Histological, histochemical and immunohistochemical study of the Effect of chronic low dose of methotrexate and folinic acid on long bone of adult male guinea pigs

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Abstract: Introduction: Methotrexate is anti-folate metabolite. It is used in low dose as first line therapy for treatment of rheumatoid arthritis. It is a main treatment for childhood acute lymphoblastic leukaemia. Several studies reported its wide range side effects such as gastrointestinal intolerance and bone defects in the form of fracture and bone growth defect**. Aim of the work:** the current study examined the structural cellular damages to the bone caused by long term low- dose methotrexate treatment, as well as the potential protective effects of folinic acid supplementary treatment. **Material and Methods:** Forty adult male guinea pigs were subjected to experiment for nineteen days. The animals were divided into 4 groups, each of 10 guinea pigs as follow: **Group I**: (control group) were injected subcutaneously (distilled water and normal saline). **Group II**: received Folinic acid (1 mg/kg b.wt./ day) was injected intraperitoneally. **Group III**: received methotrexate (0.65 mg/kg b.wt/ day subcutaneously injected) for two separate 5 days courses (5 days on/9 days off/5 days on). **Group IV**: received Folinic acid (1mg/kg b.wt./ day) intraperitoneally injected 6 hrs after the administration of same dose of methotrexate as group III.On day twenty, the guinea pigs were anaesthetized with ether then sacrificed. Right tibias were removed from guinea pig and dissected free from soft tissues and subjected to histological, histo-chemical and immunohistochemical studies. **Results**: Histologically and histochemically, tibia of methotrexate treated guinea pig showed that, long term use of low dose methotrexate can cause oesteocytes dysfunction, bone erosion, bone damage, suppress bone formation, and increase bone resorption resulting in overall bone loss, via inducing osteocytes apoptosis. There was marked increase of Masson trichrome staining in resorption cavities of bone with reduction of collagen fibers in thin cortex of bone. Immunohistochemically, in methotrexate treated group showed positive immunoreactivity for caspus 3 in bone osteocytes and positive immunoreactivity for CD 68 in numerous bone osteoclasts which increased in number when compared to control group. On the other hand, histological, histochemical and immunohistochimical examination of the tibia of guinea pig protective group treated with both folinic acid and methotrexate displayed improvement in bone structure, reduce methotrexate damage in the bone formation. It prevents osteoblasts from undergoing apoptosis and suppressing methotrexate-induced osteoclast formation. **Conclusion:** Toxic effect of methotrexate should be kept in mind during chronic usage. Folinic acid was advised to be administered in concomitant with methotrexate as it could ameliorate its adverse effect on rat testis.

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Key Words: methotrexate – folinic acid – guinea pig bone

1. Introduction

Methotrexate is anti-folate metabolite which acts by reversibly inhibiting the purine synthesis enzyme dihydrofolate reductase thus inhibiting the synthesis of DNA and RNA **(Minaur *et al.*, 2002; Madhyastha *et al.*, 2008)**. It is used in low dose as first line therapy for treatment of rheumatoid arthritis **(Strand *et al.*, 1999; Fan *et al.*, 2009).** It is a mainstay treatment for childhood acute lymphoblastic leukaemia **(Fan *et al.*, 2012)** and has been shown to be effective in other malignancies such as choriocarcinoma and osteogenic sarcoma **(Mazzantini and Munno, 2000).** Methotrexate has also become an alternative treatment of severe psoriasis due to its anti-inflammatory properties **(Clark *et al.*, 1999).** Additionally, it can be used in the management of ectopic pregnancy **(Tulandi *et al.*, 1991).**

Although methotrexate is proved to be effective in treatment of both rheumatoid arthritis and cancer chemotherapy management, discontinuation is common due to occurrence of it's adverse side effects **(Whittle and Hughes, 2004).** Although low weekly doses of methotrexate has been proven to be effective for treatment of adult and juvenile rheumatoid arthritis with some side effects **(Chan and Cronstein, 2002).** The most common side effects include gastrointestinal intolerance such as nausea, mucositis, stomatitis, diarrhea **(Xian, 2003).** Other toxicities included post-treatment fatigue, headache, dizziness and rheumatoid nodule formation. Serious toxicities such as bone marrow suppression, lung and liver toxicities were fortunately very uncommon **(Weinblatt *et al.*, 1993).** Methotrexate has also known to cause bone defect in

the form of fracture and bone growth defect especially in paediatric patient **(Mandel *et al.*, 2004).**

There is evidence that, even low dose of methotrexate reaches high concentration in the bone and synovial membrane as reported by **Bologna *et al.* (1993)** who found methotrexate concentration in cortical and trabecular bone was 13 and 11.5 fold higher respectively than plasma concentration of same drug.

Some researchers reported that, low dose of methotrexate given to rats weekly for 16 weeks resulted in significant reduction of bone mass **(May *et al.*, 1994).** On the other hand, **Mazzantini and Munno, (2000)** has been observed that, low dose methotrexate can inhibit fracture healing in an animal model and resulted in bone non-union. Moreover, **May *et al.* (1996)** revealed diminished osteoblastic cell function in dose dependent manner on cultured mouse cells exposed continuously to different concentrations of methotrexate. Also, A syndrome of bone pain, radiographic osteopenia and fractures (methotrexate osteopathy) was reported as a complication of short term methotrexate therapy **(Mazzantini and Munno, 2000; Minaur *et al.*, 2002).**

The mechanism of action of methotrexate is not completely understood. methotrexate blocks folic acid-dependent steps in the synthesis of purines and pyrimidines and thereby blocks proliferation of malignant cells. This effect is also responsible for drug toxicity (**Cronstein, 2005**). It has been reported to have negative effect on bone, probably through the inhibition of osteoblasts (**Xian *et al.*, 2008**).It has many anti-inflammatory and cytokine modulating properties. Methotrexate inhibits neutrophil chemotaxis and is able to attenuate the adhesive interaction between leucocytes and endothelial cells in post-capillary venules in acute inflammation **(Asako *et al.*, 1992).**

Folinic acid (FA) is a vitamin B. It is the formyl derivative of tetra-hydrofolic acid which is a metabolite and active form of folic acid. Folinic acid supports healthy methylation pathways, homocysteine metabolism, DNA replication and foetal development **(Van Wijingaargen *et al.*, 2013)**. It is an antidote that has been clinically used to reduce toxicity of high dose methotrexate in soft tissues and has been recently shown to have a protective effect on the skeleton of young rats receiving acute methotrexate chemotherapy **(Fan *et al.*, 2009).** However no long term studies have examined the protective effect of Folinic acid in chronic induced bone damage. Using a chronic model in adult male guinea pigs mimicking the methotrexate regimen in treating rheumatoid arthritis, the current study examined the structural cellular damages to the bone caused by long term low- dose methotrexate treatment, as well as the potential protective effects of Folinic acid supplementary treatment.

2. Material and Methods

Materials:

Animals:

Forty adult male Guinea pigs of an average weight (200 - 250 grams) were selected for this study. The animals were obtained from breeding animal house, Faculty of Science, Alexandia University (Alexandria, Egypt). The animals were acclimated to room temperature (22-25º C) for one week before start of experiment and kept under good hygienic condition. During the study, they were feed standard animal food and tap water *ad libitum*.

Drugs and chemicals:

Methotrexate:

Vial contained 50 mg/ 25ml obtained from EIMC United Pharmaceuticals Company, Cairo – Egypt.

Folinic acid:

800 microgram (0.8 mg) folinic acid tablets were obtained from BioCeuticals Australian Company The calculated dose was dissolved in 2 ml 0.9% normal saline.

Experimental protocol:

Animal experimentations were carried out in an ethical manner following guidelines set by Ethical Committee of Faculty of Science, Alexandria University. The animals were divided into 4 groups, each of 10 guinea pigs:

Group I: guinea pigs in this control group were injected subcutaneously the same amount of vehicle (distilled water and normal saline) along the time of the experiment (nineteen days).

Group II: guinea pigs in this group received Folinic acid (1 mg/kg b.wt./ day) injected intraperitoneal for 19 days **(Fan *et al.*, 2009).**

Group III: guinea pigs in this group were subcutaneously injected methotrexate once daily at a dose (0.65 mg/kg b.wt/ day) for two separate 5 days courses (5 days on/9 days off/5 days on) **(Fan *et al.*, 2012).** On day twenty animals were sacrificed after 24 hours of last dose.

Group IV: guinea pigs in this group were intraperitoneally injected Folinic acid (1 mg/kg b.wt./ day) 6 hrs after the administration of same dose of methotrexate as group III on same course (5 days on/9 days off/5 days on).

On day twenty, 24 hrs after last dose of drug administration, the guinea pigs were anaesthetized with ether then sacrificed. Right tibia was removed from guinea pig and dissected free from soft tissue. Right tibias were fixed in 10% formalin for 24 hrs, then decalcified in formic acid-based decalcifying solution (Immunocal) (Decal corporation, NY) for 7 days at 4°C and processed for paraffin blocks. For light microscopic study paraffin sections were divided and subjected to the following studies:

I-Histopathological study:

Paraffin sections of 5 µm thick were cut and stained with haematoxylin & eosin (H&E) staining for the general architecture of the bone (**Stevens and Wilson, 1996**).

II- Histochemical studies:

Paraffin sections were stained with Masson trichrome (M.T) stain which is specific for collagen fibers (**Stevens and Wilson, 1996**).

III-Immunohistochemical study:

A- Caspus 3 immunostain

Apoptosis in osteocytes was revealed by caspus 3 immunostaining. For this study, paraffin sections 4 µm thick were mounted on glass slides coated on pol-L-lysin, deparaffinized sections were dehydrated and then immersed in 10 M sodium citrate buffer (pH 6.0). Sections were then heated in a microwave oven at 60 ºC for 10 minutes. Endogenous peroxidase were inactivated by incubating sections with 3% hydrogen peroxide and nonspecific reactions were blocked by incubating sections in a solution containing 5% normal human serum and 1% normal goat serum. Then sections were incubated with primary antibody overnight at 4ºC. Activated caspase-3 expression was assessed using a peroxidase-conjugated rabbit monoclonal antibody IgG (Cell signaling Technology, Ipswich, MA) (dilution 1:200). After 3 rinses with phosphate-buffered saline, the sections were incubated with a commercial kit (Pic-Ture TM, Zymed and South San Francisco, CA) for visualization of immunoreactions. Finally, they were rinsed with distilled water and counterstained with Mayer`s hematoxylin. Normal lymphoid tissue was used as positive control. Negative control was performed by omitting primary antibody step consequently no immune-staining occurred **(Lee *et al.*, 2006).**

B- CD68 immunostain

Immunohistochemical staining was done to identify cells of the monocyte /macrophage lineage and is also expressed in osteoclasts. (CD68, is a marker of osteoclasts). CD68 antibody (clone KP1, Dako Corporation, Carpinteria, California, USA) was used in a dilution of 1:50 **(Kunisch, 2003)** then counter-stained with Mayer`s hematoxylin**.** CD68 stain osteoclast cytoplasm**.**Liver Kupffer's cells and lung macrophages were used as positive Control. Negative control was performed by omitting primary antibody step consequently no immune-staining occurred **(McGuinness *et al.*, 1999)**.

VI- Morphometric study:

CD68 immunostained sections of tibia from all groups were examined under light microscopy at high power field by using the interactive measuring menu of image analyzer (Lecia Qwin 500 image analyzer computer system, England) in Faculty of Medicine for Girls, (Al-Azhar University). The number of multinucleated osteoclast lining the endosteum were counted in 10 high power fields (HPF) in each specimen **(Naim, 2011).**

Statistical analysis

Results have been summarized using descriptive statistics. These were presented as mean±standard deviation and compared using Student`s t-test. Significance was set at P-value of less than 0.05 for all comparisons. 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 **(Peat and Barton, 2005).**

3. Results

Histopathologic results:

Control group I:

Histological examination of transverse sections of the diaphysis of the tibia stained with hematoxylin and eosin showed that, bone was presented as a compact structure in a dark acidophilic color. It was formed of an outer shell of cortical bone to which the periosteum was attached to its external surface and endosteum was attached to its internal surface (Fig. 1). The periosteum consisted of two layers. The outer fibrous layer was formed of dense collagenous fibers with fibroblasts in between, and the inner osteogenic layer contained osteo-progenitor cells and osteoblasts. Osteocytes were seen inside their lacunae with densely stained oval nuclei around centrally located Haversian canals (Figs. 2 &3). The cancellous bone was formed of a network of branching and anastomosing bone trabeculae with bone marrow spaces in between (Fig. 4).

Masson's trichrome staining of diaphysis of tibia showed a positive reaction to trichrome staining indicating a collagen-rich stroma with regular arrangement. Blue collagen fibers can be seen in the outer fibrous layer of the periosteum (Figs. 5&6).

To further consolidate our previous observations, immunostaining was done to assess the expressions of caspase-3, an indicator of apoptosis. The control group did not react to caspase-3 staining, while many bone marrow-derived cells positively reacted with caspase-3 staining. The expression of caspase-3 within the osteocytes was negative (Fig.7).

Expression of CD68 is a valuable marker for the identification of cells of the monocyte/macrophage lineage and is also expressed by authentic osteoclasts. Bone samples were immune-histochemistry stained for CD68. MNCs on bone were uniformly CD68 positive, consistent with their identity as osteoclasts. Osteoclasts were identified in their lacunae on the bone surface as immune-histochemically positive cells (Fig. 8).

Group II

Microscopic results of sections of the diaphysis of the tibia of this group resembled those of the control group (group I).

Group III

In haematoxylin and eosin staining, the methotrexate treated group showed histological characteristics of the necrotic bone. It showed pyknotic nuclei of osteocytes and empty lacunae. The numbers and size of empty lacunae were markedly increased compared to control group (Fig 9). Resorption cavities were observed in the cortical bone accompanied with faintly stained matrix (Fig. 10). Bone trabeculae appeared very thin and discontinuous. Widening of bone marrow spaces was also seen. Erosion cavities and faint staining of bone trabeculae were frequently seen (Fig. 11).

Masson's trichrome staining of this group, showed a positive reaction to trichrome staining and marked increase in resorption cavities. The cortex of bone appeared thin with a widening of the cavities and a reduction in green-stained collagen (Figs. 12&13).

Immunostaining with caspase-3 antibodies demonstrated that osteocytes in methotrexate treated group were positive to this staining, accompanied by surrounding bone marrow cell necrosis (Fig. 14).

In this group, CD 68 immunostaining of tibial diaphysis showed numerous osteoclasts in their lacunae on the bone surface compared with the control group (Fig. 15).

Group IV

Examination of the hematoxylin and eosin-stained sections of the tibia diaphysis of this group revealed that bone was apparently similar to that of the control group, but differed from group III. The bone contained osteocytes within their lacunae and many blood vessels. Networks of branching and anastomosing bone trabeculae were seen (Fig. 16).

Masson's trichrome staining of this group showed a positive reaction to trichrome staining indicating a collagen-rich stroma, but no obvious differences in staining intensity were observed compared to control groups. Moreover, resorption cavities markedly reduced. (Figs. 17 & 18).

Immunostaining with caspase-3 antibodies demonstrated that none of the osteocytes of the methotrexate and folinic acid treated group reacted to caspase-3 antibodies (Fig. 19).

In CD 68 immunostaining of this group, osteoclasts were identified in their lacunae on the bone surface which was more or less similar to the control group. (Fig. 20).

Morphometric results:

Statistical analysis showed a highly significant increase in the number of osteoclast in experimental group III (methotrexate treated) in comparison to control group I (P<0.01).

Table 1: Number of osteoclast/ high power field in the different experimental groups

Groups	Mean number of osteoclast /HPF ±SD
Control (Group I)	0.6±0.44
Folinic acid treated group (Group II)	0.7±0.81
Methotrexate treated group (Group III)	2 ± 0.65**
Methotrexate and folinic acid treated group (Group IV)	1.1±0.75

HPF: high power field, SD: Standard deviation.
Comparison was done between Group I (control group) and groups II, III &VI.
*p < 0.05, **p< 0.01, ***p< 0.001, NS: Non significant.

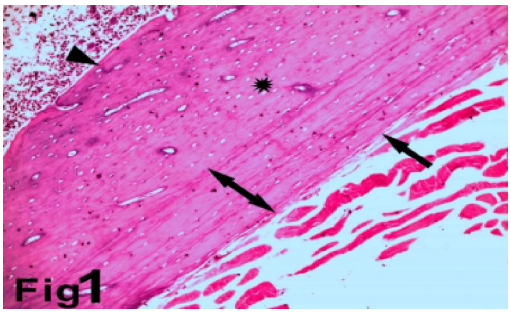

Fig.1. A photomicrograph of a section of a control adult guinea pig tibial diaphysis showing compact bone structure as dark acidophilic matrix covered by periosteum (→).The inner surface is lined by endosteum (►). Lacunae containing osteocytes could be distinguished in the bone matrix (*). The cortical bone is demonstrated **(H&E, X100)**

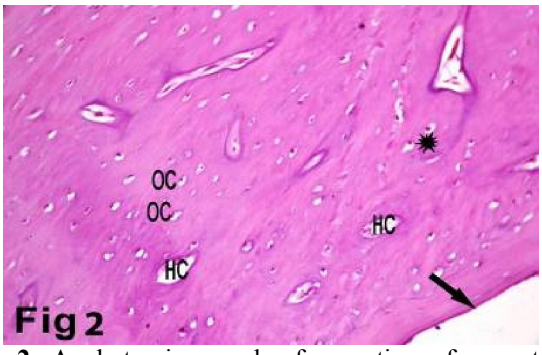

Fig.2. A photomicrograph of a section of a control adult guinea pig tibial diaphysis showing compact bone structure formed of dark acidophilic matrix covered by periosteum (→). The periosteal surface of shaft was lined by osteoprogenitor cells (OC) with flat nuclei. Lacunae containing osteocytes(*) could be distinguished in the bone matrix. Haversian canals containing blood vessels (HC) could be seen. **(H&E, X200)**

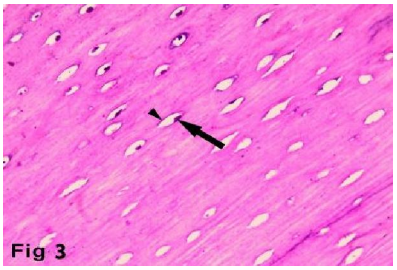

Fig.3. A photomicrograph of a section of a control adult guinea pig tibial diaphysis showing compact bone structure with its acidophilic matrix in which osteocytes (→) resided in their lacunae (►). **(H&E, X400)**

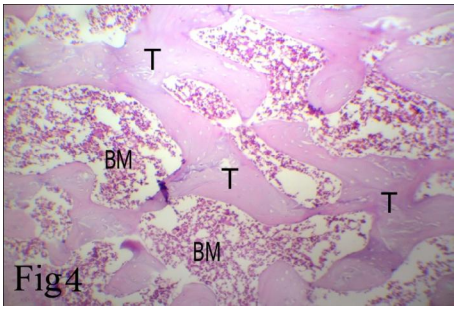

Fig.4. A photomicrograph of a section of a control adult guinea pig tibial diaphysis showing cancellous bone formed of a network of branching and anastomosing bone trabeculae (T) with bone marrow (BM) spaces in between. **(H&E, X100)**

Fig.5. A photomicrograph of a section of a control adult guinea pig tibial diaphysis showing positive reaction to trichrome staining indicating a collagen-rich stroma. The periosteal surface (►), endosteum (*) and lacunae containg osteocytes (→) and cortical bone () could be distinguished. **(Masson's trichrome, X 100)**

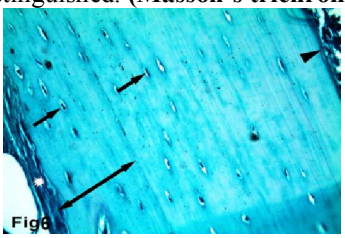

Fig.6. A photomicrograph of a section of a control adult guinea pig tibial diaphysis higher magnification than previous photomicrograph showing positive reaction to trichrome staining indicating a collagen-rich stroma. The periosteal surface (*), endosteum (►) and lacunae containg osteocytes **(→)** and cortical bone () could be distinguished. **(Masson's trichrome, X 400)**

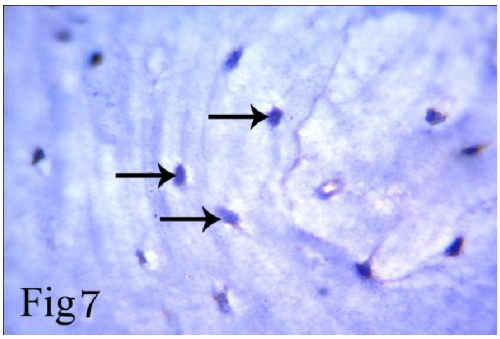

Fig.7. A photomicrograph of a section of a control adult guinea pig tibial diaphysis showing negative immunoreactivity for caspus 3 in osteocytes **(→) (Caspus 3 immunostain, X1000)**

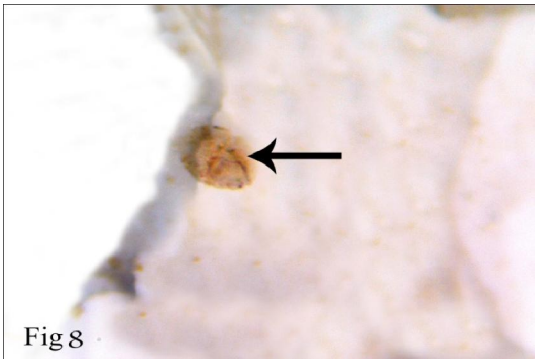

Fig.8. A photomicrograph of a section of a control adult guinea pig tibial diaphysis showing positive immunoreactivity for CD68 in Osteoclasts. They were identified in their lacunae on the bone surface **(CD6 immunostain, X1000)**

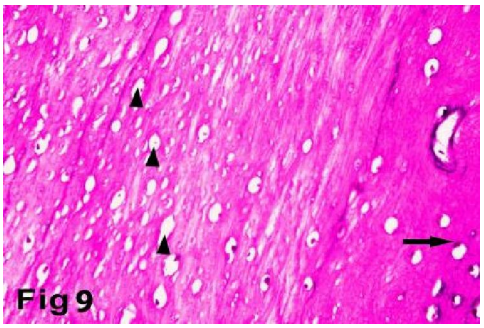

Fig.9. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate showing histological characteristics of necrotic bone. It showed pyknotic nuclei of Osteocytes **(→)** and empty lacunae (►). The numbers of empty lacunae markedly increased. **(H&E, X400)**

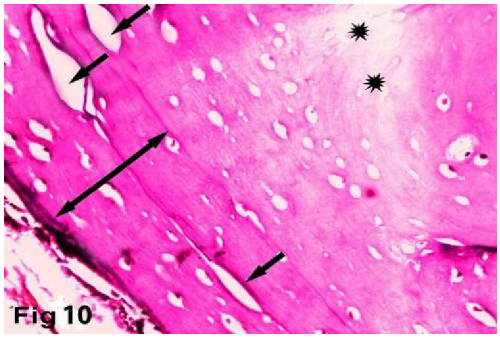

Fig.10. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate showing resorption cavities **(→)** in the cortical bone () and faintly stained matrix (*) **(H&E, X400)**

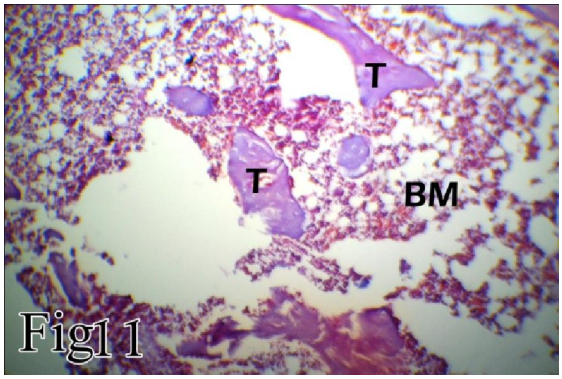

Fig.11. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate showing very thin, discontinuous, widely separated bone trabeculae (T). Widening of bone marrow (BM) spaces is also seen **(H&E, X100)**

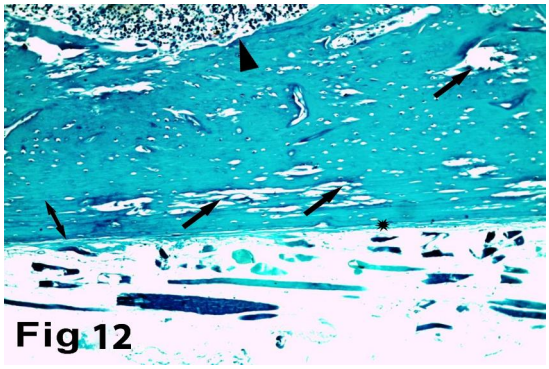

Fig.12. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate showing positive reaction to trichrome staining indicating a collagen rich stroma. The periosteal surface (*), endosteum (►) and marked increase in cavities **(→)** could be distinguished. Outer cortical bone revealed marked decrease in thickness () **(Masson's trichrome, X100)**

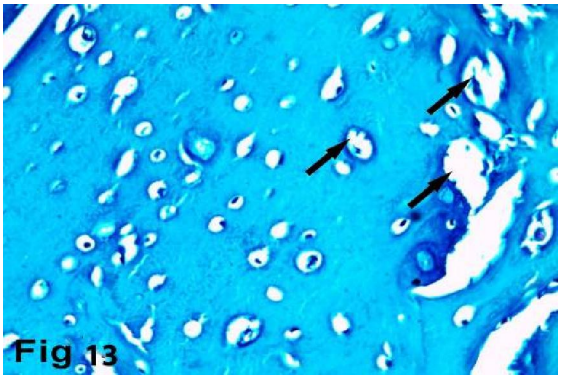

Fig.13. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate showing positive reaction to trichrome staining. Marked increase in cavities **(→)** could be distinguished **(Masson's trichrome, X400)**

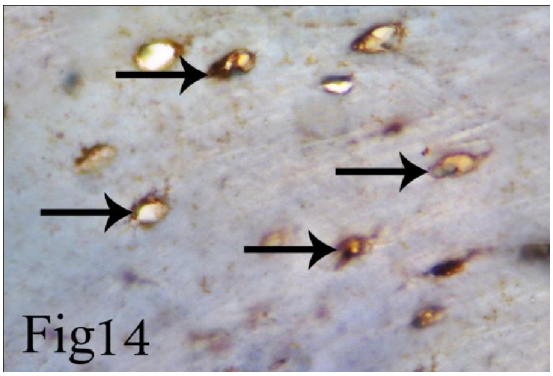

Fig.14. A photomicrograph of a section of an adult Guinea pig tibial diaphysis treated with methotrexate showing osteocytes which are positive immunostain to caspus 3 antibody (→). **(Caspus 3 immunostain, X1000)**

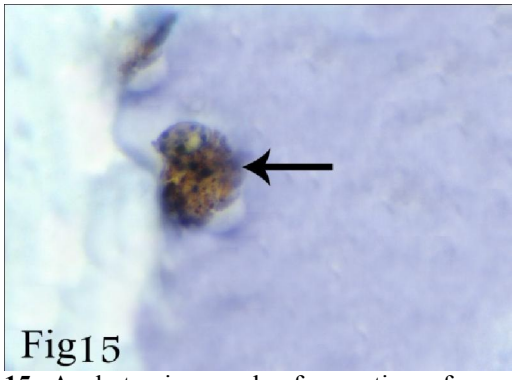

Fig.15. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate showing positive immunoreactivity for CD68 in numerous Osteoclasts which were identified in their lacunae on the bone surface compared with the control group **(CD68 immunostain, X1000)**

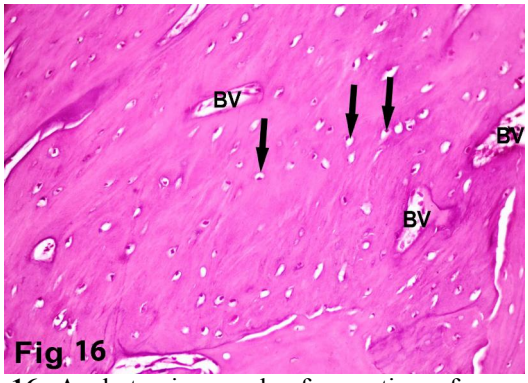

Fig.16. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate combined with folinic acid showing less osteocytes residing in their lacunae (→) and many blood vessels (BV). Note: Networks of branching and anastomosing bone trabeculae were seen. **(H&E, X400)**

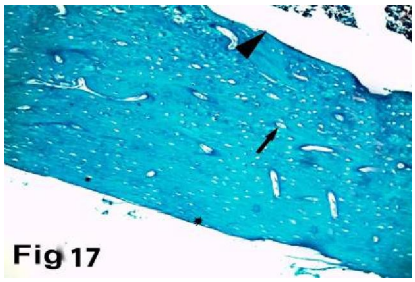

Fig.17. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate combined with folinic acid showing positive reaction to trichrome staining. Lcunae containing osteocytes (→) could be distinguished. The periosteal surface (*), enodsteum (►) could be seen **(Masson's trichrome, X100)**

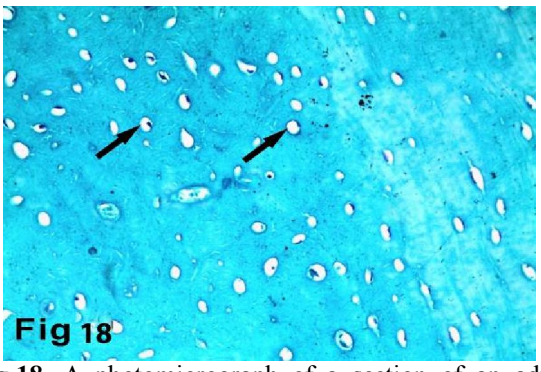

Fig.18. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate combined with folinic acid showing positive reaction to trichrome staining. Lcunae containing osteocytes (→) could be distinguished. No obvious differences in staining intensity or lacunae containin osteocytes were observed compared to control groups.
(Masson's trichrome, X400)

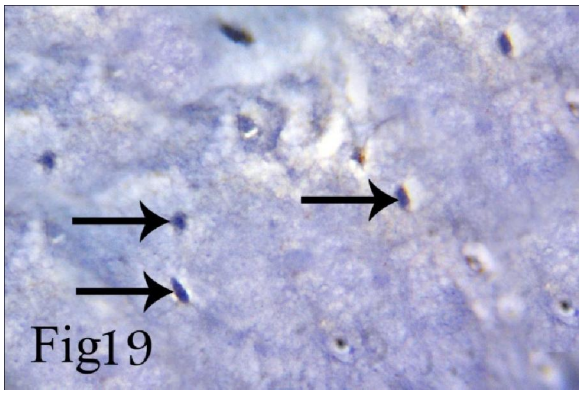

Fig.19. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate combined with folinic acid showing that osteocytes were negative against caspase-3 antibody (►) while other osteocytes were positive against caspase-3 antibody **(Caspus 3 immunostain, X1000)**

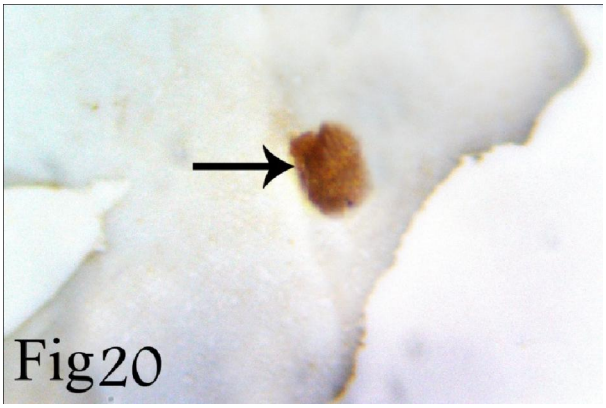

Fig.20. A photomicrograph of a section of an adult guinea pig tibial diaphysis treated with methotrexate combined with folinic acid showing osteoclasts were identified in their lacunae on the bone surface which was mor or less similar to the control group **(CD68 immunostain, X1000)**

4. Discussion

Methotrexate is a folate antagonist, commonly used at high-doses for the treatment of malignancies **(Abromowitch *et al.*,1988; Minaur *et al.*,2002)** and at lower doses for the treatment of inflammatory diseases such as rheumatoid arthritis **(Minaur *et al.*,2002; Cronstein 2005)**.Several organ toxicity had be reported during methotrexate treatment. The bone among non-target tissues that are vulnerable to side effects of this chemotherapeutic and anti-rheumatic drug **(Fan *et al.*, 2009)**. Methotrexate chemotherapy is associated clinically with bone pain, bone loss, increased fracture risks and osteoporosis which were a serious concern **(Brennan *et al.*,1999; Mandel *et al.*,**

2004). Therefore, there is a lack of adjuvant treatments for protecting bone during methotrexate chemotherapy.

The present study aimed to throw light on the potential protective effects of folinic acid supplementary treatment on bone damage caused by long term low dose methotrexate treatment.

In the present work, bone affection was found at a cellular level in methotrexate treated animals by light microscopes. The bone sections revealed marked and significant thinning of the outer shell of compact bone. Histological characteristics of the necrotic bone was shown as apoptotic osteocytes with pyknotic nuclei and empty lacunae. The numbers and size of empty lacunae relatively increased compared with control group. **Nilsson *et al.* (1984)** explained that, methotrexate chemotherapy has disturbed effect on bone metabolism through its inhibitory effect on osteoblast function without altering osteoblast numbers in vivo. Methotrexate induced osteoblastic damage led to a diminished mineralizing surface, mineral apposition rate, and bone formation rate with subsequent osteoblastic apoptosis **(Wheeler *et al.*, 1995)**. Our findings coincided with findings of **H¨ogler *et al.* (2007)** who reported significant damaging effects on bone growth in children with administration of high-dose methotrexate. Other clinical studies, have highlighted osteopenia as a complication for childhood malignancies, characterised by reduced bone marrow density and increased fracture risks **(Van der Sluis *et al.*, 2002; H¨ogler *et al.*,2007)** Moreover, **Halton *et al.* (1996); Crofton *et al.* (1998)** added that, during intensive chemotherapy, children treated with high-dose methotrexate in combination with corticosteroids showed depressed bone formation and enhanced bone resorption.

In the current study, histopathologic examination of the cancellous bone of methotrexate treated group showed very thinned bone trabeculae and discontinuous in some areas with erosion cavities and faintly stained bone marrow, widening of bone marrow spaces. These findings can be attributed to methotrexate effect on a reduction of bone marrow osteoprogenitor cells and suppressed stromal progenitor cell proliferation **(Xian *et al.*,2008; Fan *et al.*, 2009).** Osteoblasts are derived from stromal progenitor cells or mesenchymal stem cells (MSCs), which can differentiate into osteogenic cells **(Benayahu, 2000).** Similarly, **Lane *et al.* (2003)** described osteoporosis as a syndrome of excessive skeletal fragility that result from loss of trabecular bone mass and trabecular bone connectivity. In recent rat studies of methotrexate chemotherapy-induced bone defects, it has been found that apart from the reduced osteoblast number and trabecular bone volume; there is a significant increase in marrow adiposity **(Xian *et al.*, 2007; Xian *et al.*,2008).**

In this study, multiple erosion cavities were seen in the endosteal surface of compact and cancellous bone in methotrexate treated group. Many investigators reported that, in methotrexate osteoporosis, the most dramatic finding was the increase number of resorptive surfaces **(Mosekilde et al., 2000).** In this study, marked thinning of bone trabeculae with their wide separation concided with **Steiniche (1995)** findings who mentioned that, osteoporosis in cancellous bone might be manifested as thinning of trabeculae and total loss. The investigator reported that, bone loss in osteoporosis was initiated by an increase depth of erosion cavities, which would lead to focal disruption of the trabeculae, followed by progressive enlargement of the perforations, which would lead to conversion of the trabecular plates to widely separated bars and rods. This was termed as the button phenomenon and was considered as a characteristic feature of osteoporosis **(El-Morsy *et al.*, 2011)**. **Nadhanan *et al.* (2013)** concluded that, methotrexate therapy might lead to overall reduction of trabecular bone volume.

Bone trabeculae showed areas of osteolysis, which appear as faintly staining of bone trabeculae. Similar lesions were prescribed by **(Sulayman, 2007).**

In the current study, examination of bone sections of methotrexate treated ginea pigs stained with Masson trichrome revealed marked increase in collagen fibers in resorptive cavities and reduction in thinned cortex of bone. **Xian *et al.* (2007)** explained reduction of collagen fibers in bone cortex may be due to reduction of oseocytes and chondrocyte proliferation as well as the induction of osteocytes and chondrocytes apoptosis possibly through the Fas/Fas-L death receptor pathway induced by methotrexate treatment. Interestingly, high-dose corticosteroid treatment in rats was reported to promote apoptosis of chondrocytes and osteocytes via suppression of Bcl-2 and induction of Bax expression. **Mocetti *et al.* (2001)** suggested different chemotherapeutic agents may induce apoptosis by different apoptotic pathways.

More recent animal studies revealed that, while long-term low-dose methotrexate treatment caused minimal damaging effect to the bone and growth plate, long term higher dose of methotrexate caused a significant decrease in growth plate height and more serious side effects on bone **(Fan *et al.*,2009).** This perhaps could be related to the pharmacokinetic of methotrexate. Once methotrexate is administered, it is transported into tissues and become tightly bounded to proteins, and the amount of protein bound methotrexate determines the cytotoxicity **(Wheeler *et al.*, 1995). Xian *et al.* (2008)** concluded that, the effect of methotrexate on compact bone structure and function is largely dependent on the treatment dose, regimen and duration.

CD68 antigen is a 110 kDa highly glycosylated transmembrane protein which is mainly located in lysosomes. Studies have shown that, antibody stains macrophages/monocytes in the bone marrow and bone tissues mainly osteoclast **(Wayne, 2005).** Other studies have shown that, antibody stains macrophages in many human tissues including Kupffer's cells, macrophages in the red pulp of the spleen, in lung alveoli and in lamina propria of the gut **(Petrovichev, 1997).**

Microscopic examination of compact bone of methotrexate treated group stained with CD68 immunostain showed positive immunoreactivity to CD68 in osteoclast with increase in their number. These findings proved by highly significant increase in osteoclast number in the methotrexate treated group when compared to control group. These findings of increased osteoclast formation and numbers might be due to inflammatory microenvironment created in bone with methotrexate therapy with subsequent reduced bone volume **(King *et al.*, 2012).** Recent studies confirmed that, methotrexate induced a significant increase in the numbers of osteoclast at metaphysis of long bone and enhanced osteoclastogenesis in the bone marrow were observed in the methotrexate alone group **(Nadhanan *et al.*,2013).** Additionally, **May *et al.* (1994)** observed reduced bone density in associated with an increased osteoclast number with prolonged use of low dose methotrexate therapy. More recent ex vivo study using bone marrow cells obtained from rats treated with methotrexate showed an increase in the osteoclast precursor cell pool which express surface marker CD11b+ and an increase in ex vivo osteoclast formation **(Fan *et al.*,2009).**

In the current study, examination of bone sections of ginea pigs in group IV, which received methotrexate along with folinic acid revealed that, compact bone nearly restored its shape with mostly normal osteocytes in their lacunae, with collagen rich stroma in bone matrix and markedly reduced resorptive cavities. These findings denoted that, folinic acid diminished the damaging effects of methotrexate on bone. This indicate that, folinic acid contributes to suppression of pro-apoptotic molecules involved in the death receptor pathway with subsequent preventing methotrexate induced chondrocyte and osteocytes apoptosis **(Xian *et al.*, 2007).**

Since folate is essential for cell proliferation and survival **(Matherly 2001; Jhaveri *et al.*,2004)**, folate deficiency caused by repeated use of methotrexate could be one of the possible causes of skeletal defects. Folinic acid is an antidote, that has been clinically used for supplementing methotrexate therapy to reduce hepatotoxicity and gastrointestinal side effects without lowering the efficacy of methotrexate **(Hoekstra *et al.*, 2003).** Recently, Folinic acid treatment was found to protect against methotrexate induced bone damage in short-term animal study **(Xian *et al.*,2008).**

Folinic acid supplementation provided bone-protective effect through preventing osteoblast apoptosis thus preserving osteoblast density and preventing a higher density of osteoclasts (reducing bone resorption) with subsequent preservation of chondrocyte number and their columnar arrangement in methotrexate treated rats. At the metaphysis, folinic acid supplementation was able to preserve primary spongiosa heights and overall trabecular bone volume in methotrexate treated rats **(Xian *et al.*,2007).**

Consistently, folinic acid supplementation was previously shown to preserve osteoblast numbers and bone marrow stromal cell pool in rats receiving acute intensive methotrexate chemotherapy by reducing osteocyte apoptosis **(Nilsson *et al.*, 1984),** and to suppress methotrexate -induced increased osteoclast density in rats treated with methotrexate at a high dose for a short term or at a low dose for a long-term. This might be due to the ability of these supplements to not only to suppress osteoclastogenesis /osteoclast numbers but also to preserve the bone marrow osteogenesis /bone surface osteoblast numbers **(Fan *et al.*,2009).**

Conclusion:

Using a chronic model of ginea pigs mimicking the commonly used clinical protocol, the current study has identified that, chronic methotrexate therapy causes osteoporosis by reducing bone formation and increasing bone resorption and marrow adiposity, and that folinic acid supplementation can prevent these adverse effects. While folinic acid treatment has been used in methotrexate therapy to reduce toxicities of methotrexate in soft tissues. Our study suggests that, folinic acid may be potentially useful in patients who are at risk of skeletal resorption and bone loss as a result of chronic methotrexate therapy.

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