal groups. ***Banji et al.***[35] referred that decline in the serum level of Ca in glucocorticoids-exposed rats owing to increased renal excretion and change in their transport across the brush border membrane of the renal tubules.

Moreover, ***Badae et al.***[36] added that decreased serum calcium and phosphorus level is due to GCs which act on the kidney directly and indirectly, by induction of secondary hyperparathyroidism, decrease phosphate tubular reabsorption resulting in phosphaturia. Also, they act through disrupting metabolism of vitamin D reducing 1, 25- dihydroxyvitamin D receptors in bone which causes osteoporosis or by lowering absorption of intestinal calcium. They decrease synthesis of calcium binding protein and inhibit calcium release by mitochondria.

Also, ***Jilka et al.***[37] reported that GCs enhance the amiloride sensitive Na+/H+ exchange activity in the renal proximal tubule brush border vesicles and decrease the Na+ gradient-dependent phosphate uptake leading to stimulation of acid secretion with phosphaturia.

The level of serum alkaline phosphatase is an indicator of excessive bone activity, osteoblast activity and new bone formation as an increased level indicates an enhanced bone activity, resulting i accelerated loss of bone in the future. So, it is used to monitor metabolic bone diseases[38, 39].

A significant increase in serum alkaline phosphatase level of GC treated rats was noticed in the current study compared to the control group suggesting increased bone turnover due to osteoporosis induction. Similar findings have been declared in other studies; ***Raja et al.***,[39], ***Fahmy et al.***[40] and ***Hou et al.***[41] who stated that glucocorticoid group revealed an elevated serum alkaline phosphatase level estimated at 79.02%, in osteoporotic rats compared to the normal control group. Moreover, Abd El Moneim and Mahmoud,[42] reported that bone specific alkaline

phosphatase (BSAP) is localized in the plasma membrane of osteoblasts and is liberated into the circulation during bone mineralization. On the contrary, ***Badae et al.***[36] found that serum ALP was decreased significantly in glucocorticoid administrated rats which is against our finding.

Treatment with MSM remarkably increased the body weight, ameliorated the biochemical changes and significantly restored their normal values, where serum calcium and phosphorus levels were increased and serum alkaline phosphatase was decreased.

In the present study, GC treated rats showed irregular periosteum and endosteum of the tibial diaphysis with projections, erosions and subperiosteal tunnels together with a significant increase in the thickness of fibrous layer of the periosteum. These findings were in agreement with ***El-Haroun et al.***[43] who found thickened and irregular outer fibrous layer of periosteum associated with apparent decrease in cortical bone thickness in GC treated group.

El-Masry et al.[44] and ***Ali et al.***[45] also recorded an apparent increase mainly in the thickness of fibrous layer of periosteum which invaginate inside bone matrix deeply in osteoporotic group as compared with that of control group. Similarly increased periosteal thickness could be a compensatory support to the fragile osteoporotic cortical bone reducing the risk of its fracture as stated by ***Allen et al.***[46].

In our study, microscopic examination of GC treated group revealed marked thinning of the cortical bone and its trabeculae which was confirmed by morphometric study. Significant thinning of the tibial compact layer in the glucocorticoid treated rats was observed leading to widening of the gap between this compact bone and bone marrow, together with appearance of many fissures, cracks, osteoporotic and resorptive cavities that may contain osteoclasts. Similar results were declared by ***Shata et al.***[47] and ***Saad et al.***[13] who stated that these histopathological findings confirm osteoporotic bone dystrophic changes induced by GCs.

Derakhshanian et al.[48], ***Abdel Fattah et al.***[28] and ***Saad et al.***[13] also reported a reduction in trabecular and cortical thickness were associated with a significant decline in the number of osteoblasts in rats treated with glucocorticoid.

Moreover, ***Bouvard et al.***[49] and ***Badae et al.***[36] stated that in GIOP group, tibiae showed thin bone trabeculae and increase intratrabecular distance. This was supported by bone loss induced by GCs because GCs diminished bone formation of tibias, rate of both mineral deposition and bone formation with a significant decline in both of trabecular thickness and trabecular bone volume.

In the present work, changes in the bone cells were reported, there was decreae in the number of osteogenic cells and osteoblasts in the cortex of tibial diaphysis with increased number of osteoclasts. This was in agreement with ***Abdel Fattah et al.***[28] who found many osteoclasts in Howship's lacunae and lack of continuous surface osteoblasts. ***Lihui et al.***[50] also reported that cytokines and oxidative stress suppressed the bone forming osteoblasts and added that calcium and vitamin D deficiency in osteoporotic rats can contribute to secondary hyperparathyroidism with subsequent increase in osteoclastic activity and bone resorption.

Kim et al.[51] explained that GCs extend the life span of osteoclasts by direct actions on osteoclasts or through inability of osteoclasts to organize their cytoskeleton in response to macrophage colony-stimulating factor (M-CSF) which is a cytokine binds to receptors on osteoclasts inducing its differentiation. Dexamethasone particularly stops M-CSF activation of RhoA, Rac, and Vav3, each one of them regulate the osteoclast cytoskeleton. Furthermore, Komori,[52] demonstrated that GCs induce osteocyte and osteoblast apoptosis, increase reactive oxygen species, pro-apoptotic molecules and endoplasmic reticulum stress and inhibit the Wingless/ Integrated/ β-catenin pathway (Wnt/β-catenin), a critical pathway for osteoblasts differentiation; thus, osteoblastogenesis is impaired.

In the present work, many osteocytes were present inside widened lacunae and showed irregular distribution in lamellae around Haversian canals and some of them were degenerated and showed pyknotic nuclei or died leaving wide empty lacunae in GC treated group. This was in agreement with the study made by ***Elshawarbi et al.***[53] in which there was areas devoid of osteocytes or few degenerated osteocytes in bone trabeculae of osteoporotic rats while others appeared with dark nuclei. Osteoclasts were often present within erosion cavities and were seen as large irregular cells with eosinophilic cytoplasm.

Tristan et al.[54] explained decreased osteocytes by suppression of expression of many proteases required for perilacunar remodeling of osteocytes leading to osteocyte degeneration and lacunacanalicular network, also matrix hyper mineralization and collagen disorganization.

The present study reported histological changes in the Haversian systems of GC rats with remarkable changes

in the size and number of Haversian canals plus irregular distribution of large number of them, also some have irregular walls. This was in accordance with ***Nontakorn et al.***[55] who declared that cortical bone loss and resorption in osteoporotic rats causes widening of Haversian canals which was explained by ***Ali et al.***[45] as abnormal osteoid deposition in Haversian canal wall.

In GC treated rats of this study, bone marrow spaces were widened with hypocellular bone marrow that is invaded by fat cells. This was supported by ***Abdel Fattah et al.***[28] who reported an increase in fatty tissue which invade the bone marrow spaces. Moreover, ***Cawthorn et al.***[56] found that increasing the circulating glucocorticoids caused expansion of adipose tissue of the bone marrow.

Okasha and Elbakary,[57] also declared that the bone marrow had an excess fat cells and Supposed that a relationship between abnormal metabolism of lipid and osteonecrosis may be present. Also Naim,[58] said that in the bone marrow of osteoporotic rats an accumulation of fat cells was noticed, rendering the marrow yellow. Moreover, Ng and Duque,[59] suggested that, this may be due to shifting of mesenchymal stem cells into adipocytes at the expense of osteoblasts, as in osteoporosis there was reduction in lamin A/C protein expression from mesenchymal cells, therefore a predominant differentiation into adipocytes may occur.

Histological and morphometric assessment of tibias of GC group treated with MSM restored its normal cortical, trabecular thickness while the periosteum still showing some degree of thickening. Haversian system with normal arrangement, distorted, less apparent and eroded cavities were detected. Numerous osteocytes within their lacunae and were regularly arranged could be noticed. Few osteocytes had widened or empty lacunae. Increased number of osteogenic cells, osteoblasts, osteocytes and decreased osteoclast number when compared to GC group. ***Aljohani et al.***[60] reported that MSM stimulated mesenchymal stem cells differentiation into osteoblast-like cells and bone formation through investigating the effect of MSM on the osteogenic potential in vivo using an aging mice model and in vitro using stem cells from human exfoliated deciduous teeth (SHED). Moreover, ***Kim et al.***[61] stated that MSM positively regulated BMP-2-induced osteoblastic differentiation through Smad1/5/8, proposing that in bone formation MSM possessing an important role.

In the present study, sections of GC treated rats showed decreased intensity of calcium staining with Alzarine red stain within the cortical shaft as compared to control group. This was in accordance with ***Liang et al.***[62] who stated that Alzarine red staining became less apparent in osteoporotic groups indicating decreased formation of bone nodules. Moreover, ***Gao et al.***[63] explained decreased Alzarine red staining in osteoporotic groups by decreased calcification.

MSM treatment in this study increased calcium deposition in GC group treated with MSM which appeared as many calcified nodules within the cortical bone matrix as shown by Alzarine red staining. The formation of mineralized nodules confirmed an enhancement in osteogenesis by MSM. This was in agreement with ***Aljohani et al.***[60] who explained that during the mineralization process the enzyme transglutaminase 2 (TG2) had an important role in the cross-linking of proteins. The levels and activity of TG2 were increased by MSM. The enhancement in the levels of TG2 corresponded well with its interaction with the osteopontin and collagen found in the mineralized nodules. If an inhibitor Suppressed the activity of TG2 the effects of MSM would be decreased.

In this study, GC treated group revealed a significant decrease in osteopontin (OPN) expression; a bone formation marker; in osteoblasts and osteocytes indicating decreased bone formation. This result was in accordance with Shady and Nooh,[64] who observed that sections of diabetic induced osteoporotic rats revealed a marked apparent decline in osteopontin expression pointing to declined bone formation as osteopontin is a multifunctional protein supposed to have principal in bone formation. ***Saad et al.***[13] stated that GCs induce bone proteins catabolism; osteopontin and osteocalcin, leading to diminished anabolic state of bone.

In the current study, immunohistochemical staining with receptor of activator of NF-kappa b ligand (RANKL), a bone resorption marker, revealed significant increase in RANKL expression in glucocorticoid treated sections. This was in agreement with McClung,[65] who reported that osteoblasts secrete two proteins: osteoprotegerin (OPG) and RANKL. The interaction between RANKL on osteoblasts and the RANK receptor on hematopoietic osteoclast precursor cells enhances osteoclast activation. On the other hand, OPG acts as a soluble decoy receptor to RANKL and competes with RANK for RANKL binding to suppress osteoclast activation, thereby controlling osteoblast-osteoclast activity balance. He added that the ratio between OPG and RANKL adjusts the direction of bone metabolism to either bone formation or resorption.

Abdel Fattah et al.[28] demonstrated that GCs have been known to enhance bone resorption; by increasing RANKL. ***Saad et al.***[13] study demonstrated that imbalance in the OPG/ RANKL ratio is present in the GIOP group in favor of RANKL leading to increased osteoclastic bone

resorptive capacity. GCs increase bone resorption by activation and prolongation of life span of osteoclasts. This occurs through upregulating RANKL and at the same time, by downregulation of the RANKL decoy receptor OPG.

MSM treatment showed increased OPN and decreased RANKL expression in GC group treated with MSM as compared to GC group revealed enhancement of bone formation and decrease resorption. This was in line with ***Joung et al.***[66] and ***Aljohani et al.***,[60] who reported an increase in RUNX2 expression, which is a critical transcriptional factor, in MSM treated cells. Also, the expression of RUNX2 is associated with differentiation of osteoblast. This reveals that MSM may regulate the expression of its target genes, such as osteopontin, osteocalcin, collagen, and RUNX2, at both mRNA and protein level. MSM appears advantageous in increasing osteogenesis.

In the current work, GC treated group showed a significant increase in expression of TUNEL immune staining, a marker for detection of apoptosis, in the osteocytes as compared to control rats. This was in line with ***Valenti et al.***[67] who found that glucocorticoid administration induced apoptosis of osteocytes which causes suppression of bone formation and low BMD. These results revealed that increased osteocyte apoptosis contributes to bone loss induced by glucocorticoid.

Kogianni et al.[68] supposed that the dying osteocytes are able to regulate bone remodeling. The primary culprits for bone loss is the apoptotic osteocyte and osteocytes death by apoptosis acts as important signal for osteoclasts to enhance local bone resorption. Furthermore, apoptotic osteocytes released apoptotic bodies which express RANKL to recruit osteoclasts.

In addition to undergoing apoptosis, osteocytes posses a cellular homeostasis mechanism called autophagy which is essential to maintain cell viability. Decrease in cell autophagy might cause cell death. Autophagy is a significant protective process against apoptosis. cell autophagy is mandatory for osteocyte survival as osteocytes present in a potentially nutrient poor and hypoxic environment. Recently, it has been documented that autophagy protect osteocyte against the harmful effect of glucocorticoids[69].

In the present study, MSM reduced apoptosis that was observed through decreased TUNEL expression in GC group treated with MSM as compared to GC treated rats. This was in accordance with ***Kim et al.***[70] who explained that MSM suppresses the release of biomarkers of oxidative stress such as prostaglandin E2 and nitric oxide in macrophages by decreasing NF-κB signaling. furthermore, MSM work directly as free radical scavenger, that increase the efficacy of MSM as an antioxidant.

MSM has protective anti-oxidative effects through the induction of serum SOD, CAT, and glutathione peroxidase (GSH-Px) activities coupled with related reducing agents, such as increasing GSH[71, 72].

Al laham,[10] observed that treatment with MSM adjusted colonic GSH depletion and return the level toward the normal value due to its antioxidant action. Moreover, ***Lee et al.***[73] declared that the main component of MSM is sulfur, which is an important constituent of amino acids, that preserve integrity of cellular systems by influencing cellular capacity and the state of cellular redox to detoxify toxic compounds, free radicals and ROS. Also, Sulfur amino acids are incorporated in the synthesis of intracellular antioxidants (taurine and glutathione) and in the methionine sulfoxide reductase antioxidant system.

Moreover, ***Butawan et al.***[9] demonstrated that MSM is able to inhibit the release of vasodilating agents such as prostanoids and NO. decreased NO protects macrophages against apoptosis stimulated by NO. Furthermore, MSM may benefit other immune modulatory effects related to cell cycle and cell death.

Van der Merwe and Bloomer,[74] stated that MSM decrease tissue damage and the inflammation and this will prevent leukocyte apoptosis, due to its antioxidant property. It also decreases cytokines production such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) both are signaling proteins related to systemic inflammation.

In the present work, scanning electron microscopic (SEM) studies of rat bone in GC treated group revealed marked roughness of bony surface with exposure of collagen fibers. Compact bone appeared thin with presence of fissures, widened and irregular Haversian canals. Cancellous bony trabeculae were also thin with wide bone marrow spaces. Fracture of the trabeculae was also detected.

This was in agreement with ***El-Morsy et al.***[75] study, in which SEM examination of osteoporotic rats showed that there was decreased thickness of the outer cortical bone, broken trabeculae and enlarged Haversian systems. Also in Naim,[58] SEM study there was exfoliation off the surface of bone and appearance of broken trabeculae. They added that the detected exposure of collagen fibers could be due to increased osteoclastic activity that dissolve inorganic

component of bone.

In the present work, treatment with MSM remarkably ameliorated the osteoporotic changes as seen by scanning electron microscope. Restoration of the normal thickening of the compact bone with nearly normal periosteum, endosteum and bone marrow spaces. The cancellous bone of tibial epiphysis restored its normal thickness and continuity of the trabeculae. Uniform sized Haversian canals with less dissociated collagen fibers were observed.

The results gained from the present study showed that co-treatment with MSM provided a beneficial role against GC induced osteoporosis through its antioxidant and osteogenic properties.

CONCLUSION

The results of this study confirm that GCs negatively affect the bone and MSM has a protective role in treatment of GC induced osteoporosis.

FUNDS

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CONFLICT OF INTERESTS

There is no conflicts of interest.

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تأثير الكبريت العضوي على هشاشة العظام المستحثة تجريبيا في ذكور الجرذان البيضاء البالغة

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المقدمة: تعتبر العظام هي الوحدة البنائية للجهاز العظمي للانسان يوجد حوالي ٢٠٠ مليون شخص حول العالم يعانون من مرض هشاشة العظام. تنصح العديد من القواعد الإرشادية الدولية بمجموعة البيسفسفونات; مثل الألندرونات كأول عقار للوقاية من وعلاج هشاشة العظام ولها اثار جانبية مثل كسور عظمة الفخذ و تنخر عظام الفك وقد وجد أن الكبريت العضوي من المركبات الطبيعية معظم الاطعمه وهو معروف بخواصه المضادة للأكسدة، الالتهاب، الوقائية كيميائية، مضاد لتصلب الشرايين، مضاد لموت الخلايا.

الهدف من البحث: يهدف هذا العمل إلي دراسة التأثيرالعلاجي للكبريت العضوي علي مرض هشاشة العظام المستحثة تجريبيا فى ذكر الجرذ الابيض البالغ علي أسس كيميائية حيوية، هستولوجية و هستوكيميائية مناعية.

مواد وطرق البحث:

الحيوانات: أجريت الدراسة الحالية على ٣٠ ذكر جرذ ابيض بالغ وقد تم تقسيم الحيوانات إلي ثلاث مجموعات مجموعة ضابطة لكل المجموعات الاخري شملت هذه المجموعة ١٠ جرذان.

مجموعة هشاشة العظام تضمنت هذه المجموعة ١٠ جرذاًن، تم حقن كل جرذ بالديكساميثازون (٧ مللي جرام لكل كليوجرام/ مرة واحدة أسبوعياً عن طريق الحقن العضلي حوالي ١,٤ مجم/ جرذ او٠,٣٥ مللي جرام من امبول الديكساميثازون) لمدة ٤ أسابيع لاستحثاث هشاشة العظام.

مجموعة هشاشة العظام والكبريت العضوي تضمنت هذه المجموعة ١٢جرذاً، تم حقن كل جرذ بالديكساميثازون (٧ مللي جرام لكل كليوجرام/ مرة واحدة أسبوعيا عن طريق الحقن العضلي) لمدة ٤ أسابيع لاستحثاث هشاشة العظام ثم تم اعطاء الكبريت العضوي (٤٠٠ مجم/كيلوجم/يوميا) مذابة فى ٠,٢ مللى لتر من محلول الملح عن طريق أنبوبة معدية لمدة ٤ أسابيع أخرى. في نهاية التجربة تم وزن الجرذان وجمع عينات الدم لتخضع للدراسة الكيميائية ومن واستخراج عظمه الساق الكبري ثم إعداد شرائح مجهرية للدراسة الهستولوجية، الهستوكيميائية مناعية والمسح المجهري الالكتروني.

النتائج: وجدت بعض اضطرابات في التركيب الهستولوجي والدقيق لعظام الساق في جرذان هذه مجموعة هشاشة العظام ونقص ملحوظ في عدد الخلايا العظمية المتأثرة بدلائل تكون العظم (osteopontin) وزيادة عدد الخلايا العظمية المتأثرة بدلائل ارتشاف وهشاشة العظم (RANKL). كما وجد زيادة في عدد الخلايا العظمية المتأثره بدلائل وجود إجهاد تأكسدي (TUNEL)عند مقارنتها بالمجموعة الضابطة العظام وأن استخدام الكبريت العضوي أحدث تحسناً ملحوظاً في نسيج العظام وربما يرجع ذلك لوجود مجموعة الكبريت به ولخواصه المضادة للأكسدة والمحفزة لبناء العظام.