Methotrexate Effects on the Lumbar Vertebrae of Young Male Albino Rat and Possible Protective Effect of Folic acid: Light Microscopic, Electron Microscopic and Morphometric Study

Original Article

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ABSTRACT

Background: With appearance of intensive chemotherapy regimens, methotrexate is a folic acid antagonist that is commonly used for the treatment of malignancies and rheumatic disorders.

Aim of the Work: To reflect the effects of methotrexate on growing lumbar vertebrae of young male albino rat and potential protective effects of folic acid.

Materials and Methods: 30 young male Albino rats were equally divided into three groups. Control group (I), methotrexate treated group (II) was subcutaneously injected once daily at 0.75 mg/kg for two separate 5-days courses and methotrexate plus folic acid group (III) was given methotrexate as group II plus folic acid at a dose of 1mg/kg. The lumbar vertebrae were extracted and processed for the light and electron microscopic examination. Morphometric measurements were also done.

Results: Normal histological structures were determined in the control group whereas methotrexate treated group revealed cellular disorganization with degeneration. The proliferative zone showed decrease in chondrocyte number with failure of the secondary ossification center to appear and the centrum showed abundant fat cells and few bone marrow cells. Both reserve and proliferating cells revealed rarified vacuolated cytoplasm. The morphometric studies revealed a significant decrease in both proliferative cell count and midzonal thickness of growth plate. On the other hand, rats received folic acid with methotrexate revealed more or less apparent normal architecture.

Conclusion: Folic acid proved to have remarkable protective effect against harmful effects of methotrexate. So, its supplementary should be started with methotrexate.

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Key Words: Albino rat, epiphyseal plate, folic acid, lumbar vertebra, methotrexate.

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INTRODUCTION

Bone is the primary anatomical structure comprising of the human skeletal system. Functionally, it assumes a significant mechanical role by the skeleton and represents a stock of mineral salts to mobilize for maintenance of calcium and phosphorus homeostasis. Bone is comprised of mainly collagen fibers type 1 and inorganic salts[1].

The basic elements of bone tissue are cells (osteocytes, osteoblasts, osteoclasts) and extracellular matrix (ECM)[2,3]. The skeleton is made up of bones developing through two distinct processes; intramembranous and endochondral ossification. Intramembranous occurs in the calvarial bones and distal end of the clavicle and develops directly from un-differentiated mesenchymal cells. In contrast, endochondral ossification occurs in the remainder of skeleton and involves differentiation of mesenchymal cells into chondrocytes that form a cartilage template for future bone[4,5]. The epiphyseal plate situated between diaphysis and epiphysis, is composed of four distinct zones: the resting, proliferative, hyper-trophic and calcifying zones. Bone development begins as progenitor cells at resting zone are activated and enter the cell cycle at the proliferative zone[6,7].

The folic acid is needed for the de novo synthesis of the nucleoside thymidine, required for DNA synthesis and is essential for purine and pyrimidine base biosynthesis. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins[8]. While, methotrexate (MTX) is a folic acid antagonist and plays a cytotoxic role in the cell cycle, inhibits DNA synthesis, stops cell proliferation and causes apoptotic death. Methotrexate is commonly used at high-doses for the treatment of malignancies[9]. This agent is well known and used as a chemotherapeutic agent for a long time and not selective for cancer cells so, healthy cells beside cancer cells are also affected by it[10]. It is the most widely used antimetabolite in the treatment of childhood cancers especially for acute lymphoblastic leukemia (ALL)[11] by reversibly inhibiting the enzyme dihydrofolate-reductase (DHFR)[12]. While, at lower doses has an anti-inflammatory property which is

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thought to be beneficial in treating of some inflammatory and autoimmune diseases such as rheumatoid arthritis[13], psoriasis, psoriatic arthritis, Systemic lupus, sarcoidosis, Crohn's disease[14], eczema and many forms of vasculitis[15].

As shown, a number of studies have collected evidence for MTX-mediated several deficits in different organs and systems. Therefore, MTX therapy at a high dose is known to cause bone growth defects while, effects of its use at a low dose on growing skeleton remain less clear[9]. The present study was planned to reflect the effects of MTX therapy at a low dose on the structures of growth plate and formation of primary bone in lumbar vertebrae of young male albino rats and the potential protective effects of supplementary treatment with folic acid.

MATERIALS AND METHODS

Animals

A total number of 30 young male Albino rats (aged 4weaks) were obtained from animal house, Faculty of Medicine, Assiut University. The rats were left two weeks of acclimatization in suitable sized cages in a good aerated room and feed standard diet. The experiment was approved by the Institutional Ethics Committee of Assiut University.

Experimental design and chemicals

Rats were randomly equally divided into 3 groups (10 rats each):

1) Group I: Served as control group and was subdivided into two equal subgroups:

a. Subgroup Ia: Rats were received an equivalent amount of saline (1ml) subcutaneously injected.

b. Subgroup Ib: Rats were given folic acid intraperitoneally at a dose of 1mg/kg once daily (5 days on/9 days off/5 days on)[8] (clinical dosing regimen). Folic acid was provided by The Nile Company of Egypt. It available in the form of tablets and their contents were dissolved in distilled water.

2) Group II: Served as MTX treated group. The rats were subcutaneously injected with MTX once daily at 0.75 mg/kg was diluted in 1ml of saline (a therapeutic dose in pediatric patients) for two separate 5-day courses (5 days on/9 days off/5 days on)[8]. MTX was manufactured by Haupt pharma, Germay and imported by Ramco.

3) Group III: Served as MTX with folic acid treated group. Rats in this group were concomitantly treated with MTX at the same regimen in group II plus folic acid injection at a dose of 1mg/kg 6 hours after MTX. The used dose in this work was according to previous study[8].

At the end of the experiment (20 days), rats were anesthetized by ether inhalation then subjected to intracardiac perfusion of normal saline 0.9% NaCl. At each of the previous groups, lumbar vertebrae were disarticulated and extracted. These samples were carried out and used for the following studies:

Light microscopic study

Half of these specimens were fixed in 10% Formaldehyde solution for 24-72 hours, decalcified (by formic acid-sodium citrate solution for 20-30 days according to size of specimens), dehydrated (by ascending grades of alcohol), cleared (by cedar wood oil for 48 hours), embedded in paraffin and then the sections were cut at 5-7 µm and stained with H&E for a routine histological examination[16,17].

Electron microscopic study

The other half of samples were fixed in 5% gluteraldehyde for 2-48 hours and then post fixed in 1% osmic tetra oxide for 2-4 hours . Then they were dehydrated in ascending grades of alcohol, cleared by pure propylene oxide and embedded in epoxy resin. Thick 1 µm sections were mounted on glass slides and stained with toluidine blue for identification. Ultrathin sections 50-70nm were cut and double stained with uranyle acetate and lead citrate[17] and examined by a transmission Jeol-JEM-100 CXII electron microscope and photographed at the electron microscope unit of Assiut University.

Morphometric study

Crown rump length in cm was measured from top of the head to the bottom of the buttocks by using measuring tape. The image analyzer computer system; OLYMPUS DP27 digital camera attached to an OLYMPUS CX41 light microscope and PC running cellSens Standard software (version 1.7) was used to determine the number of chondrocytes in the proliferative zone of growth plate and midzonal thickness of growth plate of lumbar vertebra. The area 17948.21Mm² was chosen to enumerate chondrocytes at total magnification ×400 while the midzonal thickness at total magnification ×100. Statistical analysis of these morphometric measurements was carried out using the SPSS software, version 13 program (USA) in anatomy department, Faculty of medicine, Assiut University. Comparison of the significance between the different groups was carried out using ANOVA test.

RESULTS

Histological results

There were no differences between subgroups Ia &Ib, therefore we describe data of control subgroup (Ia) to represent the overall control groups.

Light microscope study

Examination of H&E-stained lumbar vertebrae epiphysis in the control groups revealed nearly the same normal histological structures. Epiphyseal cartilage with secondary ossification center which separates the growth plate cartilage from intervertebral disc and contain secondary ossification center (plate 1a). At the periphery of hyaline cartilage, young chondrocytes had an elliptical shape with the long axis parallel to surface. Four zones of chondrocytes could be observed. Reserve zone at the

epiphyseal side of plate showed randomly distributed small inactive chondrocytes. Proliferative zone showed chondrocytes that were increased in size and arranged in columns. Hypertrophic zone showed greatly enlarged chondrocytes. Calcifying zone showed degenerating chondrocytes and the cartilage became calcified (plate 2a). While the centrum of these lumbar vertebrae with outer cortex and inner medulla was identified. Their medulla had bone trabeculae which enclosed between them bone marrow cells and few fat cells (plate 3a).

Methotrexate treated group of nearly all examined sections revealed absence of secondary ossification center. Also they showed decrease in the overall thickness of the growth plate cartilage with loss of normal alignments of the chondrocytes (plate 1b). Areas of matrix devoid of cells and loss of their normal longitudinal arrangement of the proliferating zone with degenerated cells in the hypertrophied cell zone were seen (plate 2b). On other hand, their inner medulla of centrum revealed indistinct or few bone marrow cells and abundant fat cells (plate 3b).

Methotrexate with Folic acid treated group showed reappearance of secondary center of ossification. The epiphyseal growth plate showed increase in thickness and normal arrangement of chondrocytes (plate 1c). In the proliferating cell zone; the cells more or less normal could be observed (plate 2c). Also, the inner medulla of centrum showed restoration of most of bone marrow cells. Fat cells mostly retained its normal amount and appearance between bony trabeculae (plate 3c).

Electron microscope study

The ultra-thin sections of the control subgroups (Ia &Ib) revealed nearly the same normal appearance. Normally, the reserve cell zone was formed of uniform, small, spindle shaped chondrocytes. These cells were surrounded by narrow clear pericellular zone called groove of Ranvier. Outside these zones a fine network of thin fibrils were observed in the extracellular matrix. Their cytoplasm showed multiple cytoplasmic processes, eccentric large rounded nuclei and also well-developed rough endoplasmic reticulum (plate 4a). While in the proliferative zone, their cells were flattened, long and appeared in pairs separated from each other by transverse septa and surrounded by extracellular matrix with abundant collagen fibrils. They showed cytoplasmic processes, eccentric nuclei, rough endoplasm reticulum and lipid droplets (plate 5a).

Methotrexate treated group of nearly all examined sections revealed the reserve cells appeared enlarged or hypertrophied with indistinct or ill-defined nuclei. Rarified cytoplasm with excessive vacuolization or melting cytoplasmic materials were identified in numerous examined cells (plate 4b). Other conspicuous findings in this group were the proliferating cell appeared flattened with elongated flat nuclei and surrounded by an extracellular matrix. They showed rarified vacuolated cytoplasm and nearly absent organelles in several examined sections (plate 5b).

Methotrexate with Folic acid treated group showed their reserve cells preserved their spindle shape and surrounded by clear wide pericellular zones and also abundant extracellular matrix in numerous examined sections. They showed few short cytoplasmic processes, large rounded euchromatic nuclei, well-developed rough endoplasmic reticulum and also some vacuoles or large lipid droplets (plate 4c). Whereas, slightly enlarged proliferative cells with cytoplasmic processes projecting into clear pericellular zone and surrounded by extracellular matrix were demonstrated. They exhibited eccentric irregular shaped nuclei, slightly dilated rough endoplasmic reticulum and large vacuoles or lipid droplets in numerous sections (plate 5c). Q3

Morphometric and statistical study

Statistical analysis of the morphometric measurements of crown rump length showed significant decrease in MTX treated rats compared to control with significant improvement after folic acid treatment (Table, Chart 1). Also counting of chondrocytes per certain area revealed that the average number of growth plate chondrocytes significantly decreased in rats treated with MTX compared to control rats (Table, Chart 2) also the analysis revealed significant reduction in midzonal thickness of the growth plate (Table, Chart 3) . Folic acid treatment cause significant improvement in thickness and number compared to MTX treated rats.

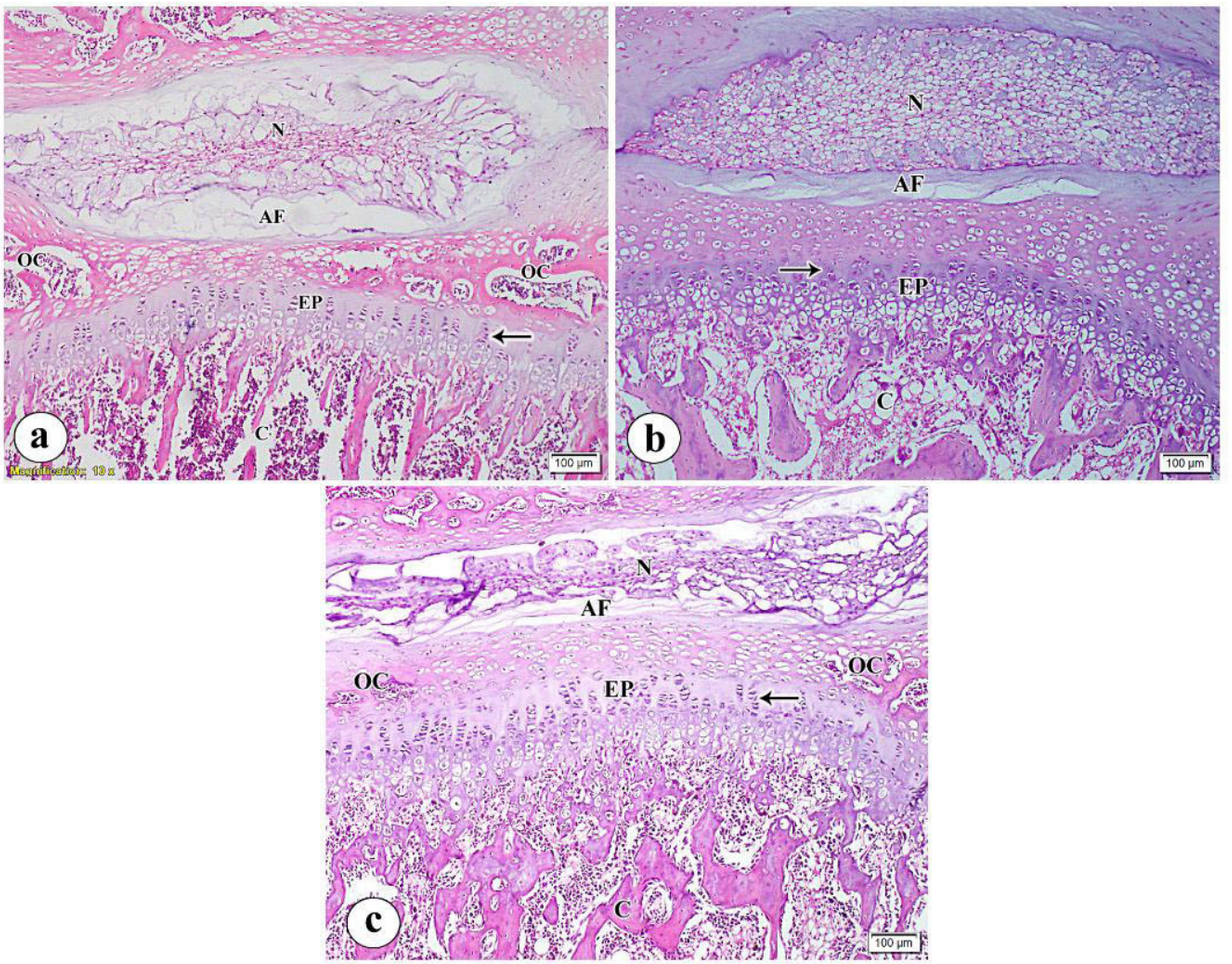

Plate 1: Photomicrographs of a sagittal section in the lumbar vertebra of young albino rat (a- control, b- MTX treated & c- MTX + folic acid treated groups) showing nucleus pulposus (N) and annulus fibrosus (AF) of the intervertebral disc. Ossified centrum of the vertebral body (C) is also noticed. (a): Normal epiphyseal plate (EP) with regular alignment of chondrocytes (arrow) and secondary ossification center (OC) are seen. (b): An apparent decrease in thickness of epiphyseal plate (EP) as compared to control group. Their chondrocytes lose their normal alignment (arrow). Noticed, no secondary ossification center. (c): The epiphyseal plate (EP) shows increase in thickness with normal alignment of chondrocytes (arrow) compared to MTX treated group. Noticed, secondary ossification center (OC) is clear.

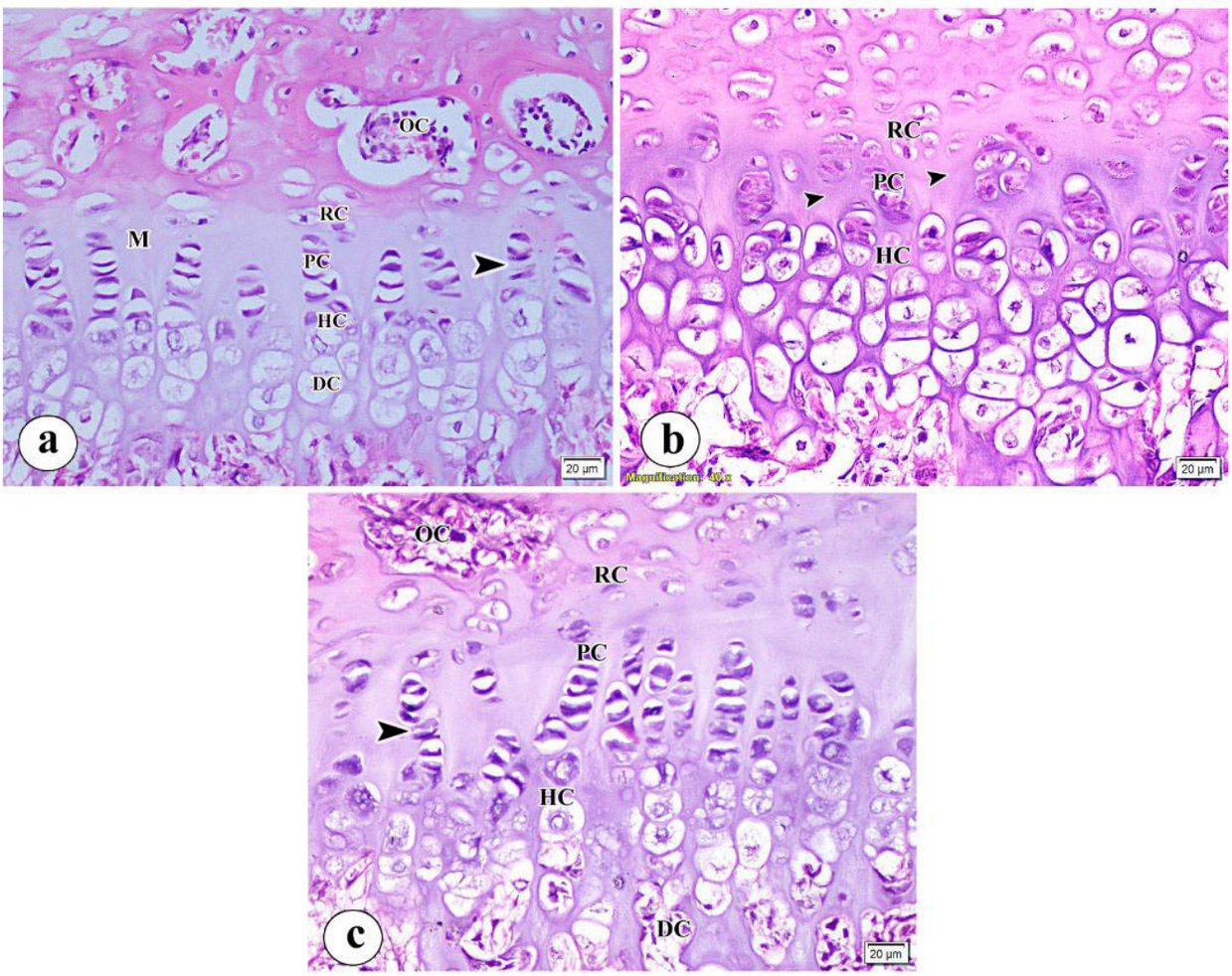

Plate 2: Photomicrographs of a sagittal section in the epiphyseal growth plate cartilage of the lumbar vertebra of young albino rat (a- control, b- MTX treated & c- MTX + folic acid treated groups). Secondary ossification center is noticed in both a & c groups. (a): The reserve cell zone (RC), proliferating cell zone (PC) arranged in longitudinal column within elliptical lacunae (arrow head) and compressed matrix (M) in between the columns, hypertrophied cells (HC) lies next to the proliferating cells. Larger and degenerating cells (DC) of calcification zone are the nearest to the centrum. (b): The reserve cell zone (RC), proliferating cell zone (PC) with loss of their normal alignment (arrow head) and hypertrophied cells (HC) with vacuolated lacunae are observed. Noticed, no secondary ossification center. (c): The proliferating cell zone which appears more or less normal (PC) and maintains its normal alignment (arrow head) as compared to control group. Note, reserve cells (RC), hypertrophic cell (HC) and degenerating cells (DC).

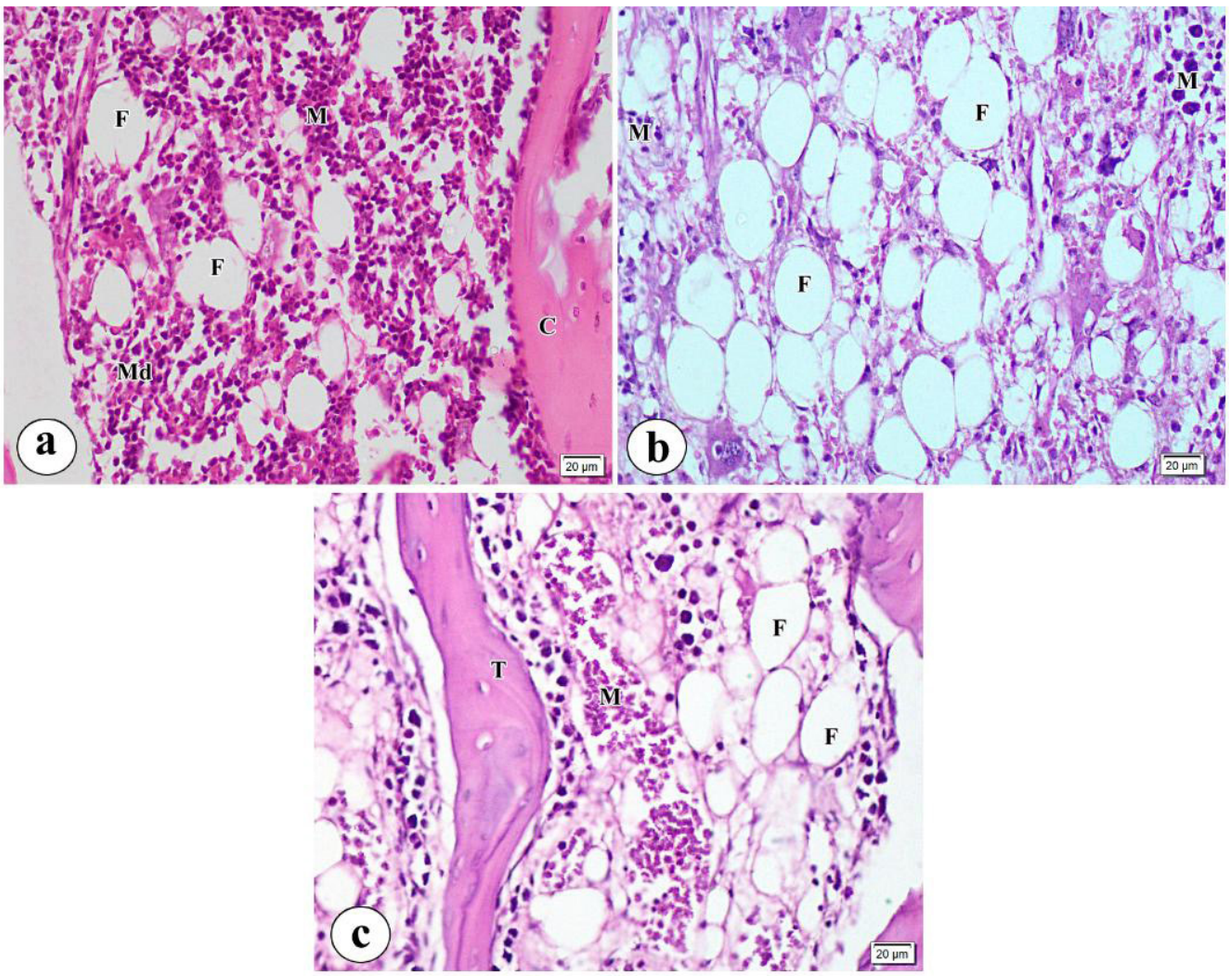

Plate 3: (a): Photomicrographs of a sagittal section in the centrum of the lumbar vertebra of young albino rat (a- control, b- MTX treated & c- MTX + folic acid treated groups). (a): The centrum shows outer cortex (C) and inner medulla (Md) which contains excess bone marrow cells (M) and few fat cells (F). (b): The inner medulla of centrum (Md) shows indistinct or few bone marrow cells (M) and abundant fat cell (F). (c): The inner medulla of the centrum reveal restoration of most of bone marrow cells (M) and decrease in fat cells (F) compared to MTX treated group. Note, bony trabeculae (T).

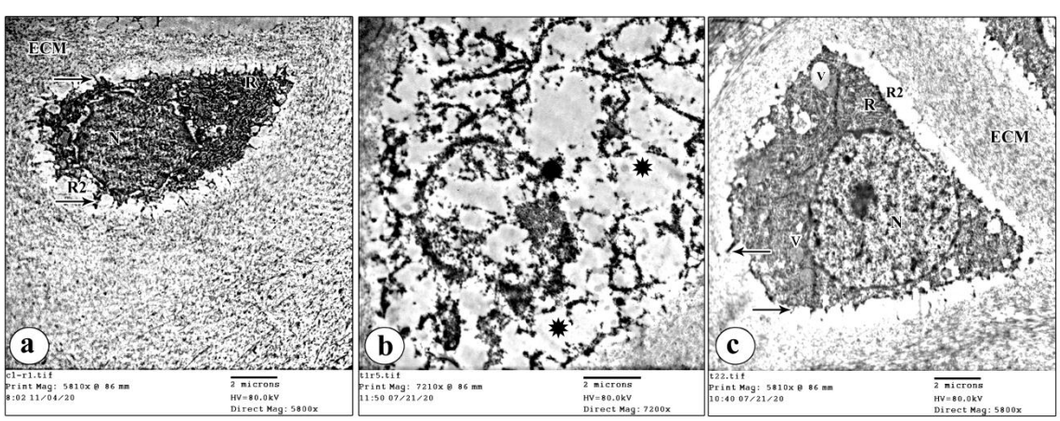

Plate 4: Electron micrographs of reserve cells in the growth plate cartilage in the lumbar vertebra of young albino rat (a- control, b- MTX treated & c- MTX + folic acid treated groups). (a): A spindle shaped reserve cell is surrounded by pericellular zone (R2) and extracellular matrix (ECM) with a large amount of collagen fibrils. This cell has multiple cytoplasmic processes (arrow), eccentric large rounded nucleus (N) and Well-developed rough endoplasmic reticulum (R) is observed. (b): Hypertrophied reserve cell with indistinct nucleus but melting cytoplasmic materials (asterisk) can be identified. (c): A reserve cell is surrounded by clear wide pericellular zone (R2) and abundant extracellular matrix (ECM). This cell has few short cytoplasmic processes (arrow), large rounded euchromatic nucleus (N), well-developed rough endoplasmic reticulum (R) and also some vacuoles (V).

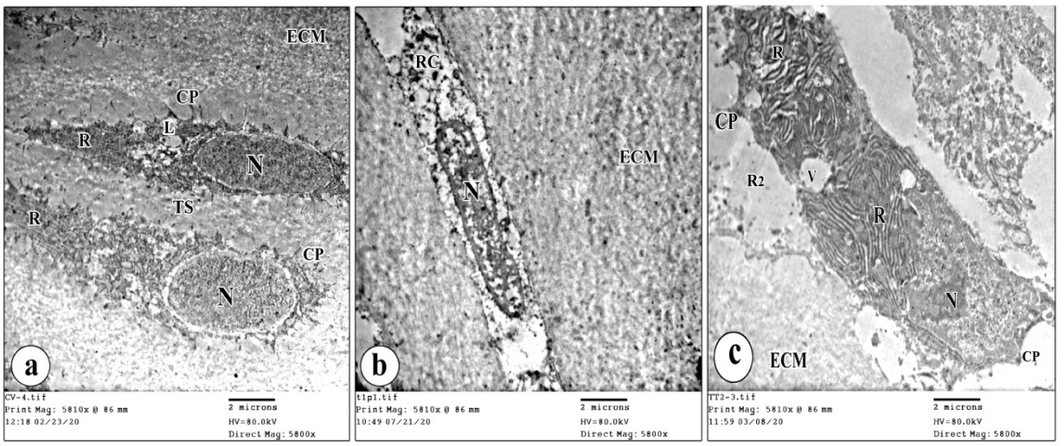

Plate 5: Electron micrographs of proliferative cells in the proliferative zone of the growth plate cartilage in the lumbar vertebra of young albino rat (a-control, b- MTX treated & c-MTX + folic acid treated groups). (a): Paired flattened proliferating cells separated from each other by transverse septa (TS) and surrounded by extracellular matrix (ECM) with abundant collagen fibrils are observed. Eccentric nuclei (N), rough endoplasm reticulum (R), lipid droplets (L) and cytoplasmic processes (CP) can be seen. (b): Flattened proliferating cell with elongated nucleus (N) and surrounded by an extracellular matrix(ECM) is observed. Noticed, the vacuolated rarified cytoplasm (RC) with nearly absent organelles. (c): Slightly enlarged proliferative cell with cytoplasmic processes (CP) projecting into clear pericellular zone (R2) and surrounded by extracellular matrix (ECM) is good seen. It exhibits an eccentric irregular shaped nucleus (N), slightly dilated rough endoplasmic reticulum (R) and large vacuoles (V).

Table & Chart 1: Comparison between the mean values of the crown rump length in cm in different studied groups. They showed a statistically significant decrease in length ($P<0.05$) after MTX when compared to the corresponding control(group I), as well as a statistically significant increase after folic acid when compared to group I and group II.

	Group I	Group II	Group III	*P-value* 1	*P-value* 2	*P-value* 3	*P-value* 4
Mean ± SD	16.00 ± 1.05	13.42 ± 1.02	15.00 ± 0.55	0.001*	0.000*	0.074	0.008*
Range	14.5-17.0	12.0-15.0	14.5-16.0				

Table & Chart 2: Comparison between the mean values of chondrocytes number in the proliferative zone of growth plate of the lumbar vertebra in different studied groups. They showed a statistically significant decrease in chondrocyte number ($P<0.05$) after MTX when compared to the corresponding control(group I), as well as a statistically significant increase after folic acid when compared to group I and group II.

	Group I	Group II	Group III	*P-value* 1	*P-value* 2	*P-value* 3	*P-value* 4
Mean ± SD	48.00 ± 2.61	26.83 ± 6.40	40.17 ± 1.94	0.000*	0.000*	0.005*	0.000*
Range	45.0-52.0	19.0-35.0	38.0-43.0				

Table & Chart 3: Comparison between the mean values of midzonal thickness (in μm) of growth plate of the lumbar vertebra in different studied groups. They showed a statistically significant decrease in thickness ($P<0.05$) after MTX when compared to the corresponding control(group I), as well as a statistically significant increase after folic acid when compared to group I and group II.

	Group I	Group II	Group III	*P-value* 1	*P-value* 2	*P-value* 3	*P-value* 4
Mean ± SD	151.69 ± 4.53	128.67 ± 5.06	155.70 ± 3.90	0.000*	0.000*	0.146	0.000*
Range	145.7-157.7	121.6-133.6	152.2-161.0				

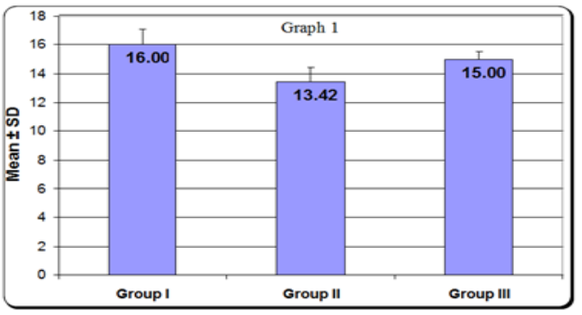

Chart 1: Showing comparison of the crown rump length in cm between control, methotrexate treated and folic acid protected groups.
*P-value*1: Comparison among all groups
*P-value*2: Comparison between groups I &II
*P-value*3: Comparison between groups I &III
*P-value*4: Comparison between Groups II &III

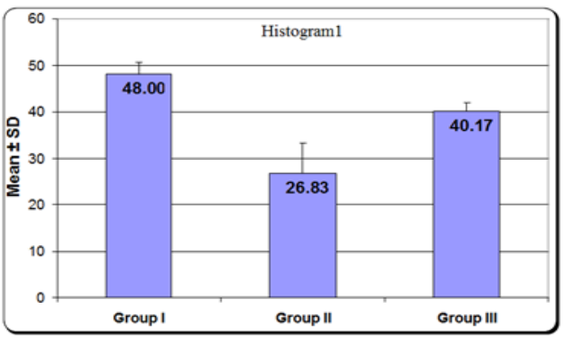

Chart 2: Comparison of number of chondrocytes in proliferative zone (area 17948,21 Mm2) of growth plate of the lumbar vertebra at magnification of-400- between control, methotrexate treated and folic acid protected groups.
*P-value*1: Comparison among all groups
*P-value*2: Comparison between groups I &II
*P-value*3: Comparison between groups I &III
*P-value*4: Comparison between Groups II &III

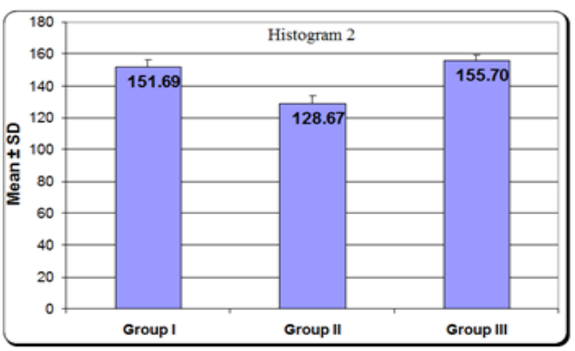

Chart 3: Comparison of midzonal thickness of growth plate of the lumbar vertebra in between control, methotrexate treated and folic acid protected groups.

*P-value*1: Comparison among all groups
*P-value*2: Comparison between groups I &II
*P-value*3: Comparison between groups I &III
*P-value*4: Comparison between Groups II &III

DISCUSSION

The rapid advances in the field of oncology with intensive high doses of MTX chemotherapy result in improved cure rates especially in pediatric patients with acute lymphoblastic leukemia (ALL) and in other malignancies as osteogenic sarcoma[18-20]. However, one of the major health threats associated with these effective regimens is the detrimental effects on bone[21]. It is associated clinically with bone pain, bone loss, fracture and osteoporosis[22]. Although it's low dose generally has been reported with a safety profile for treating rheumatoid arthritis (AR) but has been reported to cause bone marrow suppression and bone fragility in some patients[9]. While, the effects of long-term low-dose use of MTX on bone still not clear.

So, the current work was carried out to further define the effects of MTX at low dose on the young rat skeleton and to show the possible protective effect of folic acid. Also, in order to put the bases for the best way of use of these drugs to avoid hazards that may develop due to uncontrolled use. Moreover, it was performed on the lumbar vertebrae as an example of an irregular bone.

In the present study, light and ultrastructural findings of the control rats were in accordance with previous studies[23-25]. They described the lumbar vertebra with central part or centrum and two peripheral hyaline cartilage or epiphysis. The body of the vertebra showed a primary center of endochondral ossification. These epiphysis showed several zones of chondrocytes maturation. Also these chondrocytes and their division rate play a vital role in longitudinal bone growth and growth plate midzonal thickness with chondrocytes number measurements represented a direct correlation to the growth rate.

In the study of Bassiouny *et al*.,[26] who reported that epiphyseal cartilage of tibia in MTX treated rats exhibited marked reduction in thickness. Also, the epiphyseal cartilage appeared disorganized, disruption in chondrocytes arrangement and marked reduction in their number with disappearance of secondary centers of ossification. These measurements (thickness and chondrocyte number) showed a significant reduction when compared to control which were reported by other study[27]. Similar data were observed in the current work. In accordance with others[29] who found that MTX significantly reduced primary spongiosa height, secondary spongiosa trabecular bone volume and total thickness of the growth plate in young rats model. In agreement with our study, Iqbal *et al*.,[28] who stated that MTX had significantly decreased the height of the femoral and tibial growth plate. Whereas, the main effect of MTX seemed to be on the hypertrophic proliferative zone. Also, these results can be explained by other studies[15,26] who reported that MTX was shown to decrease chondrogenesis, induction of chondrocyte apoptosis and reduction of collagen-II mRNA expression at the growth plate.

In this study, the clear findings in the medulla of centrum were few bone marrow cells and appearance for numerous adipocyte or fat cells. These current findings were in harmony with others[26] who reported that bone marrow stromal cells from MTX-treated rats have lower osteogenic potential but higher adipogeneic potential. Also, other studies revealed that MTX caused decrease bone marrow cellularity and a significant switch of bone marrow stromal progenitor cells from osteogenesis to adipogenesis[29-32].

In the current study several changes in the ultrastructure of both nuclei and organelles could occur following administration of MTX. Some nuclei showed irregularity, condensed or clumps of heterochromatin. Other cytoplasmic changes were in the form of ill-define its organelles or absent and vacuolations with dilatation of endoplasmic reticulum. Also these changes were similar to those described by other studies[33-36] who studied MTX effects on hepatocytes and also with another study[37] who revealed mitochondrial and nuclear irregularity in different cells of ovarian follicles. These destructive changes of MTX could be explained by the toxicity studies of MTX which highlighted the role of oxidative stress in causing damages on the bone, liver and other organs[38,39]. Also Fan *et al*.,[8] reported that once MTX is administered, it is transported into tissues and tightly bounded to proteins. These protein-bound MTX amount determines the cytotoxicity. Therefore, prolonged use MTX gradually build up these bonded proteins and increase its cytotoxicity.

Folic acid is now used clinically to reduce MTX toxicity in soft tissues. So, it is considered as MTX antidote as it can reduce its side effects and compensate folate deficiency caused by MTX. Previous studies in rats and mice had shown that supplementary with folic acid was able to reduce the growth inhibition caused by MTX[12]. Folic acid supplementary in rats receiving low-dose MTX was shown able to preserve primary spongiosa bone volume[8,12]. The protective role of folic acid was documented by several studies[26,41] that reported that folic acid is essential for cell proliferation as well as synthesis of nucleic acid required by rapidly growing cells and caused suppression of pro-inflammatory cytokines.

So, in the present study, the rats treated with MTX and folic acid showed reduction or improvement in the cellular structures and also a positive significant impact on both growth plate thickness and chondrocytes number. These findings were in agreement with other studies who detected relatively normal ultrastructures of granulosa cells of ovary[37] and hepatocyte[40] in the rats received folic acid with methotrexate. In current work, folic acid supplementation was able to preserve bone marrow stromal cell pool and bone mass during chemotherapy. These findings suggest that folic acid can reduce bone resorption and preserves bone volume[41].

From this work when all results were evaluated, we can suggest that MTX seems to induce damages in growth plate of bone. On other hand, folic acid proved to have remarkable protective effect against its harmful effects. So, its supplementary should be started with methotrexate. Further studies are needed to characterize the MTX damaging effects, the underlying mechanisms and to investigate benefits and mechanisms of folic acid in protecting bone growth.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Baig MA, Bacha D. Histology, Bone. StatPearls [Internet]. 2019 Oct 19. Treasure Island (FL): StatPearls Publishing; 2020 Jan.
2. Strum JM, Gartner LP, Hiatt JL. Cell biology and histology. Hagerstwon, MD: Lippincott Williams & Wilkins. 5th edition 2007;83.
3. Wojnar R. Bone and cartilage–its structure and physical properties. Biomechanics of hard tissues. 2010:1-75.
4. Marino R. Growth plate biology: new insights. Current Opinion in Endocrinology, Diabetes and Obesity. 2011 Feb 1;18(1):9-13.
5. Spitalnik PF. Histology Laboratory Manual. Caitlin Alexander edited the text and created many of the diagrams and micrographs as part of the Columbia College of physicians and surgeons Scholarly Project Program. 2017; Chapter 4:(17-24).
6. Cormack DH. Essential histology. Lippincott Williams & Wilkins; 2001; Chapter 8:190.
7. Telser AG, Young JK, Baldwin KM. Cartilage and bone, Elservier¸s integrated histology, Mosby, Inc, Mosby, Inc, USA. 2007;125-130.
8. Fan C, Cool JC, Scherer MA, Foster BK, Shandala T, Tapp H, Xian CJ. Damaging effects of chronic low-dose methotrexate usage on primary bone formation in young rats and potential protective effects of folinic acid supplementary treatment. Bone. 2009 Jan 1;44(1):61-70.
9. Swierkot J, Szechinski J. Methotrexate in rheumatoid arthritis. Pharmacol. Rep. 2006;58:473–492.
10. Ozkorkmaz EG, Gul N, Ozluk A, Ozay Y. Ultrastructural alterations of liver tissue cells in methotrexate-treated Balb/c Mice. Journal of microscopy and ultrastructure. 2018 Oct;6(4):192.
11. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. The Lancet. 2008 Mar 22;371(9617):1030-43.
12. Xian CJ, Cool JC, Scherer MA, Fan C, Foster BK. Folinic acid attenuates methotrexate chemotherapy-induced damages on bone growth mechanisms and pools of bone marrow stromal cells. Journal of cellular physiology. 2008 Mar;214(3):777-85

13. Cronstein BN. Low-dose methotrexate: a mainstay in the treatment of rheumatoid arthritis. Pharmacological reviews. 2005 Jun 1;57(2):163-72.

14. Herfarth HH, Long MD, Isaacs KL. Methotrexate: underused and ignored?. Digestive Diseases. 2012;30(Suppl. 3):112-8.

15. Rossi S, ed. Australian Medicines Handbook (2013 ed.). Adelaide: The Australian Medicines Handbook Unit Trust. ISBN 978-0-9805790-9-3, 2013.

16. Drury RA, Wallington EA. Preparation and fixation of tissues. Carleton's histological technique. 1980;5:41-54.

17. Bancroft JD, Gamble M. Theory and practice of Histological Techniques. The 7th edition . Philadelphia: Churchill Livingstone of Elsevier. 2013; 172-186.

18. Peters GJ, Van der Wilt CL, Van Moorsel CJ, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacology & therapeutics. 2000 Aug 1;87(2-3):227-53.

19. Abromowitch M, Ochs J, Pui CH, Fairclough D, Murphy SB, Rivera GK. Efficacy of high-dose methotrexate in childhood acute lymphocytic leukemia: analysis by contemporary risk classifications. Blood. 1988; 71 (4): 866-869.

20. King TJ, Shandala T, Lee AM, Foster BK, Chen KM, Howe PR, Xian CJ. Potential effects of phytoestrogen genistein in modulating acute methotrexate chemotherapy-induced osteoclastogenesis and bone damage in rats. International journal of molecular sciences. 2015 Aug;16(8):18293-311.

21. Tseng PC, Hou SM, Chen RJ, Peng HW, Hsieh CF, Kuo ML, Yen ML. Resveratrol promotes osteogenesis of human mesenchymal stem cells by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis. Journal of Bone and Mineral Research. 2011 Oct;26(10):2552-63.

22. Mandel K, Atkinson S, Barr RD, Pencharz P. Skeletal morbidity in childhood acute lymphoblastic leukemia. Journal of Clinical Oncology. 2004 Feb; 22(7):1215-21.

23. Williams BL, Banister LH, Berry MM, Collins B, Dayson M, Dayson M, Dussek JE, Ferguson MW. Gray's Anatomy Chuchill Livingstone, Edinburgh. Thirty eight edition. 1995; 283-290.

24. Ross MH, Romrell LJ, Kaye GI. Histology A text and Atlas. Williams &Wilkins, Maryland. 3rd edition. 1995; 132-1995.

25. Hassanien HM. Histological and morphometric studies on the postnatal development of bones under the effect of hypothyroidism and thyroxin replacement in the albino rats. Thesis submitted for M.D. of anatomy, Assiut University. 2018.

26. Bassiouny HS, Afifi Amin NM, Ibrahim ER, Hassan RM. Comparative histological study on the protective effect of folinic acid versus fish oil on the growing bone of methotrexate-treated young rats. Egyptian Journal of Histology. 2019 Jun 1;42(2):262-75.

27. Lee A, Shandala T, Soo PP, Su YW, King TJ, Chen KM, Howe PR, Xian CJ. Effects of resveratrol supplementation on methotrexate chemotherapy-induced bone loss. Nutrients. 2017 Mar;9(3):255.

28. Iqbal MP, Ahmed M, Umer M, Mehboobali N, Qureshi AA. Effect of methotrexate and folinic acid on skeletal growth in mice. Acta Pædiatrica. 2003 Dec;92(12):1438-44.

29. Nadhanan RR, Skinner J, Chung R, Su YW, Howe PR, Xian CJ. Supplementation with fish oil and genistein, individually or in combination, protects bone against the adverse effects of methotrexate chemotherapy in rats. PloS one. 2013 Aug 12;8(8):e71592.

30. Georgiou KR, Nadhanan RR, Fan CM, Xian CJ. Methotrexate-Induced bone marrow adiposity is mitigated by folinic acid supplementation through the regulation of wnt/β-Catenin signalling. Journal of cellular physiology. 2015 Mar;230(3):648-56.

31. Georgiou KR, Scherer MA, Fan CM, Cool JC, King TJ, Foster BK, Xian CJ. Methotrexate chemotherapy reduces osteogenesis but increases adipogenic potential in the bone marrow. Journal of cellular physiology. 2012 Mar;227(3):909-18.

32. Fan CM, Foster BK, Hui SK, Xian CJ. Prevention of bone growth defects, increased bone resorption and marrow adiposity with folinic acid in rats receiving long-term methotrexate. PloS one. 2012 Oct 5;7(10):e46915.

33. Elwakeel EE, Mohamed AZ. The Hepatotoxic effect induced by methotrexate therapy and protective role of bone marrow-derived mesenchymal stem cells in adult male albino rats. Histological and ultrastructural study. Jounal Am Sci. 2018;14(5).

34. Khafaga AF, El-Sayed YS. Spirulina ameliorates methotrexate hepatotoxicity via antioxidant, immune stimulation, and proinflammatory cytokines and apoptotic proteins modulation. Life sciences. 2018 Mar 1;196:9-17.

35. Kadry SM, Ashry MA, Abuzeid NR, Algaw NA. Hepatotoxicity of Methotrexate and the Possible Ameliorative Effect of L-Carnitine: Ultrastructural Study. Journal of Scientific Research in Science. 2016 Sep 1;33(part1):307-16.

36. Ali SY, Hassan IM, Sadek S. Ultrastructural changes in rat livers perfused in *vitro* and in *vivo* with a high dose of methotrexate. Histol. Histopathol. 2005;20(4):1131-1145.

37. Shohda AA, El-banna A, Abdel Fattah F. The possible protective effect of folic acid against methotrexate induced ovarian damage in female albino rats.«Light and electron microscopic study. Bulletin of the National Nutrition Institute of the Arab Republic of Egypt. 2017 Nov 1;49(1):1-24.

38. Tousson E, Hafez E, Zaki S, Gad A. P53, Bcl-2 and CD68 expression in response to amethopterin-induced lung injury and ameliorating role of l-carnitine. Biomedicine & Pharmacotherapy. 2014 Jun 1;68(5):631-9.

39. Erdogan E, Ilgaz Y, Gurgor PN, Oztas Y, Topal T, Oztas E. Rutin ameliorates methotrexate induced hepatic injury in rats. Acta cirurgica brasileira. 2015 Nov;30(11):778-84.

40. Soliman ME. Evaluation of the possible protective role of folic acid on the liver toxicity ınduced experimentally by methotrexate in adult male albino rats. Egypt J Histol. 2009;32(1):118-28.

41. Yang XJ, Chen YP, Wang HC, Zhao J, Zheng FY. Protective effect of calcium folinate against methotrexate-induced endosalpinx damage in rats. Fertility and sterility. 2011 Mar 15;95(4):1526-30.

الملخص العربى

تأثيرات الميثوتريكسيت علي الفقرات القطنية لذكر الجرذ الابيض الصغير و التأثير الواقي المحتمل لحمض الفوليك دراسة مجهرية ضوئية والكترونية وقياسية

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المقدمة: إن واحدا من الاضرار الصحية الكبيرة الناتجة من العلاج الكيماوي هو الأثار الجانبية الجسيمة علي العظام .ويعد عقار الميثوتريكسيت المضاد لحمض الفوليك من العلاجات الكيميائية التي تستخدم علي نطاق واسع في علاج السرطان . وقد عرف عنه أنه يسبب أضرار علي العظام في صورة كسور وتأخر في النمو العظمي وخاصة في الاطفال.

الهدف من البحث: أجريت هذه الدراسة لتوضيح الأثار الناتجة من عقار الميثوتريكسيت علي العظام والدور الوقائي لحمض الفوليك.

المواد والطرق المستخدمة: تم إجراء البحث علي٣٠ من ذكور الجرذان البيضاء وتم تقسيمها عشوائيا الي ثلاث مجموعات :مجموعة ضابطة (١أ) تم إعطاؤها محلول ملح, مجموعة ضابطة (٢ب) تم إعطاؤها حمض الفوليك بنفس الطريقة في المجموعة (٣). مجموعة تجريبية(٢): تم إعطاء هذه المجموعة عقار الميثوتريكسيت بجرعة ٧٥,ملجم/ كيلوجرام لمدة ٥ وتم إيقاف الحقن لمده ٩ أيام ثم معاودة الحقن لمدة ٥ أيام أخري. مجموعة تجريبية (٣):الجرذان في هذه المجموعة تم إعطاؤها عقار الميثوتريكسيت بنفس الطريقة في المجموعة (٢) بالإضافة الي حقن حمض الفوليك بعد ٦ ساعات من حقن الميثوتريكسيت بجرعة ١ملجم/ كيلو جرام. في كل مجموعة من المجموعات السابقة تم التضحية بالجرذان وأخذ عينات من عظام الفقرات القطنية و تقطيعها وتحضيرها للفحص بالميكروسكوب الضوئى و الالكترونى. كما تم اجراء دراسة مورفومترية.

النتائج: اوضح الفحص الهستولوجي لمنطقة المشاش في الفقرات القطنية في الجرذان المعالجة بعقار الميثوتريكسيت عدم انتظام الخلايا وخاصة في الطبقة النامية كما أظهرت بعض المناطق تحطم في خلاياها مع عدم ظهور مركز التعظم الثانوي مقارنة بالمجموعة الضابطة والمجموعة المعالجة بحمض الفوليك. وتم فحص جسم الفقرة والذي أوضح تحطم في معظم خلايا النخاع العظمي مع زيادة تكوين الخلايا الدهنية . كما أظهر الفحص المجهري الالكتروني لمنطقة الاحتياط انتفاخ الخلايا مع زيادة عدد الفجوات في السيتوبلازم كما لوحظ أن الانوية لها شكل غير طبيعي مع عدم وضوح الغشاء النووي مع نقص في عدد الشبكة الاندوبلازمية الخشنة او اختفاءها كما أن الياف الكولاجين في المصفوفة خارج الخلية كانت غير واضحة اما في منطقة الانتشار فلوحظ أن النواة لها شكل ومكان غريب مع زيادة عدد الفجوات في السيتوبلازم. الا أن العلاج بحمض الفوليك يعكس معظم هذه التغيرات عند إعطاؤه مع عقار الميثوتريكسيت وبالتالي فهو له دور واقي ضد معظم الاضرار الناتجة عنه.

الخلاصة: تناول حمض الفوليك أثناء المعالجة الكيميائية ب الميثوتريكسيت يقلل من الخلل و الاضرار الناتجة عنه علي النمو والتكوين العظمي.