The Protective Role of Simvastatin on Methotrexate-Induced Bone Injury in Adult Albino Rat

Original Article

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ABSTRACT

Background: Methotrexate (MTX) is a folic acid antagonist and chemotherapeutic agent widely used in cancer treatment. It is used as first line therapy in treatment of rheumatoid arthritis (RA). MTX was known to cause bone defects in the form of reduced mineral density (BMD) and bone fractures. Simvastatin (SIM) is widely used for cardiovascular diseases.

Aim of Work: To investigate the possible protective role of SIM on MTX induced bone injury in rats.

Material and Methods: Forty adult male albino rats were divided into four groups; Control group, SIM-treated group, MTX group and MTX + SIM group. MTX was given by subcutaneous injection as 0.65 mg/kg/day for two separate 5 days. SIM was administered orally as 25 mg/kg/day one month prior to and during the MTX course. At the end of the experiment, blood samples were collected for levels of osteocalcin (OSC) and alkaline phosphatase (ALP). All rats were sacrificed and specimens from their femurs were examined.

Results: examination of MTX group showed marked thinning of the periosteum, and widening of bone marrow spaces. There was an apparent decrease in the number of osteocytes, together with an apparent increase in the number of osteoclasts. There was a significant decrease in the serum level of OSC together with a highly significant increase in the serum level of ALP in the MTX group as compared to the control group. On administration of SIM with MTX, there was marked improvement in most of the histological and serological parameters.

Conclusion: SIM could be used as a protective measure for MTX-induced bone defects.

INTRODUCTION

The use of chemotherapy to treat cancer began at the start of the twentieth century[1]. Both clinical and animal studies reported that chemotherapy can cause adverse effects on bone, mainly on bone remodeling and bone mass[2].

Methotrexate (MTX) is a folic acid antagonist and chemotherapeutic agent widely used in cancer treatment[3]. It is the most commonly used anti-metabolite agent for childhood cancers, and it was proved to be effective in other malignancies such as choriocarcinoma and osteogenic sarcoma[4, 5]. MTX is used as first line therapy in treatment of rheumatoid arthritis (RA) and other inflammatory diseases such as psoriasis and dermatomyositis[6].

Although MTX was proved to be effective in the treatment of both RA and many cancers, discontinuation is common due to occurrence of its adverse effects[7]. There are many evidences that low dose of MTX can reach high concentration in bone as reported by Bologna *et al*., who found that MTX concentration in cortical and trabecular bone was 13 and 11.5 fold higher than plasma concentration of same drug[8]. MTX was known to cause bone defects in the form of fractures and bone ingrowth defects especially in pediatric patient[9]. High MTX doses could contribute to bone growth impairment, reduced bone mineral density (BMD) and bone fractures[10,11]. Osteoporotic fractures associated with MTX treatment were reported for the first time in 1970 in patients treated with a high dosage of MTX for acute leukemia. This "MTX osteopathy" was characterized by osteoporosis, bone pain, and compression

fractures[12]. Also, women given chemotherapy consisting of MTX for breast and ovarian cancers may suffer from bone loss[13].

In addition, due to increased usage of anti-cancer drugs and their significant effect on skeletal health, it becomes important to explore potential supplementary treatments that may be useful in protecting bone during cancer chemotherapy[14]. Folinic acid, soy products rich in isoflavone, genistein, and fish oils rich in omega-3 polyunsaturated fatty acids were previously used to improve bone health[15- 18].

Statins are best known as competitive inhibitors of hydroxyl-methyl-glutaryl-CoA (HMG-CoA) reductase that reduce cholesterol synthesis, therefore they are widely used for the treatment of hypercholesterolemia[19]. Pacheco-Pantoja and Alvarez-Nemegyei assumed that the use of statins in the treatment of metabolic bone diseases in humans has not yet reached the status of solid scientific dogma, even though it had been about fifteen years since the first experimental evidence in an animal model of the osteomodulador effect of statins was reported by Mundy *et al.*[20, 21].

This study aimed to investigate the possible protective role of Simvastatin on MTX induced bone injury in adult male albino rats.

MATERIAL AND METHODS

• *Animals*

Forty adult male albino rats of the Sprague Dawely strain obtained from the animal house of Medical Research Centre, Faculty of Medicine, Ain Shams University. They were aged 46- months with average weight 200- 250 gm. All rats were bred and provided with food and water. All animal procedures were performed in accordance with the recommendations for the proper use and care of laboratory animals.

• *Drugs*

MTX vial was obtained from EIMC United Pharmaceuticals Company, Cairo-Egypt. The dose was calculated as 0.65 mg/kg/day for two separate 5 days courses (5 days on/9 days off), and given by subcutaneous injection[4].

SIM was obtained in the form of tablet (Zocor ®, MSD). The dose was 25 mg/kg/day orally by gastric tube for 2 months[22].

• *Experimental Design*

Animals were divided into four groups; control group (Group A; N=5), SIM-treated group (Group B; N=5),

MTX group (Group C; N=15) and MTX + SIM group (Group D; N=15). SIM (25 mg/kg/day) was administered orally by gastric tube one month prior to and during the MTX course. At the end of the experiment, blood samples were collected and serum levels of osteocalcin (OSC) and alkaline phosphatase (ALP) were determined. The animals were sacrificed and femurs were dissected free of soft tissue, washed with saline. Upper ends (including the proximal part of metaphysis) were cut and fixed immediately in glutaraldehyde at 4ºC for 24 hours. Un-decalcified sections were obtained by dehydrating the bones in acetone for 36 hours, followed by immersion in xylene for 24 hours. Thereafter, the bones were embedded in methyl methacrylate for 3 days. 10 µm Sections were cut by a Polycut S microtome (Reichert-Jung microtome, Model 2050, Leica, Deerfield, IL, USA)[23]. Sections were then stained with Haematoxylin and Eosin[24, 25]. Bcl-2 used for immunohistochemical analysis. Immunohistochemical staining for Bcl-2

Formalin-fixed and paraffin-embedded bone sections were de-waxed in xylene and then dehydrated with a graded ethanol series. Endogenous peroxidase was inactivated by incubation in 3% H2O2 for ten minutes. Sections were placed in citrate buffer for antigen retrieval by microwave heating. Non-specific binding was blocked by incubation in goat serum, and then sections were incubated with rabbit anti-Bcl-2 polyclonal antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes in phosphate buffer saline (PBS) for 15 minutes, the slides were incubated with the appropriate secondary antibody at room temperature for 30 minutes. Avidinbiotin- peroxidase complex was used to amplify the reactions, and 3, 3'- diaminobenzidine hydrochloride/ H2O2 (Sigma Aldrich Co.) to develop them. The sections were finally counterstained with hematoxylin. Under the optical microscope (Olympus, Tokyo, Japan) brown particles or patches seen in the cytoplasm indicated positive staining[26].Statistical analysis

The mean serum levels of OSC and ALP of all the studied groups of rats were presented as mean and standard deviation, and were compared using Student`s t-test. The level of statistical significance was defined as $P \leq 0.05$ where Scheffe's multiple comparison was used to indicate the significant differences among all the studied groups. The statistical analysis of data was carried out using Excel and statistical package for the Social Science Software, version 11 (SPSS, Inc., Chicago, USA.) on an IBM compatible computer[27].

RESULTS

Histological Findings

Examination of bone sections from the control group and SIM-treated group (Groups A & B) showed similar results. Under the periosteum, bones were formed of

outer shell of compact bone and inner trabeculae of cancellous bone (Fig. 1). The periosteum was formed of an outer fibrous layer and an inner osteogenic layer. The outer fibrous layer was formed of dense collagen fibers with fibroblasts in between, and the inner osteogenic layer was made up of spindle shaped osteoprogenitor cells. The sub-periosteal area showed grooves contained osteoprogenitors, osteoblasts, and blood capillaries. The compact bone showed many osteocytes inside the lacunae arranged around centrally located Haversian canals (Fig. 2). The cancellous bone was formed of network of thick branching bone trabeculae composed of irregular bone lamellae and bone marrow spaces between them. Rounded active osteogenic cells were observed lining the bone trabeculae indicating bone formation (Figs 3 & 4).

Examination of sections of bony tissues from MTX group (Group C) showed marked thinning of the periosteum especially the fibrous layer. The outer compact bone showed an apparent decrease in the number of osteocytes as compared to control group. Some osteocytes had wide lacunae and others showed pyknotic nuclei (Figs. 5 & 6). The inner cancellous bone in most of specimens lost their normal architecture and showed thin widely separated trabeculae and widening of bone marrow spaces. Some trabeculae showed refractile areas indicating bone loss and necrosis. Most of the

osteogenic cells linning the trabeculae appeared flat indicating inactive bone formation (Figs. 7 & 8). Many multinucleated acidophilic osteoclasts were observed at the site of bone resorption lining the irregular bone trabeculae (Fig. 9).

Examination of sections of bone tissues from MTX + SIM group (Group D) showed that the periosteum return nearly to its normal thickness with apparent increase in the number of osteocytes (Fig. 10). The bone trabeculae still thin but the osteoclasts were few as compared to MTX group (Fig. 11).

The control & SIM-treated groups (Groups A & B) showed negative reaction to Bcl-2. There waspositive reaction for Bcl-2 in the mesenchymal cellsresponsible for bone formation in the experimentalgroups, which was strong in MTX group (Group C) andweak in MTX + SIM group (Group D) (Figs. 12- 14).

Serological Findings

The mean OSC and ALP among the studied groups of rats are presented in Table-1. A significant decrease in the serum level of OSC was noticed in the MTX group as compared to both the control and MTX+SIM groups. Also, there was a highly significant increase in the serum level of ALP in both MTX and MTX+SIM groups as compared to the control group.

Table 1: Comparison of serum OSC and ALP among the studied rat groups

Parameters	Control (Group A) Mean ± SD (n= 5)	SIM-treated group (Group B) Mean ± SD (n= 5)	MTX group (C) Mean ± SD (n= 15)	MTX + SIM group (D) Mean ± SD (n= 15)	P value
Osteocalcin (OSC) (ng/ml)	4.515± 0.258	4.42± 0.27	2.428± 0.734	4.112± 1.689	P1=0.0361* P2=0.912 P3=0.0349*
Alkaline phosphatase (ALP) (u/l)	140.85±12.351	140.76± 12.338	250.48± 7..979	245.13±6.079	P1=0.002** P2=0.002** P3=0.7790

P1: Group A version group C.
* Significant; *P*<0.05.
P2: Group A version group D.
**Highly significant; *P*<0.01.
P3: Group C version group D.
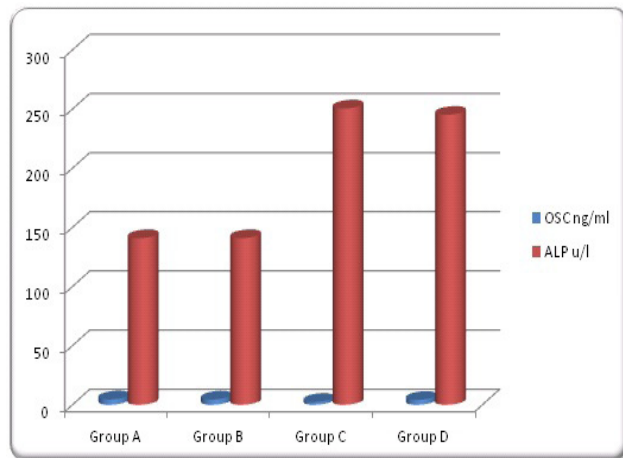
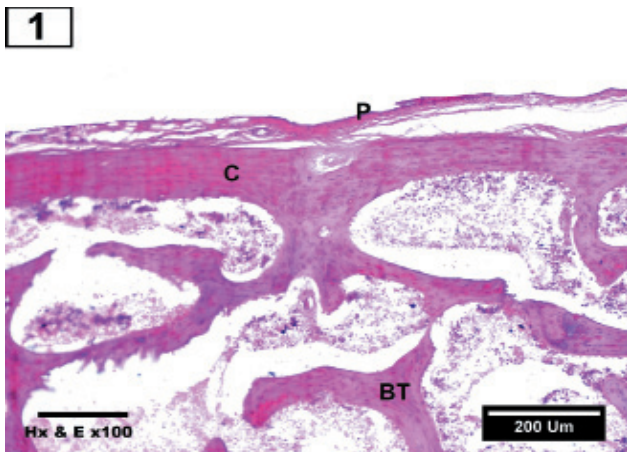**Histogram 1:** Serum OSC and ALP among the studied rat groups.

Fig. 1: A photomicrograph of a section of the shaft of femur of a control rat, showing outer shell of compact bone (C), covered by periosteum (P) and inner trabeculae of cancellous bone (BT).
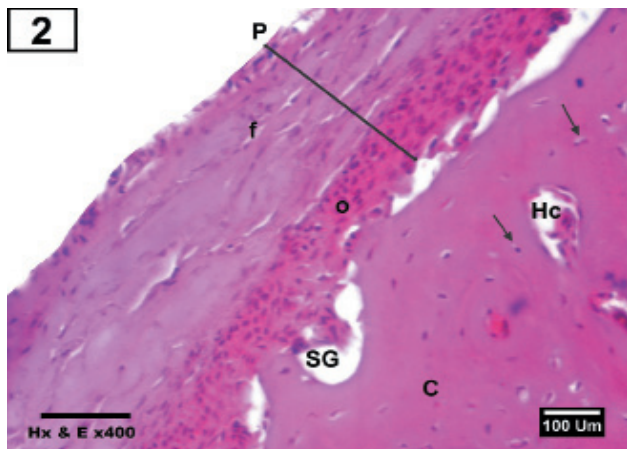H&E X 100

Fig. 2: A photomicrograph of a section of the shaft of femur of a control rat, showing the periosteum (P) formed of an outer fibrous layer (f) and an inner osteogenic layer (o). Note the subperiosteal grooves (SG). Note also osteocytes (↑) inside their lacunae around a centrally located Haversian canal (Hc) in the compact bone (C).
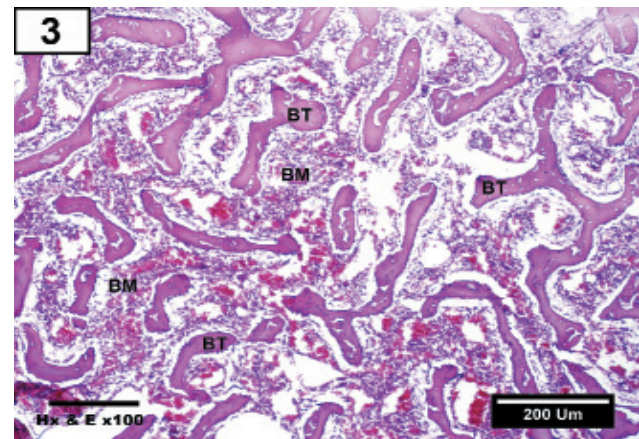H&E X400

Fig. 3: A photomicrograph of a section of the shaft of femur of a control rat, showing the inner cancellous bone formed of branching bone trabeculae (BT), with bone marrow (BM) spaces in between.
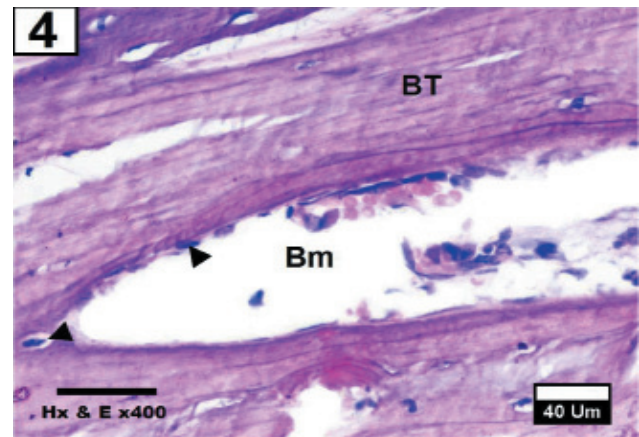H&E X 100

Fig. 4: A photomicrograph of a section of the shaft of femur of a control rat, showing thick branching bone trabeculae (BT) enclosing the bone marrow (Bm) spaces. Note the rounded osteogenic cells lining the trabeculae (▲).
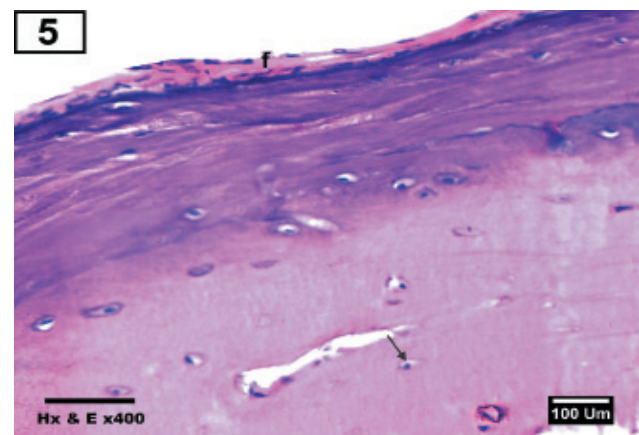H&E X 400

Fig. 5: A photomicrograph of a section of the shaft of femur of a rat of MTX group (C), showing marked thinning of the outer fibrous layer of the periosteum (f). Notice an apparent decrease in the number of osteocytes (↑) compared with that of the control group.
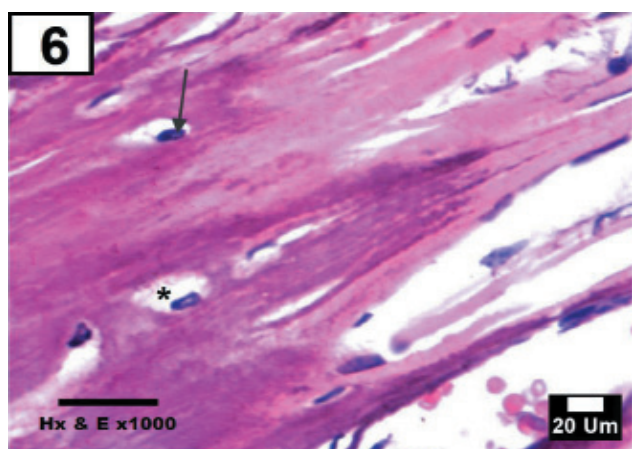H&E X 400

Fig. 6: A photomicrograph of a section of the shaft of femur of a rat of MTX group (C), showing osteocytes had wide lacunae (*). Notice an osteocyte with pyknotic nucleus (↑). H&E X 1000
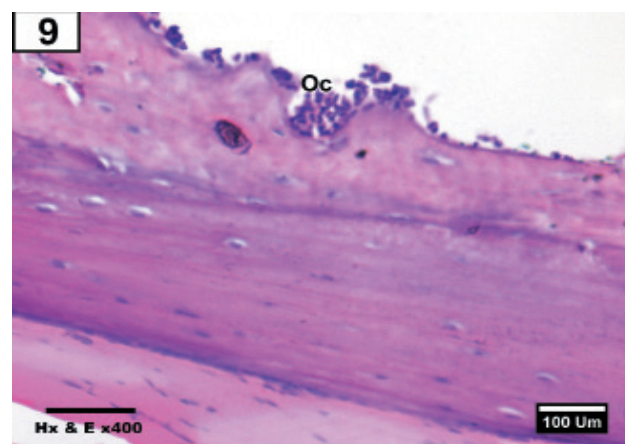

Fig. 9: A photomicrograph of a section of the shaft of femur of a rat of MTX group (C), showing multinucleated acidophilic osteoclasts (Oc) inside cavities at the site of bone resorption.
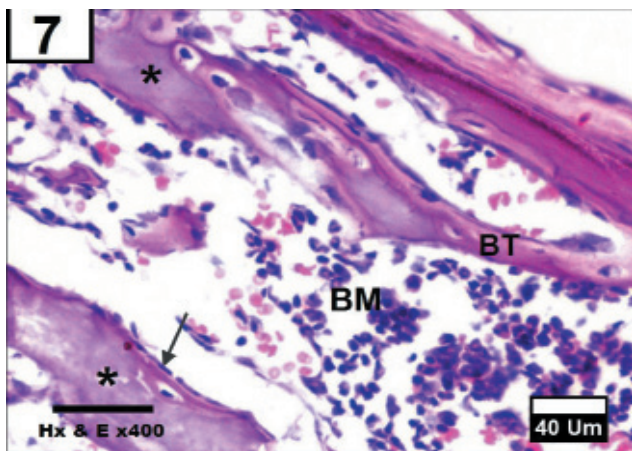H&E X 400

Fig. 7: A photomicrograph of a section of the shaft of femur of a rat of MTX group (C), showing thin bone trabeculae (BT) surrounding wide bone marrow spaces (Bm). Notice refractile areas (*) appeared inside the trabeculae. Note also flat osteogenic cells lining the trabeculae (↑).   H&E X 400
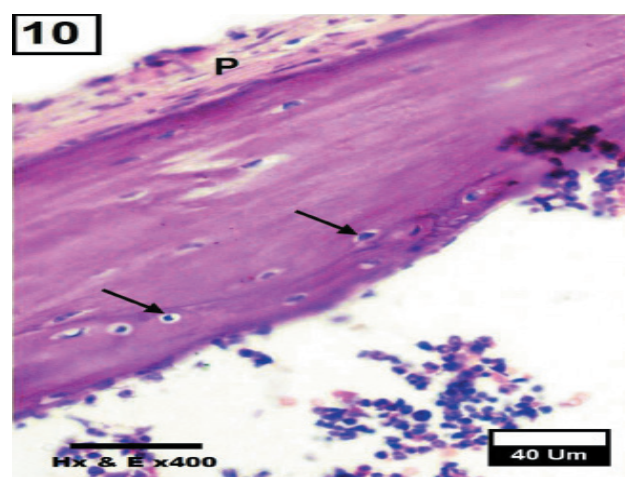

Fig. 10: A photomicrograph of a section of the shaft of femur of a rat of MTX + SIM group (D), showing normal thickness of the periosteum (p), with an apparent increase in the number of osteocytes (↑) compared with that of the MTX group (C).
H&E X 400
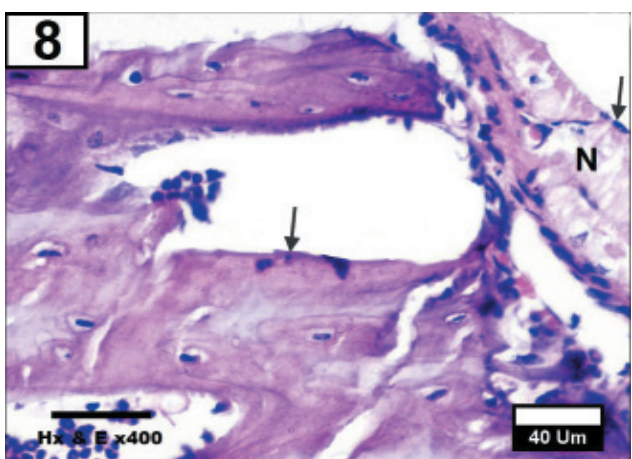
Fig. 8: A photomicrograph of a section of the shaft of femur of a rat of MTX group (C), showing areas of bone necrosis (N) inside the trabeculae. Note flat osteogenic cells (↑) lined the trabeculae.
H&E X 400

Fig. 11: A photomicrograph of a section of the shaft of femur of a rat of MTX + SIM group (D), showing that the bone trabeculae (BT) still thin but the osteoclasts (↑) were apparently decreased in number as compared to MTX group (C).
H&E X 400

Fig. 12: A photomicrograph of a section of the shaft of femur of a control rat, showing negative reaction to Bcl-2 in the bone marrow.   Bcl-2 X 400

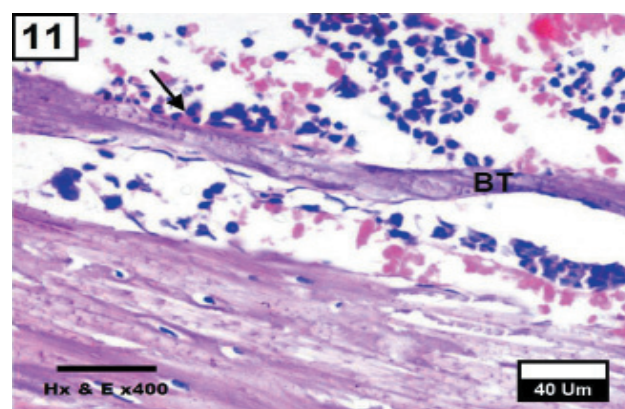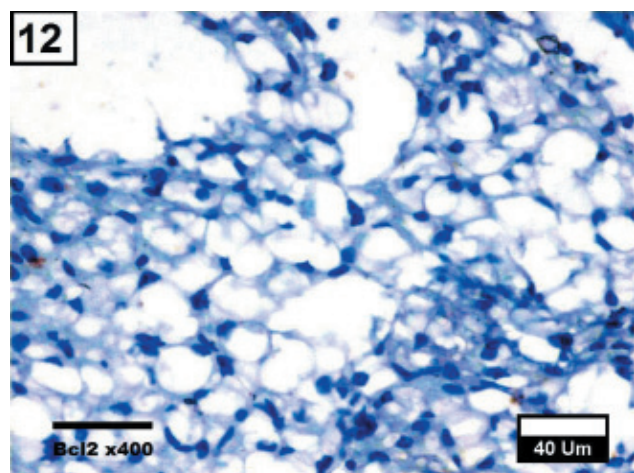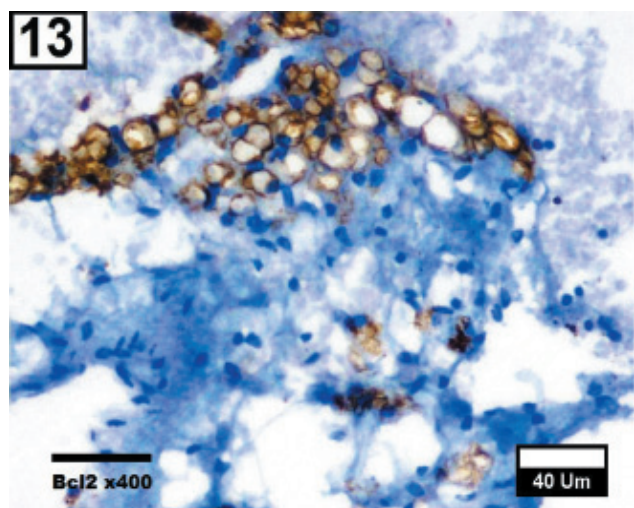**Fig. 13:**.A photomicrograph of a section of the shaft of femur of a rat of MTX group (C), showing strong positive reaction to bcl2 in the mesenchymal cells responsible for bone formation in the bone marrow.   Bcl-2 X 400
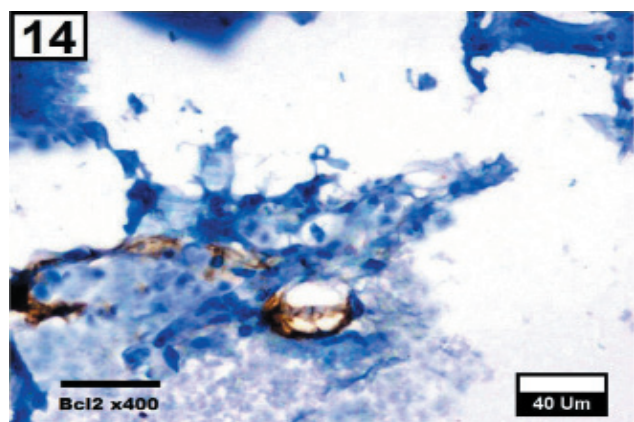

Fig. 14: A photomicrograph of a section of the shaft of femur of a rat of MTX + SIM group (D), showing weak positive reaction to bcl2 in the mesenchymal cells responsible for bone formation in the bone marrow.   Bcl-2 X 400

DISCUSSION

MTX, a folate antagonist, is commonly used at high doses for the treatment of malignancies[28]. It is associated clinically with bone pain, bone loss, increased fracture risks and osteoporosis which are a serious concern[9, 29].

In the present study, sections from the upper end of femur of the control and SIM-treated groups revealed the same histological pattern as described by El-Morsi and his colleagues[30].

In our study, bone affection appeared at cellular level in MTX group (Group C). There was marked thinning of the periosteum specially the fibrous layer. An apparent decrease in the number of osteocytes was observed and some of them showed apoptotic nuclei. Xian *et al*. attributed the reduction of collagen fibers in the bony cortex to reduction of osteocytes proliferation and induction of osteocytes apoptosis through the Fas/Fas-L death receptor pathway induced by MTX treatment[31].

In the current research, histological examination of the cancellous bone of MTX group showed thin, widely separated bone trabeculae with widening of bone marrow spaces. Previous studies assumed that osteoporosis could be manifested as thinning of bone trabeculae or as removal of some bone trabeculae with remaining trabeculae of normal thickness[30, 32, 33]. Parfitt *et al*. had reported that bone loss in osteoporosis was initiated by increased depth of erosion cavities, which would lead to focal disruption of the trabeculae, followed by progressive enlargement of the perforations and subsequently, would lead to conversion of the trabecular plates to widely separated rods and bars. This was termed as the button phenomenon and considered as a characteristic feature of osteoporosis[34]. Reiner and Bertha described this phenomenon as if the trabeculae were formed of small islands of bone[35]. Also, MTX therapy might lead to overall reduction of trabecular bone volume[36]. Experimental studies of MTX induced bone defects revealed that apart from the reduced osteoblast number and trabecular bone volume; there was a significant increase in marrow adiposity[31, 37].

We found an apparent increase in the number of osteoclasts accompanied with resorptive activity. Noteworthy, Mosekilde *et al*. considered the increased number of resorptive surfaces as the most dramatic finding in MTX osteoporosis[38]. Another possible mechanism for MTX-induced decrease in bone mass is the increased formation of bone resorptive cells (osteoclasts) and the alteration to the bone remodeling balance in favor of bone resorption[39].

SIM is widely used for cardiovascular diseases[40]. However, statins have pleiotropic therapeutic effects including vasodilatory, antithrombotic, antioxidant, anti-inflammatory, and immunosuppressive actions[41]. Mundy was first reported statins as potent stimulators of bone formation invitro. Here, we further demonstrate the protective effect of SIM on a rat model of MTX-induced bone injury[21].

Histological findings of bony sections from MTX + SIM group (Group D) showed that the periosteum retained its normal thickness with an apparent increase in the number of osteocytes in the bone lamella, together with decreased number of osteoclasts as compared to MTX group. These findings were in coincidence with Hanayama *et al*., where Fluvastatin significantly attenuated osteoclast differentiation and activation through a blockade of the classical mevalonate pathway and an antioxidant action, leading to prevention of osteoporosis[42]. Similarly, Moon *et al*. proved that SIM acted as an osteoclastogenesis inhibitor by suppressing reactive oxygen species-mediated signaling pathways. Therefore, SIM has both anti-catabolic and anabolic effect on bone metabolism[43]. The potential positive effect of statins on bone formation can be explained by three mechanisms; (a) Promotion of osteogenesis. (b) Suppression of apoptosis of osteoblasts. (c) Inhibition of osteoclastogenesis[44].

On the other hand, Reid and Hague looked retrospectively at 9014 patients treated by pravastatin for ischemic heart disease and found no significant effect of statins on fracture risk or BMD when analyzed after follow-up of 6 years[45].

The Bcl-2 gene encodes a protein located in the nuclear membrane, on the inner surface of mitochondria, and the endoplasmic reticulum. This gene has been reported to prolong the survival of cells by specifically inhibiting apoptosis[46]. Moreover, the Bcl-2 protein plays an important role in preserving stem cells[47]. In the current work, bone sections of the MTX group (Group C) showed strong positive staining for the anti-apoptotic Bcl2 protein that is supposed to be increased in case of bone injury to protect the mesenchymal cells responsible for bone formation in the bone marrow by inhibiting apoptosis. On the other hand, SIM administration with MTX could inhibit apoptosis indicated by weak staining for Bcl-2 in the control group. In context, MTX induced apoptosis at days 1 and 2 after MTX treatment, as judged by morphological criteria and TUNEL staining[48]. Similarly, apoptosis analysis by insitu nick translation labeling revealed that MTX treatment induced apoptosis among osteoblasts in bone metaphysis[39].

OSC, synthesized in the skeleton is considered a highly sensitive marker for bone formation[49].

We found a significant decrease in serum OSC in the MTX group compared to the control and MTX+SIM groups. In context, serum levels of OSC were significantly lower in the patients with postmenopausal osteoporosis than in the control subjects[50]. On the contrary, Watanabe *et al*. investigated the effects of fluvastatin on patients treated for hypercholesterolemia over a period of one year and found that serum OSC did not change even after a year[51].

ALP, a non specific bone formation marker, is present in all tissues throughout the entire body, but particularly concentrated in liver, bile duct, kidney, bone, intestinal mucosa and the placenta[52, 53]. Patel *et al*. noticed a highly significant increase in the level of ALP in MTX-treated rats which is consistent with our findings. Such significant increase in ALP may be attributed to damage of the body tissues by MTX resulting in liberation of ALP in serum[54].

CONCLUSION

SIM could be considered as an effective treatment for MTX-induced bone defects. However, this needs more studies with appropriate experimental designs, sample sizes suitable for relevant results and analysis.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

الدور الوقائى لعقار السيمفيستاتين على اصابات العظام الناتجة عن تعاطى عقار الميثوتريكسيت فى الجرذان البالغين

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المقدمة: يعتبر عقار الميثوتريكسيت مضاد لحمض الفوليك و من العلاجات الكيميائية التى تستخدم على نطاق واسع في علاج السرطان. و يتم استخدامه كعلاج الخط الأول لعلاج التهاب المفاصل الروماتويدي و الأمراض الالتهابية الأخرى مثل مرض الصدفية و التهاب الجلد والعضلات. ومن المعروف أن عقار الميثوتريكسيت يسبب تشوهات العظام في شكل تخفيض كثافة العظام المعدنية و كسور العظام. يستخدم عقار السيمفيستاتين على نطاق واسع كعلاج لأمراض القلب والأوعية الدموية. و تشتهر مجموعة الستاتين بأن لها العديد من الآثار العلاجية و تشمل توسيع الاوعيه الدمويه، و كذلك تعتبر كمضادات للأكسدة و للالتهابات و لها تأثير معدل للعظام.

الهدف: توضيح الدور الوقائى المحتمل لعقار السيمفيستاتين على اصابات العظام الناتجة عن تعاطى عقار الميثوتريكسيت فى الفئران البالغين.

مواد وطرق البحث: استخدم فى هذه الدراسة أربعون من الجرذان الذكور البالغين، تم تقسيمهم إلى اربع مجموعات : مجموعتان ضابطتان مكونتان من خمسة جرذان لكل منهما، و مجموعة مكونة من خمسة عشر جرذاً تم إعطائها عقار الميثوتريكسيت فقط عن طريق الحقن تحت الجلد بجرعة 0،65 ملغ / كغ / يوم على دورتين كل منهما لمدة 5 أيام حقن و 9 أيام إيقاف، ومجموعة رابعة مكونة من خمسة عشر جرذاً تم إعطائها عقار السيمفيستاتين عن طريق الفم بجرعة 25 ملغ / كغ شهر واحد قبل و أثناء اعطاء عقار الميثوتريكسيت. و بعد نهاية التجربة تم تجميع عينات الدم من الجرذان من المجموعات المختلفة للدراسة المصلية لنسبة الأوستيوكالسين و الفوسفاتيز القاعدى بالدم. ثم تم ذبح الجرذان و تم اخذ عينات من عظم الفخد و تجهيزها للفحص المجهرى.

النتائج: أظهر الفحص المجهرى لعظام الجرذان التى أعطيت عقار الميثوتريكسيت فقط وجود ترقق ملحوظ في السمحاق، و كذلك هناك ترقق و تباعد ملحوظ فى الترابيق العظمية مع اتساع مساحات نخاع العظام. و قد لوحظ ايضا وجود انخفاض واضح في عدد الخلايا العظمية، جنبا إلى جنب مع زيادة واضحة في عدد الخلايا الآكلة في موقع ارتشاف العظام المبطن للترابيق العظمية. كما أظهرت النتائج أيضا" وجود زيادة ذو دلالة إحصائية فى نسبة الأوستيوكالسين و الفوسفاتيز القاعدى فى دم الفئران. و قد لوحظ تحسن معظم المعلمات الهيستولوجية و المصلية فى الفئران التى اعطيت عقار السيمفيستاتين مع عقار الميثوتريكسيت بما في ذلك؛ شكل و تنظيم الخلايا و الترابيق العظمية، و كذلك عدد كل من الخلايا العظمية و الخلايا الآكلة ونسبة الأوستيوكالسين فى الدم.

الاستنتاج: إعطاء عقار السيمفيستاتين يعتبر علاجا وقائيا لاصابات العظام الناتجة عن تعاطى عقار الميثوتريكسيت فى الجرذان البالغين.