Original Article

Comparative Histological Study on the Protective Effect of Folinic Acid versus Fish Oil on the Growing Bone of Methotrexate-Treated Young Rats

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ABSTRACT

Background: Folinic acid (FA) is used to reduce Methotrexate (MTX) toxicity during treatment of childhood acute lymphoblastic leukemia (ALL). However, FA has been shown to reduce MTX treatment efficacy and cure rates of ALL. Recent studies suggested that fish oil (FO) supplementation may protect bone during MTX chemotherapy.

Aim of Work: to compare the protective effect of FA versus FO on the growing bone of MTX-treated young rats monitored by histological, immunohistochemical, morphometric and laboratory methods.

Materials and Methods: Forty two, 6 weeks-old-male albino rats were divided into: group I (control), group II (MTX-treated), group III (MTX and FA-treated) and group IV (MTX and FO-treated). MTX was injected subcutaneously, once daily for 5 consecutive days, 0.65 mg/kg, followed by 9 days of rest, then 1.3 mg/kg twice weekly for 4 weeks. FA was injected intraperitoneally, 6 hours after each dose of MTX, 0.87 mg/kg, then1.3 mg/kg twice weekly. FO was given orally daily for 6 weeks, 0.5 ml/100 gm. Left knee joints were processed for measuring RANKL/ OPG ratio (Receptor Activator of Nuclear factor Kapp-B Ligand/ Osteoprotegerin). Right knee joint sections were stained with H&E, Masson's Trichrome and immunohistochemical staining for Caspase-3. Morphometric measurements and statistical analysis were done.

Results: MTX-treated group sections revealed disruption in the growth plate structure with subsequent reduction in endochondral bone formation. Supplementation with FA and FO preserved growth plate integrity and bone formation.

Conclusion: Fish oil showed better effect than Folinic acid in ameliorating growth plate disruption and retarded bone formation encountered during MTX chemotherapy in young rats.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common cancer in the pediatric age group, accounting for almost one third of newly diagnosed pediatric cancer cases[1].

Childhood leukemia survivors are at risk of a reduced bone density. Clinical studies have highlighted osteoporosis as a complication of chemotherapy for childhood malignancy. This osteoporotic condition increases bone fracture risk with a fracture incidence during leukemia treatment as high as 39%. The poor bone quality persists into adult life and may increase bone fracture risk at an older age. Thus, understanding the bone growth arrest and osteoporosis side effects is important[2].

Methotrexate (MTX) is an anti-folate that competes for the folate binding site of the enzyme dihydrofolatereductase (DHFR), thus disrupting reduction of folic acid to tetrahydrofolic acid, responsible for DNA synthesis and cell replication. Methotrexate is a mainstay treatment for childhood ALL and has been shown to be effective in other malignancies such as choriocarcinoma and osteogenic sarcoma. Follow up studies highlighted the effect of the drug in inducing bone growth arrest, diminished bone mineral density (BMD) and bone defect in the form of fractures, especially in pediatric patients, as well as in long-term follow up in survivors[3].

Owing to significant impact of MTX on skeletal health, it has become critically important to develop treatments to ensure bone health during the course of treatment. Folinic acid (FA) is readily converted to active forms of folate, as this folic acid analogue doesn't require action of the DHFR in the folate metabolism cycle; thus FA is now used clinically to reduce MTX toxicity in soft tissues as it is considered as MTX antidote[4].

However, the use of FA has been shown to reduce MTX treatment efficacy and cure rates in childhood ALL, cause cancer relapse and possibly support tumor growth[5].

Accordingly, cancer sufferers are increasingly turning to alternative treatments including natural products for better bone health and improved life quality. Recent studies

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suggested that oral supplementation of fish oil (FO) may protect bone during MTX treatment in rats[4].

This study was designed to compare the protective effect of folinic acid versus fish oil on the growing bone of Methotrexate-treated young rats; monitored by histological, immunohistochemical, morphometric and laboratory methods.

MATERIALS AND METHODS

Drugs

a. Methotrexate (MTX): Trade name: Methotrexate, manufactured by Shanxi PUDE pharmaceutical Co., Ltd, China (imported by Techno Pharma, Egypt), in the form of vial 50 mg. The drug was dissolved in 0.9%sodium chloride.

b. Folinic Acid (FA): Trade name: Calcium Folinate, manufactured by Shanxi PUDE pharmaceutical Co., Ltd, China (imported by Techno Pharma, Egypt), in the form of vial 50 mg. The drug was dissolved in 0.9%sodium chloride.

c. Fish oil (FO): Omega-3- fish oil raw material, manufactured by Henry Lamotte, France (imported by Pharma Treat, Egypt).

Animals

The study was conducted at the Animal House of Kasr-Al Aini Faculty of Medicine, according to the ethical guidelines for the care and use of laboratory animals. Forty two male albino rats, 6 weeks old, were used in this study, their weight ranging from 90-100 (95±1.25 gm). They were housed in hygienic stainless steel cages and kept in clean well-ventilated room. They were fed standard chow diet and allowed free access to water.

Experimental design

Animals were divided into the following groups:

I- Group I (Control Group): included 12 rats.

Control animals received the solvent of the drugs by the same route of administration and for the same experimental period as for the corresponding experimental groups. The subgroup of animals corresponding to those receiving fish oil received vegetable oil.

Control rats were subdivided equally into three subgroups; 4 rats each:

- Subgroup Ia: 4 rats received 0.5 ml of 0.9% sodium chloride (solvent of MTX) by subcutaneous (S.C.) injection.
- Subgroup Ib: 4 rats received 0.5 ml of 0.9% sodium chloride (solvent of MTX and FA) by S.C. as well as intraperitoneal (I.P) injection.
- Subgroup Ic: 4 rats received 0.5 ml of 0.9% sodium chloride (solvent of MTX) S.C. and 0.5 ml of vegetable oil orally (corresponding to fish oil therapy).

Control rats were sacrificed with the corresponding experimental groups.

II- Group II (MTX-Treated Group): included 10 rats which received MTX in two phases mimicking regimen for ALL treatment[6].

- During the induction phase, rats were subcutaneously injected with MTX, once daily for 5 consecutive days, at a dose of 0.65 mg/kg, followed by 9 days of rest.
- During the maintenance phase (from the end of week 2 till the end of week 6), rats received MTX S.C. at a dose of 1.3 mg/kg twice weekly.

III- Group III (MTX and FA-Treated Group): included 10 rats which received MTX in the same regimen as in group II, in addition:

- During the induction phase, FA was injected I.P., 6 hours after each daily dose of MTX, at a dose of 0.87 mg/kg.
- During the maintenance phase, rats received FA at a dose of 1.3 mg/kg, I.P, 6 hours after MTX, twice weekly[6].

IV- Group IV (MTX and FO-Treated Group): included 10 rats which received MTX in the same regimen as in group II. Concomitant with MTX therapy, FO was given orally daily, using a gastric tube, at a dose of 0.5 ml/100 gm body weight daily[7].

Measurement of the Length (in cm) and Weight (in gm) of each rat

This was done at the start of experiment and at the end of week 6. The length of each rat was measured as the distance from the tip of the nose to the end of the tail[8]. Statistical analysis was done for these measurements.

Sample Collection and Processing

At the end of 6 weeks, rats were anaesthetized by I.P injection of Phenobarbital at a dose of 80 mg/kg[9]. Rats were sacrificed and both knee joints from each rat were dissected out.

Left Knee Joints were Processed for Biochemical Analysis

Left knee joints from all animals were dissected. Left tibiae were analyzed for studying gene expression of RANKL, OPG and measuring RANKL/OPG ratio (Receptor Activator of Nuclear factor Kappa-B Ligand/ Osteoprotegerin) [This was done at the Biochemistry Department, Faculty of Medicine, Cairo University]. Statistical analysis was done for these measurements.

Gene expression of RANKL and OPG was done by Real Time PCR which included RNA extraction, DNA synthesis and amplification steps[10].

Right knee joints were processed for the Light Microscopic Study

Right knee joints from all rats were dissected, decalcified using 10% nitric acid for 14 days, then, were

processed into paraffin blocks. Sections were subjected to the following stains:

A) Hematoxylin& Eosin (H&E) stain[11].

B) Masson's Trichrome stain[12], for demonstration of mineralized and unmineralized bone matrix in the bony trabeculae of the secondary spongiosa.

C) Immunohistochemical staining using the avidin-biotin peroxidase complex technique[13], for detection of:

Caspase-3 (CASP-3): Rabbit polyclonal antibody (NEO markers, Thermo scientific Laboratories, USA, catalogue number RB-1197-R7). CASP-3 positive cells show brown cytoplasmic deposits.

Morphometric Study

Using "Leica Qwin 500 C" image analysis computer system Ltd, (Cambridge, UK), the following parameters were measured.For each group, five slides of five different specimens were examined. From each slide, ten non-overlapping fields were examined at a magnification of x100 for all parameters (Optical density x400).

The following parameters were measured

1. Mean thickness of growth plate cartilage in H&E-stained sections.
2. Mean area % of adipocytes in BM cavities in H&E-stained sections.
3. Mean optical density of mineralized bone in the bony trabeculae of secondary spongiosa in Masson's Trichrome-stained sections.
4. Mean number of immunoreactive chondrocytes for anti-caspase-3 in the proliferative zone of the growth plate cartilage in CASP-3 immunostained sections.

Statistical Analysis

All measurements were subjected to statistical analysis using Student T test and ANOVA test using (SPSS) software version 16 Chicago USA[14].

RESULTS

1. Laboratory Results

Measurements of RANKL, OPG and RANKL/OPG Ratio (Table 1)

2.Histological Results

A- Hematoxylin and Eosin-Stained Sections (Plate. 1 and 2)

Histological examination of longitudinal sections at the upper metaphyseal ends of tibiae showed that the growth plate consisted of a cartilaginous portion exhibiting various histologic zones "epiphyseal cartilage" and a bony metaphyseal portion which comprised two distinct regions; the primary spongiosa and the secondary spongiosa.

Histological examination of control sections revealed normal histological architecture. The epiphyseal cartilage appeared well-organized and comprised histologically distinct zones including the resting zone with quiescent chondrocytes, the proliferative zone with many chondrocytes organized into adjacent columns of stacked cells, hypertrophy zone, with enlarged chondrocytes. In the provisional calcification zone, empty lacunae appeared, denoting loss of chondrocytes by apoptosis. This was accompanied by calcification of the cartilage matrix spicules, which appeared more basophilic. In the metaphyseal portion, at the ossification zone, primary spongiosa was formed of irregular, fine longitudinally-oriented trabeculae which were separated by narrow bone marrow spaces (Plate. 1A).

The secondary spongiosa was composed of enlarged, calcified, acidophilic bony trabeculae remodeled from the primary spongiosa, with osteocytes imprisoned inside their lacunae. Trabeculae were separated by multiple irregular bone marrow cavities, occupied mostly by developing hemopoietic stem cells (Plate. 1B).

Sections of group II revealed that the epiphyseal cartilage exhibited marked reduction in thickness as compared to the control sections. The epiphyseal cartilage appeared disorganized, exhibiting disruption in chondrocytes columnar arrangement and marked reduction in chondrocytes number within the proliferative zone. Some homogenous areas of cartilaginous matrix were devoid of chondrocytes. Metaphyseal changes were in the form of marked reduction in the height of trabeculae of the primary spongiosa (Plate. IC). Bony trabeculae of the secondary spongiosa appeared few, discontinuous and widely separated with multiple irregular bone marrow cavities, occupied mostly by adipocytes, replacing the developing hemopoietic stem cells (Plate. ID).

Histological examination of sections of group III, which received MTX and FA, revealed some improvement in the histological picture, where the epiphyseal cartilage appeared well-organized with restoration of its normal thickness, as well as restoration of chondrocytes arrangement in the different histologic zones. Apparent increase in chondrocytes number within the proliferative zone was observed, as compared to sections of MTX-treated rats, but some homogenous areas devoid of chondrocytes were still evident. Trabeculae of the 1ry spongiosa exhibited relative increase in height (Plate. 2A). Bony trabeculae of secondary spongiosa appeared relatively thick with intervening multiple irregular bone marrow cavities which were occupied mostly by developing hemopoietic stem cells and some adipocytes (Plate. 2B).

Histological examination of sections of group IV, which received MTX and FO, showed marked improvement in the histological architecture. The epiphyseal cartilage appeared highly organized with apparently normal thickness. Preserved cellularity and regular organization of chondrocyte columnar arrangement were also evident, with marked increase in chondrocytes number within

the proliferative zone. Apparent increase in the height of trabeculae of the primary spongiosa was evident (Plate. 2C). Bony trabeculae of secondary spongiosa appeared relatively thick. Intervening bone marrow cavities were occupied mostly by developing hemopoietic stem cells with few adipocytes (Plate. 2D).

B. Masson's Trichrome (MT)-Stained Sections (Plate. 3)

Examination of Masson's trichrome-stained longitudinal sections at the metaphyseal ends of tibiae of control rats revealed that, the bony trabeculae of secondary spongiosa were formed mostly of mineralized bone matrix which appeared red in staining and few areas of unmineralized bone matrix (osteoid) which appeared blue in staining with intervening bone marrow cavities (Plate. 3A).

Sections of group II showed that, the thin discontinuous bony trabeculae were formed mostly of unmineralized bone matrix and few areas of mineralized bone matrix (Plate. 3B). Sections of group III revealed more improvement in the trabecular mineralization pattern where the bony trabeculae were formed mostly of mineralized bone matrix and few areas of unmineralized bone matrix with intervening bone marrow cavities (Plate. 3C). Sections of group IV showed marked improvement in trabecular mineralization pattern where the bony trabeculae were formed mostly of mineralized bone matrix and few areas of unmineralized bone matrix with intervening bone marrow cavities (Plate. 3D).

C. Immunohistochemical Results (Plate. 4)

Examination of longitudinal sections at the upper metaphyseal end of tibiae of control rats stained with anti- Caspase-3 antibody, revealed that the majority of chondrocytes within the proliferative zone of the epiphyseal cartilage exhibited negative caspase-3 immunoreactivity, while very few chondrocytes exhibited positive immunoreactivity. Chondrocytes of the hypertrophic zone expressed positive immunoreactivity (Plate. 4A). Examination of sections of group II revealed positive caspase-3 immunoreactivity in most of the chondrocytes within the proliferative zone of the epiphyseal cartilage (Plate. 4B). Examination of sections of group III revealed few positive caspase-3 immunoreactive chondrocytes within the proliferative zone (Plate. 4C). Examination of sections of group IV showed few positive caspase-3 immunoreactive chondrocytes within the proliferative zone (Plate. 4D).

3. Quantitative Morphometric Results

Mean Rats' Weight in gm in the Studied Groups

Group II showed a statistically significant decrease *(P<0.05)* when compared to the corresponding control as well as to groups III and IV. Group III showed a statistically significant decrease *(P<0.05)* when compared to the corresponding control and group IV. The highest value was reported for the control group and the lowest value was reported for group II (Table 2).

Mean Rats' Length in cm in the Studied Groups

Group II showed a statistically significant decrease *(P<0.05)* when compared to the corresponding control as well as to groups III and IV. Group III showed a statistically significant decrease *(P<0.05)* when compared to the corresponding control and group IV. The highest value was reported for the control group and the lowest value was reported for group II (Table 3).

Mean Thickness of Growth Plate Cartilage in µm in H&E stained sections

Group II showed a statistically significant decrease *(P<0.05)* when compared to the corresponding control, as well as to groups III and IV. Group III showed a statistically significant decrease when compared to the corresponding control and group IV. The highest value was reported for the control group and the lowest value was reported for group II (Table 4).

Mean area% of adipocytes in BM cavities in H&E stained sections

Group II showed a statistically significant increase *(P<0.05)* when compared to the corresponding control, as well as to groups III and IV. Group III showed a statistically significant increase when compared to the corresponding control and group IV. The highest value was reported for group II and the lowest value was reported for the control group (Table 5).

Mean optical density of mineralized bone in the bony trabeculae of secondary spongiosa in Masson's Trichrome-stained sections

Group II showed a statistically significant decrease *(P<0.05)* when compared to the corresponding control, as well as to groups III and IV. Group III showed a statistically significant decrease when compared the corresponding control and group IV. The highest value was reported for the control group and the lowest value was reported for group II (Table 6).

Mean number of immunoreactive chondrocytes for anti-caspase-3 in the proliferative zone of the growth plate cartilage

Group II showed a statistically significant increase *(P<0.05)* when compared to the corresponding control, as well as to groups III and IV. The highest value was reported for group II and the lowest value was reported for control group and group IV (Table 7).

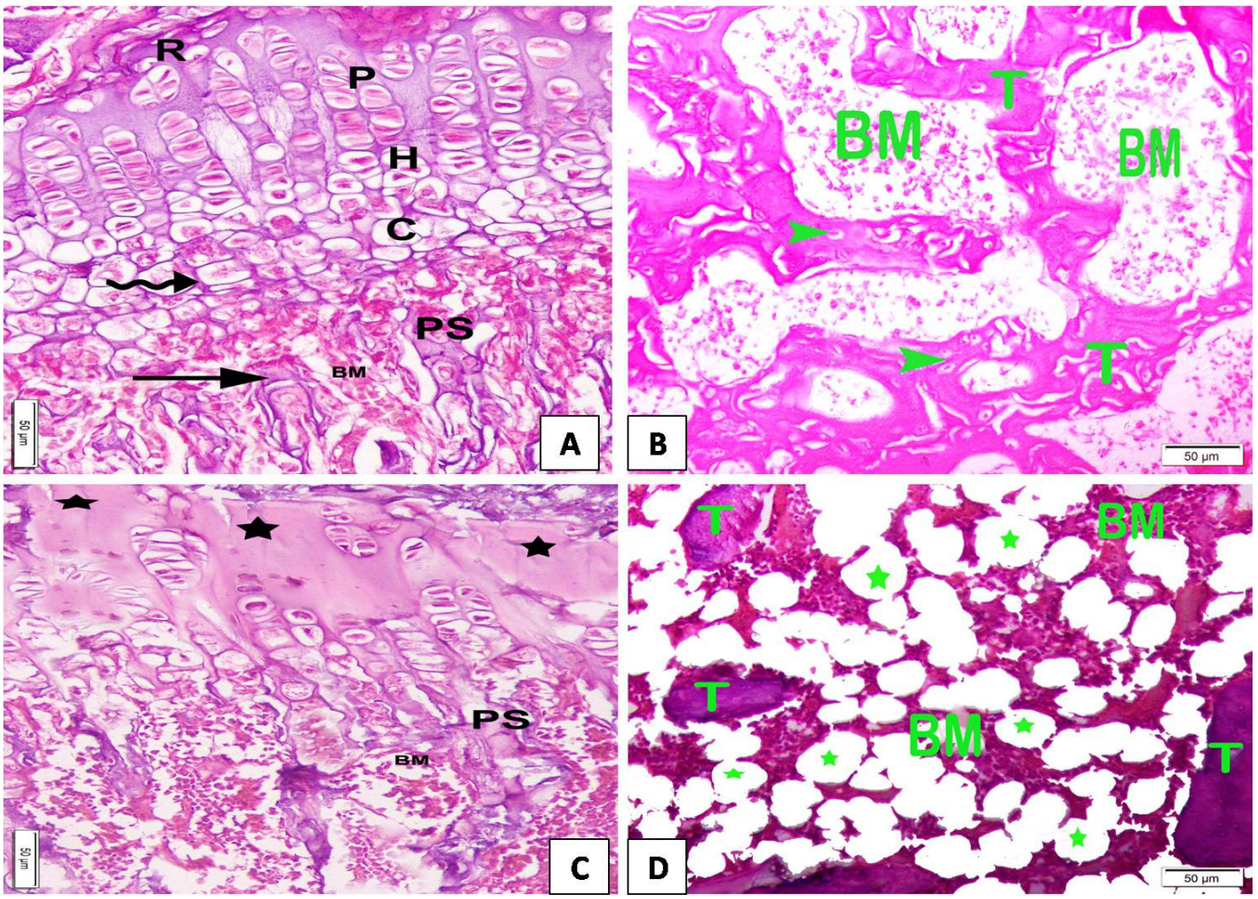

Plate. 1: [A] A photomicrograph of a longitudinal section at the upper metaphyseal end of tibia of the control group (group I) showing well-organized epiphyseal cartilage comprising the different zones; resting zone (R), proliferative zone (P) showing adjacent columns of proliferating chondrocytes, hypertrophy zone (H) with enlarged chondrocytes, calcification zone (C) with empty lacunae and surrounding basophilic calcified cartilage matrix (wavy arrow). Primary spongiosa (PS) consists of thin irregular trabecular spicules with mixed staining pattern (arrow) and intervening narrow bone marrow cavities (BM). (H& E X200). [B] A photomicrograph of a longitudinal section at the metaphyseal end of tibia of group I showing secondary spongiosa formed of a network of branching and anastomosing bony trabeculae (T) containing osteocytes imprisoned inside their lacunae (arrowheads). The intervening multiple irregular bone marrow spaces (BM) are occupied mostly by developing hemopoeitic stem cells. (H& E X200). [C] A photomicrograph a longitudinal section at the upper metaphyseal end of tibia of MTX-treated group (group II) showing disorganized epiphyseal cartilage, disruption in chondrocytes columnar arrangement, reduction in chondrocytes number in the proliferative zone and many homogenous areas devoid of chondrocytes (black stars). Trabeculae of primary spongiosa (PS) are separated with multiple bone marrow cavities (BM). Note the reduced thickness of the growth plate cartilage and the height of trabeculae of primary spongiosa (PS). (H& E X200). [D] A photomicrograph of a longitudinal section at the metaphyseal end of tibia of group II showing secondary spongiosa formed of thin bony trabeculae (T), widely separated by multiple irregular bone marrow cavities (BM) occupied mostly by adipocytes (green stars). (H& E X200).

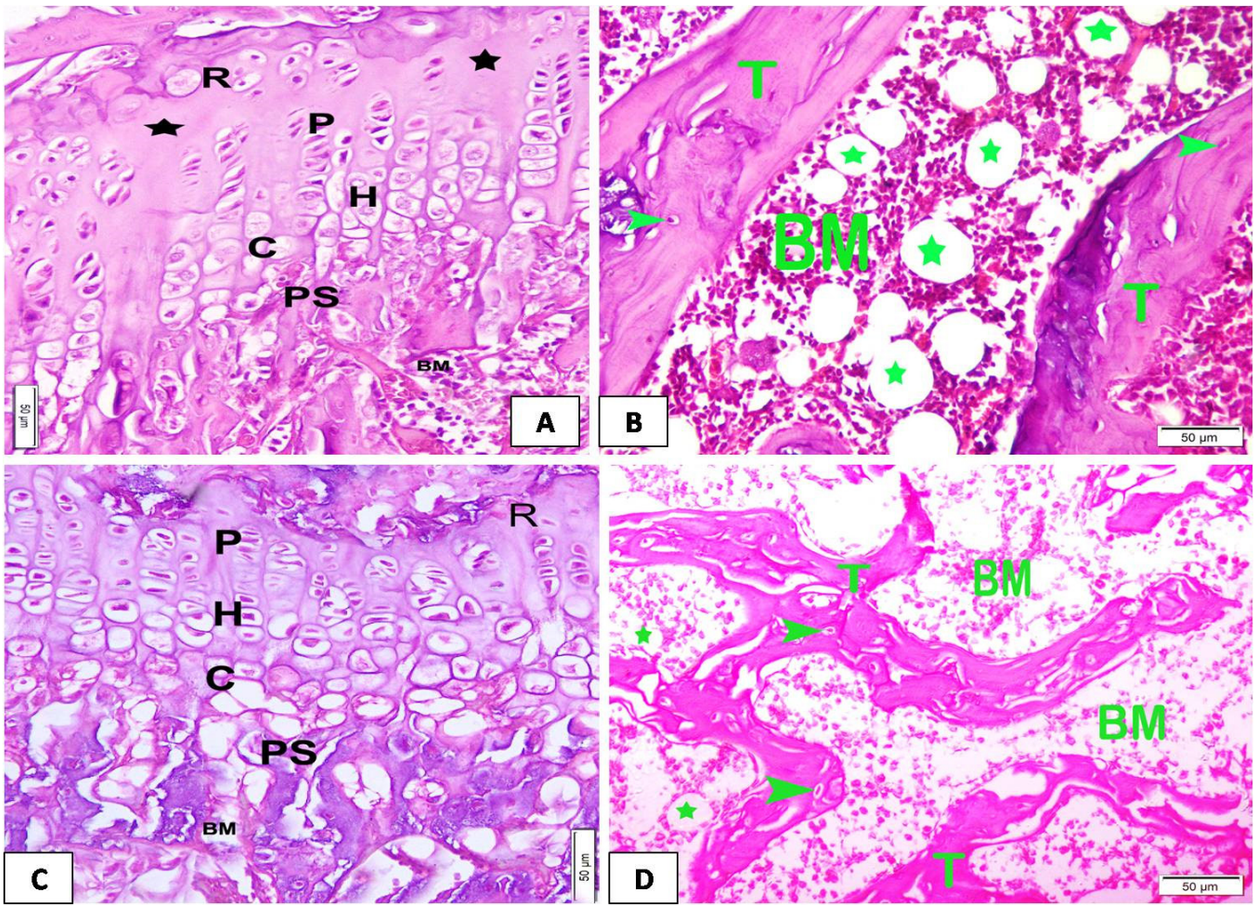

Plate. 2: Plate. 2: [A] A photomicrograph of a longitudinal section at the upper metaphyseal end of tibia of MTX and FA-treated group (group III) showing well-organized epiphyseal cartilage and preservation of resting zone (R), proliferative zone (P) with increased number of stacked chondrocytes, hypertrophy zone (H), calcification zone (C). Some areas of cartilage matrix are devoid of chondrocytes (black stars). Trabeculae of primary spongiosa (PS) are separated with multiple bone marrow cavities (BM). (H& E X200). [B] A photomicrograph of a longitudinal section at the metaphyseal end of tibia of group III showing secondary spongiosa formed of thick bony trabeculae (T) with osteocytes inside their lacunae (arrowheads). Trabeculae are separated by multiple irregular bone marrow cavities (BM), mostly occupied by developing hemopoietic stemcells and some adipocytes (green stars). (H& E X200). [C] A photomicrograph of a longitudinal section at the upper metaphyseal end of tibia of MTX and FO-treated group (group IV) showing highly organized epiphyseal cartilage, well-organized chondrocytes in the different histologic zones; resting zone (R), proliferative zone (P) with increased number of stacked chondrocytes, hypertrophy zone (H) and calcification zone (C). Trabeculae of the primary spongiosa (PS) are separated with bone marrow cavities (BM). (H& E X200). [D] A photomicrograph of a longitudinal section at the metaphyseal end of tibia of group IV showing secondary spongiosa formed of thick bony trabeculae (T) with osteocytes inside their lacunae (arrowheads). The multiple irregular bone marrow cavities (BM) are occupied mostly by developing hemopoietic stem cells and few adipocytes (green stars). (H& E X200).

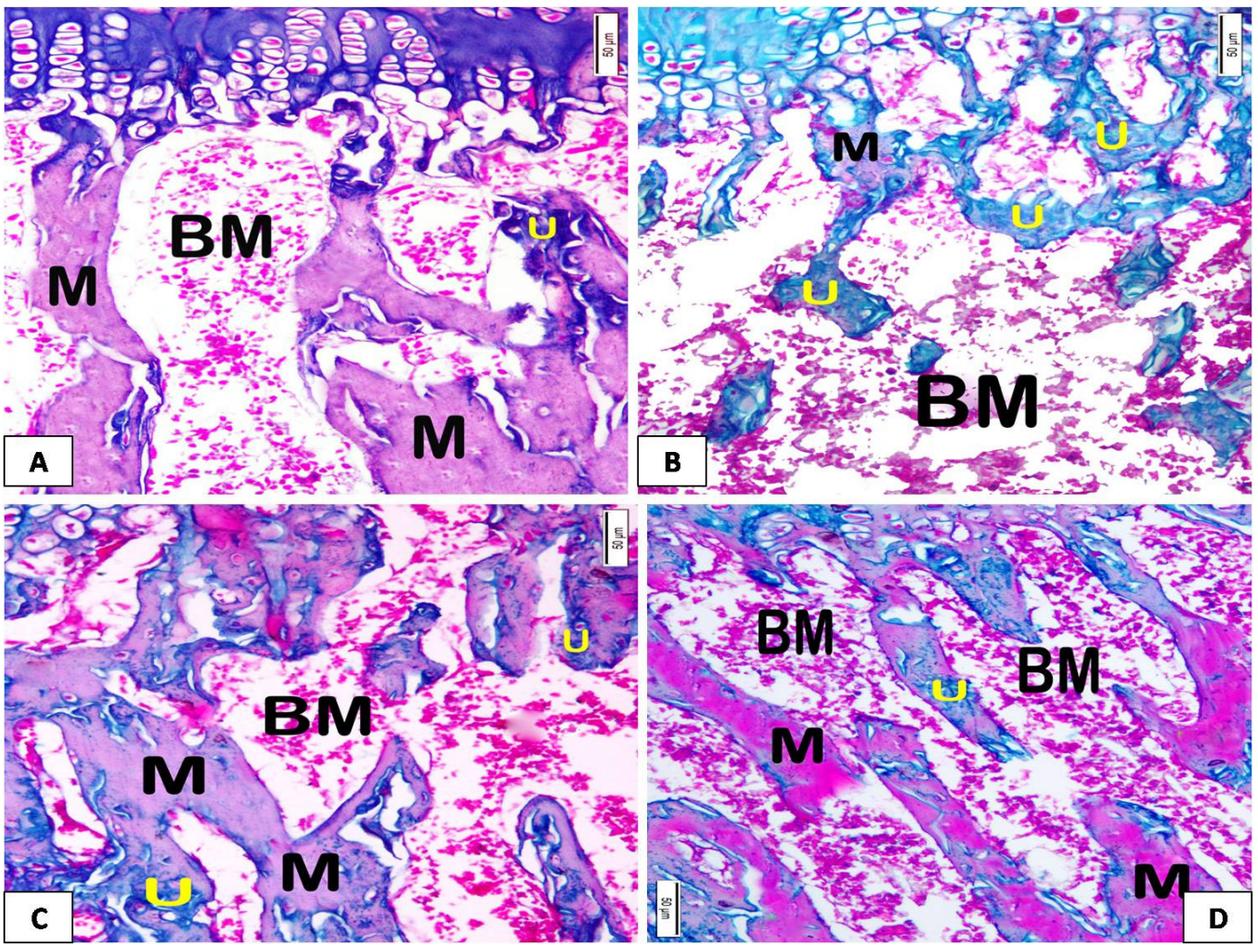

Plate. 3: [A] A photomicrograph of a longitudinal section at the metaphyseal end of tibia of a control rat (group I) showing the bony trabeculae formed mainly of mineralized bone appearing red in staining (M) and few areas of unmineralized bone appearing blue in staining (U). Bony trabeculae are separated by multiple irregular marrow cavities (BM). (MT, X200). [B] A photomicrograph of a longitudinal section at the metaphyseal end of tibia of MTX-treated rat (group II) showing bony trabeculae formed mostly of unmineralized bone matrix (U) and few areas of mineralized bone matrix (M). The intervening bone marrow cavities (BM) appear relatively widened. (MT, X200). [C] A photomicrograph of a longitudinal section at the metaphyseal end of tibia of MTX & FA-treated rat (group III) showing bony trabeculae formed mostly of mineralized bone matrix (M) and few areas of unmineralized bone matrix (U) with intervening bone marrow cavities (BM).(MT, X200). [D] A photomicrograph of a longitudinal section at the metaphyseal end of tibia of MTX and FO-treated rat (subgroup IV) showing bony trabeculae formed mostly of mineralized bone matrix (M) with few areas of unmineralized bone matrix (U) with intervening bone marrow cavities (BM). (MT, X200).

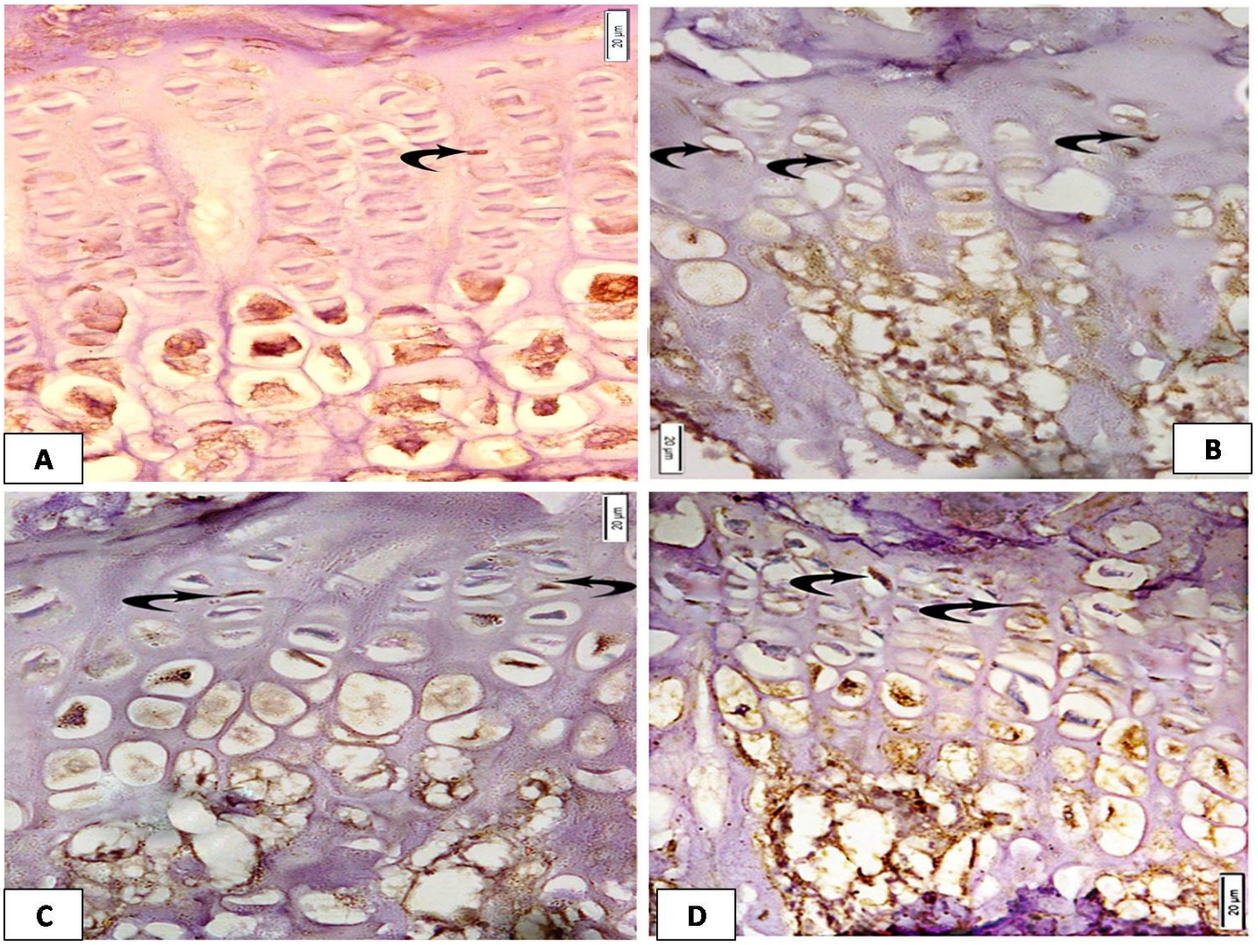

Plate. 4: [A] A photomicrograph of a longitudinal section of the upper metaphyseal end of tibia of a control rat (group I) showing that the majority of chondrocytes in the proliferative zone of the epiphyseal cartilage exhibit –ve Caspase-3 immunoreactivity. One immunoreactive chondrocyte is observed (curved arrow). Note that chondrocytes of hypertrophic zone express positive immunoreactivity. (CASP-3 immunostaining X400). [B] A photomicrograph of a longitudinal section of the upper metaphyseal end of tibia of MTX-treated rat (group II) showing Caspase-3 immunoreactivity in most of the chondrocytes in the proliferative zone of the epiphyseal cartilage (curved arrows). (CASP-3 immunostaining X400). [C] A photomicrograph of a longitudinal section of the upper metaphyseal end of tibia of MTX and FA-treated rat (group III) showing few Caspase-3 immunoreactive chondrocytes in the proliferative zone of the epiphyseal cartilage (curved arrows). (CASP-3 immunostaining X400). [D] A photomicrograph of a longitudinal section of the upper metaphyseal end of tibia of MTX & FO-treated rat (group IV) showing few Caspase-3 immunoreactive chondrocytes in the proliferative zone of the epiphyseal cartilage (curved arrows). (CASP-3 immunostaining X400).

Table 1: Mean values of RANKL, OPG and RANKL/OPG Ratio in the studied groups

Group	RANKL	OPG	RANKL/OPG Ratio
Group I	1	1	1
Group II	7.53±0.41	0.24±0.38	31.37*□
Group III	1.90±0.12	0.78±0.03	2.40*•
Group IV	1.31±0.31	0.90±0.03	1.40•□

*Significantly different from the corresponding value of the control group at *P<0.05*.
•Significantly different from the corresponding value of group II at *P<0.05*.
□Significantly different from the corresponding value of group III at *P<0.05*.

Table 2: Mean values of rats' weight in gm (±SD) in the studied groups

Group	Mean ± SD
Group I (control)	161±2.38
Group II	96±3.42*□
Group III	149±1.92*•
Group IV	158±1.25•□

*Significantly different from the corresponding value of the control group at *P<0.05*.
•Significantly different from the corresponding value of group II at *P<0.05*.
□Significantly different from the corresponding value of group III at *P<0.05*.

Table 3: Mean values of rats' length in cm (±SD) in the studied groups

Group	Mean ± SD
Group I (control)	23.34±0.5
Group II	14.34±0.37*□
Group III	21.26±0.79*•
Group IV	22.67±0.45•□

*Significantly different from the corresponding value of the control group at *P<0.05*.
•Significantly different from the corresponding value of group II at *P<0.05*.
□Significantly different from the corresponding value of group III at *P<0.05*.

Table 4: Mean thickness of growth plate cartilage (±SD) in the studied groups.

Group	Mean ± SD
Group I (control)	313.17±10.21
Group II	133.13±7.33*□
Group III	285.53± 7.86*•
Group IV	304.88± 10.26•□

*Significantly different from the corresponding value of the control group at *P<0.05*.
•Significantly different from the corresponding value of group II at *P<0.05*.
□Significantly different from the corresponding value of group III at *P<0.05*.

Table 5: Mean area% of adipocytes in BM cavities (±SD) in the studied groups.

Group	Mean ± SD
Group I (control)	4.27±0.94
Group II	73.92±2.47*□
Group III	11.99±2.59*•
Group IV	4.78±1.15•□

*Significantly different from the corresponding value of the control group at *P<0.05*.
•Significantly different from the corresponding value of group II at *P<0.05*.
□Significantly different from the corresponding value of group III at *P<0.05*.

Table 6: Mean optical density of mineralized bone in the bony trabeculae of secondary spongiosa (±SD) in the Studied Groups.

Group	Mean ± SD
Group I (control)	0.78±0.02
Group II	0.16±0.02*□
Group III	0.63±0.05*•
Group IV	0.75±0.03•□

*Significantly different from the corresponding value of the control group at *P<0.05*.
•Significantly different from the corresponding value of group II at *P<0.05*.
□Significantly different from the corresponding value of group III at *P<0.05*.

Table 7: Mean number of immunoreactive chondrocytes for anti-caspase-3 in the proliferative zone of the growth plate cartilage (±SD) in the Studied Groups.

Group	Mean ± SD
Group I (control)	1.40±0.54
Group II	5.60±0.54*□
Group III	2.20±0.83*•
Group IV	1.40±0.54•□

*Significantly different from the corresponding value of the control group at *P<0.05*.
•Significantly different from the corresponding value of group II at *P<0.05*.
□Significantly different from the corresponding value of group III at *P<0.05*.

DISCUSSION

The present study was designed to compare the protective effect of folinic acid versus fish oil on the growing bone of MTX-treated young rats, mimicking the clinical MTX acute and maintenance phase's protocol for ALL.

Monitoring rats' weight and length are regarded as important indicators for evaluating rats' growth rate[8]. MTX-treated group showed a statistically significant decrease in the mean values of rats' weight and length, when compared to the other experimental groups. These findings are consistent with[3] who stated that, rats receiving chemotherapeutic doses of MTX suffered from gastrointestinal irritation, malnourishment, poorly-

tolerated food intake and increase of water loss with subsequent reduction in body weight. In addition,[15] attributed weight loss to the susceptibility of intestinal stem cell to the toxicity of chemotherapeutic drugs, owing to its character of rapid turning-over, leading to cell apoptosis in gut tissue.

MTX-treated group showed a statistically significant decrease in the mean values of rats' length, when compared to the other experimental groups. These results could be explained clearly, based on findings observed on histological examination of the epiphyseal growth plate cartilage and metaphysis, which would possibly result in subsequent retarded bone growth.

In the current study, H&E-stained sections of MTX-treated group showed marked reduction in thickness of growth plate cartilage and marked reduction in chondrocytes number within the proliferative zone. These findings were explained by[16] who reported that MTX treatment was shown to decrease chondrogenesis, accompanied by increased apoptosis of chondrocytes in the growth plate cartilage. Also, significant reduction in the growth plate thickness following MTX treatment, as compared to the control, was reported by[17].

Examination of Caspase-immunostained sections of MTX-treated group revealed several immunoreactive chondrocytes within the proliferative zone of the growth plate. Increased rate of chondrocyte apoptosis would possibly explain the reduced growth plate thickness encountered in the current work. Recent studies have reported that, rats receiving MTX therapy showed an increase in the release of free radicals, including the OH radical that causes direct damage to DNA and a significant increase in the rate of abnormal chromosomal aberration leading to cell apoptosis[18].

Mirroring the growth plate damage demonstrated in the MTX-treated sections in the present study, metaphyseal sections showed marked reduction in the height of the primary spongiosa trabeculae. Secondary spongiosa trabeculae appeared thin, discontinous and widely separated with multiple BM cavities occupied mostly by adipocytes. In accordance with our results, it has been reported that, MTX induced osteoporosis in children treated with MTX[19]. Tracing out the role of MTX-chemotherapy in reducing primary bone formation, it was suggested to be due to decreased osteoblasts function as well as increased osteoclasts formation and function[20].

Demonstrated that MTX treatment can create an inflammatory microenvironment in bone that coincided with increased osteoclasts formation through upregulation of RANKL/OPG ratio[21].

To evaluate the role achieved by MTX in modulating the key osteoclastogenic signal, RANKL/OPG ratio was assessed in the current work. In MTX-treated group, a statistically significant increase in the mean value of RANKL/OPG ratio was reported, as compared to the other experimental groups. These findings are in accordance with[17] who suggested the important role of this osteoclastogenic factor in promoting osteoclasts formation and bone resorption, following MTX-chemotherapy. Moreover,[22] demonstrated that, MTX could increase osteoclasts density and reduce osteogenic differentiation; accompanied by reduction in mRNA expression of osteogenic factors. Similarly,[17] observed, upregulation of RANKL/OPG ratio and proinflammatory cytokines such as TNF- α, IL-1 and IL-6 in MTX-treated rats.

It has been reported that, BM stromal cells from MTX-treated rats have lower osteogenic potential, but higher adipogeneic potential. This implies that BM stromal progenitor cells tend to differentiate in favor of adipocyte lineage over osteoblast lineage due to an increased expression of the early adipogenic regulator factors[23].

It is noteworthy to mention that, BM adipocytes do not just simply fill the marrow spaces, but can act as negative regulators of the BM microenvironment and have direct functions on hematopoietic cells[24].

Examination of Masson's Trichrome-stained sections of MTX-treated group showed thin discontinuous bony trabeculae which were formed mostly of unmineralized bone matrix with few areas of mineralized bone matrix. Previous findings were reported by[25] who confirmed that, children with ALL showed a significant decrease in the bone mineral density (BMD) after MTX-chemotherapy.[26] explained that MTX-induced osteoblastic damage caused diminished mineralizing surface, mineral apposition rate and bone formation rate.

Experimental studies performed revealed that mineralized old trabecular bone and unmineralized new bone were stained red and blue, respectively, using Masson's Trichrome stain[27 and 28].

Folinic acid can reduce MTX side effects and compensate folate deficiency caused by MTX, as folate is essential for cell proliferation and survival[26]. MTX and FA-treated group showed a statistically significant increase in the mean values of rats' weight and length, as compared to MTX-treated group. In agreement with our results,[6] reported that, FA could reduce MTX-associated gastrointestinal side effects, thereby preserving rat growth.

In the present work, examination of H&E-stained sections of MTX and FA-treated group revealed more improvement in the histological architecture of the growth plate cartilage as bone protective effects of FA were clarified by[6] who reported that, FA supplementation could diminish the damaging effects of MTX at the growth plate by preventing MTX-induced chondrocytes apoptosis, via the suppression of proapoptotic molecules involved in the death receptor pathway.

Examination of Caspase immunohistochemical-stained sections of MTX and FA- treated group in this work, revealed few immunoreactive chondrocytes within the proliferative zone of the growth plate.

Examination of metaphyseal bone sections revealed that FA supplementation proved to preserve primary and secondary spongiosa bone trabeculae with BM cavities occupied mostly by developing hemopoietic stem cells. Our results were confirmed by[29] who proved that FA supplementation is responsible for reestablished marrow cellularity, preservation of bone volume and attenuation of BM adiposity following MTX treatment.

Although, FA supplementation in this study showed an improvement in the histological architecture of the bone of young growing rats as it preserved epiphyseal cartilage thickness, chondrocyte number and columnar arrangement, in spite of the presence of some homogenous areas of cartilaginous matrix devoid of chondrocytes. FA couldn't attenuate the MTX-induced damaging effects completely. FA supplementation proved to suppress osteoclastogenesis through modulation in RANKL/OPG ratio. Similar findings were observed by[7] who reported that FA supplementation was associated with down regulation of RANKL/OPG ratio.

Examination of Masson's Trichrome-stained sections of MTX and FA-treated group, revealed more improvement in the trabecular calcification pattern. FA prevents MTX chemotherapy-induced skeletal complications in childhood survivors. FA may be potentially useful in pediatric patients who are at risk of skeletal growth suppression and bone loss as a result of MTX chemotherapy[6].

However, the use of FA is controversial, as its overuse can reduce MTX treatment effects and cause cancer relapses. Thus, developing safe/effective solutions for MTX-induced bone defects is required for pediatric oncology[16].

Omega-3 PUFAs have significant health benefits, including anti-inflammatory and antioxidant properties[30]. In the current study, MTX and FO-treated group showed a statistically significant increase in the mean values of rats' weight and length, as compared to MTX and FA-treated group. These findings were confirmed by[31] who reported a gradual increase in the body weight of rats supplemented with FO, which was comparable to the control rats and explained these findings by the anabolic effect of FO.

In the present study, examination of H&E-stained sections of MTX and FO-treated group showed marked improvement in the histological architecture of the epiphyseal cartilage. Since MTX chemotherapy has proved to be associated with an inflammatory condition in bone, with upregulation of proinflammatory cytokines, it was hypothesized that the anti-inflammatory effect of FO supplementation could potentially attenuate chemotherapy-induced deleterious effects on cartilage and bone[32].

Examination of Caspase immunohistochemical stained-sections of MTX and FO-treated group, revealed few immunoreactive chondrocytes within the proliferative zone. Our results were in accordance with[30] who attributed the bone protective effects of omega-3 to their anti-oxidant potential and a decrease in the rate of chromosomal aberration, which confirms the role of omega-3 PUFAs in protecting the cell from the impact of free radicals.

Metaphyseal bone sections of MTX and FO-treated group showed marked improvement in the histological architecture of primary and secondary spongiosa trabeculae with BM cavities occupied mostly by developing hemopoietic stem cells. These findings were in agreement with[24] who reported that daily omega-3 PUFAs supplementation during MTX chemotherapy could enhance osteogenic differentiation through induction of osteogenesis factors and also inhibiting expression of osteoclastogenesis as well as adipogenesis factors.

Omega-3 PUFAs have the ability to inhibit osteoclasts formation through their anti-inflammatory properties[33]. Omega-3 PUFAs also promote production of IGF-1 which stimulates growth of bone, thereby helping to attenuate MTX chemotherapy–induced bone loss as well as preserving bone formation in rats[24].

In the present study, MTX and FO-treated group showed a statistically significant decrease in the mean values of RANKL/OPG ratio when, compared to MTX and FA-treated group.[4 and 7] confirmed our results by reporting that, FO supplementation showed suppression of MTX treatment-induced expression of the pro-inflammatory cytokines and RANKL/OPG ratio.

Examination of Masson's Trichrome-stained sections of MTX and FO-treated group, revealed marked improvement in the trabecular calcification pattern which was confirmed by[34] who reported an evidence of a positive role of omega-3 PUFAs or FO on BMD, bone mineral content and bone calcium levels, in addition to enhancing intestinal calcium absorption.

From this study we can conclude that fish oil proved to have better effect than folinic acid in ameliorating growth plate disruption and retarded bone formation encountered during MTX chemotherapy in young rats.

CONFLICT OF INTEREST

The authors have no conflicting financial interest.

REFERENCES

1. Abdel-Khalek ER, Sherif LM, Kamal NM, Gharib AF and Shawky HM: Acute lymphoblastic leukemia: Are Egyptian children adherent to maintenance therapy?. J Cancer Res Ther. (2015) 1: 54 - 68.
2. Wilson CL and Ness KK: Bone mineral density deficits and fractures in survivors of childhood cancer. Curr Osteoporos Rep. (2013)11: 329-337.
3. King TJ, Shandala T, Lee AM, Foster BK, Chen K, Howe PR and Xian CJ: Potential effects

of phytoestrogen genistein in modulating acute methotrexate chemotherapy-induced osteoclastogenesis and bone damage in rats. Int. J. Mol. Sci. (2015) 16: 18293-18311.

4. Raghu Nadhanan R, Skinner J, Chung R, Su YW, Howe PR and Xian CJ: Supplementation with fish oil and genistein, individually or in combination, protects bone against the adverse effects of methotrexate chemotherapy in rats. Journal Pone. (2013) 10: 1371-1383.
5. Skarby TV, Anderson H, Heldrup J, Kanerva JA, Seidel H and Schmiegelow K: High leucovorin doses during high-dose methotrexate treatment may reduce the cure rate in childhood acute lymphoblastic leukemia. Leukemia. (2006) 20:1955-1962.
6. Fan CM, Foster BK, Hui SK andXian CJ: Prevention of bone growth defects, increased bone resorption and marrow adiposity with folinic acid in rats receiving long-term methotrexate. Journal Pone. (2012) 7: 1371-1382.
7. Raghu Nadhanan R, Fan CM, Su YW, Howe PR, Xian CJ: Fish oil in comparison to folinic acid for protection against adverse effects of methotrexate chemotherapy on bone. J Orthop Res. (2014) 32: 587-596.
8. Xian CJ, Cool CJ, Scherer MA, Macsai CE, Fan C, Covino M and Foster BK: Cellular mechanisms for methotrexate chemotherapy-induced bone growth defects. Bone. (2007) 41: 842-850.
9. Pourghasem M, Nasiri E and Shafi H: Early renal histological changes in alloxan-induced diabetic rats. Int J Mol Cell Med. (2014) 3:11-15.
10. Pffafl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. (2001) 29: 45-55.
11. Kiernan JA: Histologic and histochemical methods: Theory and practice. Third edition. Arnold publisher, London, New York and New Delhi (2001) p. 111-162.
12. Bancroft JD and Gamble MN: Connective tissue stains. In: Theory and practice of histological techniques. Sixth edition. Elsevier health Sciences, Churchill living stone, Edinburgh, London, Oxford, New York, Philadelphia, St. Louis, Sydney and Toronto. (2008) p. 150.
13. Bancroft JD and Gamble MN: Theory and practice of histological techniques. Sixth edition. Churchill Living stone, London, Edinburgh, New York, Philadelphia, St Louis, Sydney and Toronto. (2007) p. 276 and p. 476-477.
14. Emlsey R, Dunn G and White IR: Mediation and moderation of treatment effects in randomized controlled trials of complex intervention. Stat. Methods Med. Res. (2010) 19: 237-270.
15. Natarajan K, Abraham P and Kota R: Activation of the mitochondrial apoptotic pathway contributes to methotrexate-induced small intestinal injury in rats. Cell Biochem Funct. (2017) 35: 378-391.
16. Su Y-W,Chen K-M, Hassanshahi M, Tang Q, Howe PR and Xian CJ: Childhood cancer chemotherapy – induced bone damage: pathbiology and protective effects of resveratrol and other nutraceuticals. Ann. N.Y. Acad. Sci. (2017) 1403: 109–117.
17. Lee AMC, Shandala T, Soo PP, Su Y, King TJ, Chen K, Howe PR and Xian CJ: Effects of resveratrol supplementation on methotrexate chemotherapy-induced bone loss. Nutrients. (2017) 9: 255-269.
18. Daham HH, Rahim SM and Al-Hmesh MJ: The effect of radiotherapy and chemotherapy in several physiological and biochemical parameters in cancer patients. Tikrit J Pure Sci. (2012) 17: 83–91.
19. Elsaid AG and Sadek AS: The protective role of simvastatin on methotrexate-induced bone injury in adult albino rat. The Egyptian Journal of Histology. (2017) 40: 105-115.
20. Kunstreich M, Kummer S, Laws H, Borkhardt A and Kuhlen M: Osteonecrosis in children with acute lymphoblastic leukemia. Haematologica. (2016) 101:1295-1305.
21. King TJ, Georgiou KR, Cool JC, Scherer MA, Ang ESM, Foster BK, Xue J and Xian CJ: Methotrexate chemotherapy promotes osteoclast formation in the long bone of rats via increased pro-inflammatory cytokines and enhanced NF- kB activation. Am J Pathol. (2012) 181: 121–129, doi: 10.1016/ j.ajpath.2012.03.037. Epub 2012 May 27.
22. Tchetina EV, Demidova NV, Markova GA, Taskin EA, Glukhova SI and Karateev DE: Increased baseline RUNX2, caspase 3 and p21 gene expressions in the peripheral blood of disease-modifying anti-rheumatic drug-naïve rheumatoid arthritis patients are associated with improved clinical response to methotrexate therapy. International Journal of Rheumatic Diseases. (2017) 20: 1468-1480 .
23. Georgiou KR, Scherer MA, Fan CM, Cool JC, King TJ, Foster BK and Xian CJ: Methotrexate chemotherapy reduces osteogenesis but increases adipogenesis potential in the bone marrow. J. Cell. Physiol. (2012) 227: 909–918.
24. Fan C, Su Y, Howe PR and Xian CJ: Long chain omega-3 polyunsaturated fatty acid supplementation protects against adriamycin and

cyclophosphamide chemotherapy-induced bone marrow damage in female rats. Int. J. Mol. Sci. (2018) 19: 484-494.I

25. Inaba H, Cao X, Han AQ, Panetta JC, Ness KK, Metzger ML, Rubnitz JE, Ribeiro RC, Sandlund JT, Jeha S, Cheng C, Pui C, Relling MV and Kaste SC: Bone mineral density in children with acute lymphoblastic leukemia. Cancer. (2018) 124: 1025-1035.
26. Bashandy MA and Elharoun H: 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. J Am Sci. (2014) 10: 47-57.
27. Xu L, Guo H, Li C, Xu J, Fang W and Long X: A time-dependent degeneration manner of condyle in rat CFA-induced inflamed TMJ. Am J Transl Res. (2016) 8: 556-567.
28. Han X, Du J, Liu D, Liu H, Amizuka N, and Li M: Histochemical examination of systemic administration of eldecalcitol combined with guided bone regeneration for bone defect restoration in rats. J Mol Hist. (2017) 48: 41–51.
29. Georgiou KR, Ghunadhanan R, Fan C and Xian CJ: Methotrexate-induced bone marrow adiposity is mitigated by folinic acid supplementation through the regulation of wnt/ b-catenin signaling. J. Cell. Physiol. (2015) 230: 648–656.
30. Ali IN, Awad MM and Mahmood AS: Effect of methotrexate and omega-3 combination on cytogenetic changes of bone marrow and some enzymatic antioxidants: An experimental study. Yemeni J Med Sci. (2017) 11: 1-7.
31. Al-Attar AM and Al-Rethea HA: Chemoprotective effect of omega-3 fatty acids on thioacetamide induced hepatic fibrosis in male rats. Saudi Journal of Biological Sciences. (2017) 24: 956-965.
32. Raghu Nadhanan R, Abimosleh SM, Su YW,Scherer MA, Howarth GS and Xian CJ: Dietary emu oil supplementation suppresses 5 fluorouracil chemotherapy-induced inflammation, osteoclast formation, and bone loss. Am. J. Physiol. Endocrinol. Metab. (2012) 302: 1440–1449.
33. Choi E and Park Y: The association between the consumption of fish/shellfish and the risk of osteoporosis in men and postmenopausal women aged 50 years or older. Nutrients. (2016) 8: 113-123.
34. Lavado-García J, Roncero-Martin R, Moran JM, Pedrera-Canal M, Aliaga I, Leal-Hernandez O, Rico-Martin S and Canal-Macias ML: Long-chain omega-3 polyunsaturated fatty acid dietary intake is positively associated with bone mineral density in normal and osteopenic Spanish women. PLOS ONE.(2018)13: 1371-1380.

الملخص العربى

دراسة هستولوجية عن التأثير الوقائى لحمض الفولينيك مقارنة بزيت السمك على نمو العظام فى الفئران صغيرة السن المعالجة بالميثوتريكسيت

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الخلفيه: يستخدم حمض الفولينيك للحد من سمية الميثوتريكسيت أثناء علاج سرطان الدم الليمفاوي الحاد في مرحلة الطفولة. ومع ذلك، فقد تبين أن حمض الفولينيك يحد من فعالية العلاج بالميثوتريكسيت ومعدلات علاج سرطان الدم الليمفاوي. اقترحت دراسات حديثة أن مكملات زيت السمك قد تحمي العظام خلال العلاج الكيميائي بالميثوتريكسيت.

الهدف من العمل: مقارنة التأثير الوقائى لحمض الفولينيك مقابل زيت السمك على نمو العظام فى الفئران صغيرة السن المعالجة بالميثوتريكسيت، و رصدت النتائج بالوسائل الهستولوجية, الهستوكيميائية المناعية، المورفومترية و المعملية.

المواد والأساليب: قُسِّمت اثنين وأربعين من ذكور الجرذان البيضاء البالغة من العمر ٦ أسابيع إلى: المجموعة الأولى (الضابطة)، المجموعة الثانية (المعالجة بالميثوتريكسيت)، المجموعة الثالثة (المعالجة بالميثوتريكسيت وحمض الفولينيك) والمجموعة الرابعة (المعالجة بالميثوتريكسيت و زيت السمك). تم حقن الميثوتريكسيت تحت الجلد، مرة واحدة يومياً لمدة ٥ أيام متتالية، ٠.٦٥ مجم / كجم، تليها ٩ أيام من الراحة ، ثم ١.٣ مجم / كجم مرتين أسبوعياً لمدة ٤أسابيع. تم حقن حمض الفولينيك في الغشاء البريتونى، بعد ٦ ساعات من كل جرعة من الميثوتريكسيت، ٠.٨٧ مجم / كجم ثم ١.٣ مجم / كجم مرتين أسبوعياً. تم إعطاء زيت السمك يومياً لمدة ٦ أسابيع، ٠.٥ مل / ١٠٠ جرام. تمت معالجة مفاصل الركبة اليسرى لقياس نسبة RANKL / OPG (المنشط المستقبلي للعامل النووي / Kapp-B Ligand Osteoprotegerin). صبغت مقاطع مفاصل الركبة اليمني بالهيماتوكسيلين والإيوسين, ماسون ثلاثي الألوان وصبغة هستوكيميائية مناعية ضد Caspase-3.

النتائج: كشفت مقاطع المجموعة المعالجة بالميثوتريكسيت خلل في بنية صفيحة النمو مع انخفاض في تكوين العظام. مع مكملات حمض الفولينيك و زيت السمك أمكن الحفاظ على سلامة صفيحة النمو وتكوين العظام.

الاستنتاج: أظهر زيت السمك تأثيراً أفضل من حمض الفولينيك في تخفيف خلل صفيحة النمو وتأخر تكوين العظم الذي لوحظ أثناء المعالجة الكيميائية بالميثوتريكسيت في الفئران الصغيرة.