

PREVENTION OF METHOTREXATE
CHEMOTHERAPY-INDUCED BONE
GROWTH ARREST AND
OSTEOPOROSIS WITH FOLINIC ACID

**A THESIS SUBMITTED IN TOTAL FULFILMENT OF
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THESIS SUMMARY

During childhood and adolescence, bone continues to lengthen through endochondral ossification, which occurs within the growth plate and the adjacent metaphysis. As the production of calcified cartilage scaffold for bone deposition relies on the regulation of growth plate chondrocyte activities, any disruption to this carefully controlled process will result in bone growth defects. Methotrexate (MTX), an inhibitor of dihydrofolate reductase and DNA synthesis, is a commonly used chemotherapeutic agent in childhood oncology, and has been shown to induce bone growth defects in paediatric cancer patients and in short-term experimental young rats. Moreover, current knowledge on substances available to preserve bone growth during chemotherapy of childhood malignancies is limited.

Previous animal studies have shown the short-term damaging effects of MTX on bone, and revealed that short-term MTX treatment in young rats can cause growth plate structural damages via suppression of chondrocyte proliferation and induction of chondrocyte apoptosis, which lead to metaphyseal bone loss. However, the underlying mechanisms for the structural and cellular damages remain unknown, particularly in the chronic treatment setting. Therefore, this PhD study, using chronic rat chemotherapy models, firstly aimed to compare and examine the damaging effects of low-dose vs. high-dose MTX on the skeleton and marrow progenitor cells of young rats. This was followed by mechanistic studies using immunostaining and real time RT-PCR with specimens from a chronic high-dose MTX chemotherapy trial, to identify underlying cellular and molecular mechanisms for MTX-induced growth plate and metaphyseal damages. In addition, this study also focused on the potential protective effects of

supplementary anti-dote folinic acid (FA) against chronic MTX-induced skeletal damages.

This study revealed chronic low-dose MTX treatment resulted in no damaging effects in the growth plate and nor significant suppression in primary spongiosa heights at the metaphysis. However, both short-term and chronic high-dose MTX treatment caused severe growth plate and metaphyseal damages. These results suggest MTX-induced skeletal toxicity in growing long bones is dose-dependent.

Mechanistic studies using a chronic high-dose MTX chemotherapy model revealed that chronic MTX chemotherapy can result in severe structural and cellular damages at the growth plate. MTX was able to induce chondrocyte apoptosis, which was confirmed by real time RT-PCR analysis showing up-regulation of the apoptotic molecules. In addition, more cartilage resorptive cells “chondroclasts” were found along the cartilage-bone transitional zone after MTX treatment, which could affect the conversion of growth plate cartilage template into bone. In the metaphysis, MTX significantly reduced bone volume by inducing osteoblast apoptosis, adipocyte and osteoclast formation. However, molecular analysis within bone samples revealed no significant changes for molecules involved in bone cell differentiation, suggesting possible recovery of progenitors/precursors after intense induction phase. However, some cytokines were found upregulated in blood plasma of treated rats. Finally, supplementary treatment with FA was able to reverse MTX-induced cellular damages at both the growth plate and metaphysis, suggesting FA supplementary treatment may be promising for reducing bone toxicity in young patients during chronic MTX chemotherapy.

DECLARATION

This work contains no material which has been accepted for the award of any other degrees or diplomas in any university or other tertiary institution to Chiaming Fan and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due references has been made in the text.

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Fan C, Foster BK, Wallace WH, Xian CJ. Pathobiology and prevention of cancer chemotherapy-induced bone growth arrest, bone loss, and osteonecrosis. *Curr Mol Med* (2011): 11(2): 140-151.

Fan C, Georgiou KR, King TJ, Xian CJ. Methotrexate toxicity in growing long bones of young rats: a model for studying cancer chemotherapy-induced bone growth defects in children. *Biomed Biotechnol* (2011): 903097 Epub

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): 44(1): 61-70.

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ABBREVIATIONS

α -MEM	Alpha minimal essential medium
ALL	Acute lymphoblastic leukemia
ALP	Alkaline phosphatase
Bax	Bcl-2-associated X protein
Bcl-2	B-cell leukemia-2 protein
BMD	Bone mineral density
BM-MNCs	Bone marrow mononuclear cells
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
BrdU	5'-bromo-2'-deoxyuridine
BV	Bone volume
BV/TV %	Bone volume/total volume %
cDNA	Complementary deoxyribonucleic acid
CFU-f	Colony forming units-fibroblast
CFU-GM	Colony forming unit-granulocyte/macrophage
CTR	Calcitonin receptor
Cyc-A	Cyclophilin A
DAB	Diaminobenzidine
DMARDs	Disease-modifying anti-rheumatic drugs
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FA	Folinic acid

FADD	Fas-Associated protein with Death Domain
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fas-L	Fas ligand
GH	Growth hormone
GP-130	Glycoprotein 130
GPOF	Growth plate-orienting factor
H&E	Haematoxylin and eosin
HD	Hodgkin's disease
HSC	Hematopoietic stem cells
IGF	Insulin-like growth factor
IL-1 β	Interleukin-I beta
IL-6	Interleukin-6
IL-11	Interleukin-11
ISNT	<i>in situ</i> nick translation
MMP-9	Matrix metalloproteases-9
MMP-13	Matrix metalloproteases-13
MMP-3	Matrix metalloproteinase-3
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
M-CSF	Macrophage/monocyte-colony forming factor
MTX	Methotrexate
MTX+FA	Methotrexate with Folinic acid
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin's lymphoma

°C	Degree Celsius
OCN	Osteocalcin
OCPs	Osteoclast precursors
OPG	Osteoprotegerin
OSCAR	Osteoclast-associated receptor
Osx	Osterix
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
Pen/Strep	Penicillin/streptomycin
PPAR γ	Peroxisome proliferator-activated receptor gamma
PTHrP	Parathyroid hormone-related peptide
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RUNX-2	Runt-related transcriptional factor 2
SEM	Standard error of the mean
TdT	Terminal deoxynucleotidyl transferase
TGF- β	Transforming growth factor beta
TNF- α	Tumor necrosis factor-alpha
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TRAP	Tartrate-resistant acid phosphatase

TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TV	Total volume
VEGF	Vascular endothelial growth factor
μ CT	Micro-computed tomography

CHAPTER ONE

LITERATURE REVIEW & PROJECT AIMS

1.1 Introduction to literature review

The development of more successful chemotherapy regimens has produced a growing population of childhood cancer survivors. However, the intensified use of chemotherapy treatment has also led to the emergence of cancer survivors facing long-term skeletal defects and bone health. It has become increasingly apparent that children grow poorly during and after the cancer chemotherapy, and osteopenia and osteoporosis are often found in adult survivors of high dose chemotherapy, a condition which predispose to a higher risk of fracture. Many clinical studies have outlined these problems, and more recently some *in vivo* experimental studies have investigated mechanisms how chemotherapy damages bone growth. This review summarizes clinical studies which examined skeletal risks in paediatric cancer survivors during and after chemotherapy treatment, recent animal studies aiming to understand the underlying mechanisms for chemotherapy-induced bone growth defects, and possible supplementary treatments or recommendations that have been investigated for preventing the skeletal complications during chemotherapy.

1.2 Osteogenesis

Bones are important connective tissues of the body and have important physiological functions, including supporting body tissues, protection, mineral storage and haemopoiesis. In children, a long bone consists of epiphysis, growth plate, metaphysis and diaphysis (**Figure 1.1**). Bones are vascular connective tissues consisting of cells and calcified extracellular matrix (ECM), which may be dense or sponge-like. Sponge-like bones, or “trabecular bones”, are composed of fine bone plates “trabeculae” surrounded with bone marrow, fat containing marrow and blood vessels. Trabecular bones are always surrounded by denser bones called “compact bones”,

which makes up approximately 80% of total bone mass. Compact bone is most abundant in the shafts of long bones, and has mechanical functions.

NOTE:
This figure is included on page 3 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1 Long bone structure.

The long bone consists of four regions, namely the epiphysis, the growth plate, metaphysis and diaphysis. Image is from:

<http://www.mountnittany.org/wellness-library/healthsheets/documents?ID=5416>

Bone growth is the process involving fascinating changes in morphology and biochemistry during fetal and postnatal development and growth, which gradually ceases until adolescence ends. During the first few weeks of embryonic development, bone formation and skeletal growth undergo morphological and biochemical changes. It begins when mesenchymal cells differentiate, condense, and transform into chondrocytes that form a cartilaginous model of the future skeleton (Calmar and Vinci 2002). By the end of the 8th week after conception, the skeletal pattern is formed in both the cartilage and connective tissues, and ossification begins. Paediatric skeleton is created by two distinct processes, which are “intramembranous ossification” and “endochondral ossification” (Calmar and Vinci 2002).

1.2.1 Intramembranous and endochondral ossification

Intramembranous ossification involves the replacement of sheet-like connective membranous tissue with bony tissue. This occurs in certain flat bones of skull, and some irregular bones including pelvis, scapula, clavicles and skull as well as the cortical dense bone of long bones (de Baat, Heijboer et al. 2005). Intramembranous ossification arises when mesenchymal cells differentiate into bone-forming cells “osteoblasts” which then begin to elaborate bone matrix and forming trabecular bone without an intermediate stage of cartilage formation (Rabie, Dan et al. 1996). As more trabeculae form, they become interconnected and fused to form cancellous bone, which will then be remodelled and become compact bone (Xian and Foster 2005). The region where mesenchymal cells did not participate in intramembranous ossification will remain to be the soft tissues of the bone such as periosteum (a dense membrane structure that wraps around all bones) and endosteum (the thin layer of cells lining the marrow medullary cavity of the bone) (Xian and Foster 2005).

Endochondral ossification occurs at the growth plate, which is responsible for the lengthening of long bones during fetal development and throughout childhood until bone growth ceases (Kronenberg 2003). The growth plate then stops functioning when skeletal maturity is achieved, and undergoes closure (Thomas, Byers et al. 2005). A more detailed description of growth plate structure and endochondral bone formation will be discussed in sections 1.2.2 and 1.3.

1.2.2 Growth plate structure and function

Growth plate is a layer of cartilage found at the end of growing long bones between epiphysis and metaphysis, which is the basic structure for endochondral ossification, whereby cartilaginous template is formed and remodelled into bony tissue at the metaphysis via the process of endochondral ossification. Growth plate consists of three distinct zones: the resting zone, proliferative zone and hypertrophic zone (**Figure 1.2**). The process of endochondral ossification begins when progenitor cells or stem cells at resting zone are activated, which enter the cell cycling at proliferative zone (Pateder, Eliseev et al. 2001), where the matrix is rich in collagen-II and aggrecan (Kronenberg 2003). The hypertrophic chondrocytes then direct the mineralisation of their surrounding matrix for chondrocyte maturation and hypertrophy (Robson 1999), attract chondroclasts for cartilage resorption, and secrete matrix rich in collagen-X and undergo calcification. As new blood vessels invade the calcified cartilage, hypertrophic chondrocytes then undergo apoptotic cell death. The left over cartilage matrix can then provide a scaffold for bone formation as osteoblasts and blood vessels invade the cartilage mould (Kronenberg 2003).

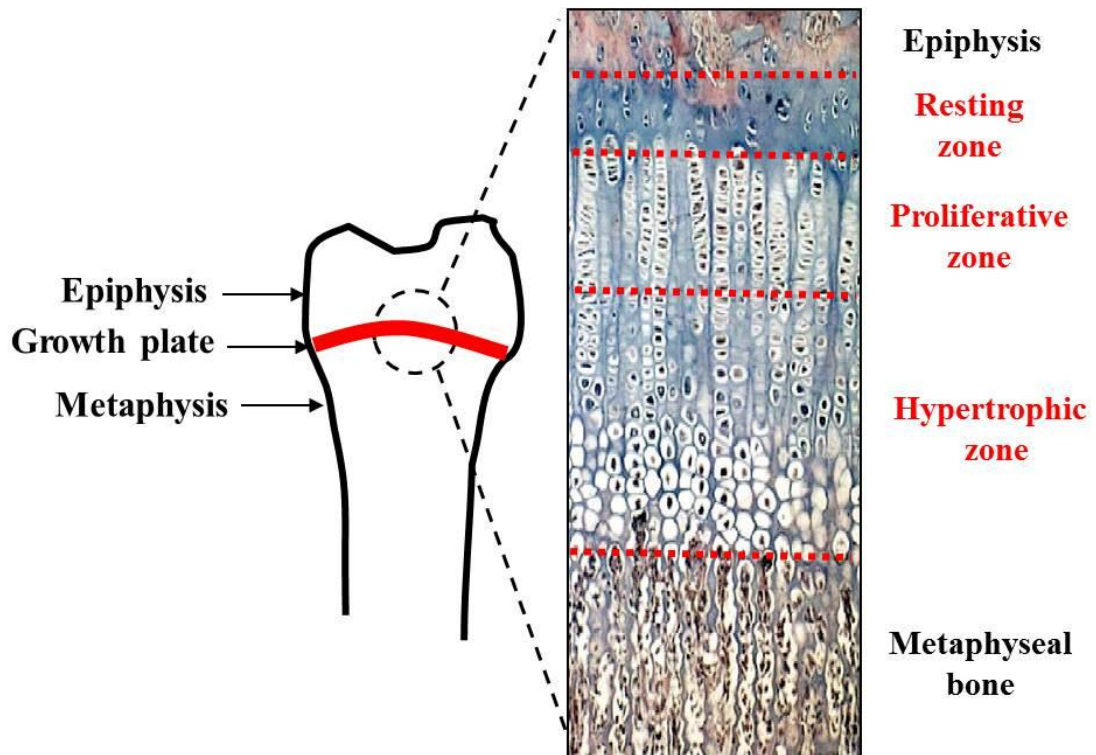


Figure 1.2 Distinct zones of the growth plate.

Growth plate is located at the end of long bones in between epiphyseal bone and metaphyseal bone, consisting of three distinct zones: the resting zone, proliferative zone and hypertrophic zone. Images obtained from own lab.

1.2.2.1 Resting zone

Progenitor cells, or pre-chondrocytes in the resting zone, are irregularly distributed in the bed of cartilage matrix (Iannotti 1990). Cellular proliferation in the resting zone is sporadic, and cells within this zone have very low intracellular and ionized calcium content, hence resting zone does not have much contribution to longitudinal bone growth (Iannotti 1990). The function of resting zone is not well understood as the cells are inactive in both proliferation and matrix turnover. Recent experiments suggested that resting zone cartilage may contribute to endochondral ossification at the growth plate as follows (Abad, Meyers et al. 2002):

1. Resting zone contains stem-like cells that give rise to clones of proliferative chondrocytes.
2. Resting zone cells can produce growth plate-orienting factor (GPOF), a morphogen that diffuse into the proliferative zone, which guides the alignments of proliferative columns.

Resting zone might also produce a factor that inhibits terminal differentiation of proliferative zone into hypertrophic chondrocytes, thus may contributes to the organization of the growth plate into distinct zones of proliferation and hypertrophy (Abad, Meyers et al. 2002).

1.2.2.2 Proliferative zone

The proliferative zone plays a crucial role in the process of endochondral ossification, and is characterised by longitudinal columns of chondrocytes, with their roles being matrix production and cell division. These together contribute to

longitudinal bone growth (Thomas, Byers et al. 2005). When chondrocytes in the proliferative zone divide, they give rise to two daughter cells which line up along the long axis of the bone, which results in columnar arrangement of chondrocyte clones (van der Eerden, Karperien et al. 2003). This special orientation directs the growth in a specific direction, therefore is responsible for elongation and shape of endochondral bone formation.

Apart from undergoing cell division, chondrocytes from proliferative zone also produce ECM macromolecules including collagens II, IX, XI and proteoglycans in an aggregated form (Pateder, Eliseev et al. 2001). Collagen-II is the predominant collagen in the matrix of proliferative zone, and collagen-XI helps to regulate collagen-II fibril diameter size (Orth 1999). Collagen IX contains proteoglycan moiety that links to collagen II fibrils, and may have the function to interact with other extracellular proteins (Orth 1999). Aggrecans, the major proteoglycans in aggregated form, act to inhibit matrix mineralisation and give the growth plate its structure (Thomas, Byers et al. 2005).

1.2.2.3 Hypertrophic zone

Cells in the proliferative zone eventually stop dividing, and enter a pre-hypertrophic stage where they are committed to become hypertrophic chondrocytes (Beier 2005). In the hypertrophic zone, the energy derived from mitochondrial electron transport is mainly for calcium accumulation, storage and release. Since the hypertrophic chondrocytes contain large amount of calcium and a great pool of stored calcium in their mitochondria, the primary function of hypertrophic zone is to prepare the matrix for calcification (Iannotti 1990). Hypertrophic chondrocytes secrete type X collagen, which

helps to facilitate matrix mineralisation and remodeling at the transition point between cartilage and bone. Hypertrophic chondrocytes also express Runx2, matrix metalloproteases 9 and 13 (MMP-9 and MMP-13), as well as proteins that are involved in the mineralisation of the ECM, these include osteocalcin, osteopontin and alkaline phosphatase (Orth 1999). Proteolytic enzymes such as collagenase-3 (MMP-3) are expressed for matrix degradation and chondrocyte expansion prior to mineralisation (Orth 1999). The proportion and size of aggrecans are much smaller in hypertrophic zone compared to the resting and proliferative zones. These changes may suggest the role of cartilage mineralisation at the hypertrophic zone. During the endstage of endochondral bone formation, ECM becomes calcified due to the expression of vascular endothelial growth factor (VEGF) released by hypertrophic chondrocytes, which triggers the invasion of blood vessels from the underlying metaphyseal bone, bringing bone cells osteoblasts and osteoclasts for bone deposition (van der Eerden, Karperien et al. 2003). Moreover, chondroclasts recruitment is known to play an important role at the cartilage-bone transitional zone and express MMP-9, which involves in in chondrocyte apoptosis, vascularization and ossification (van der Eerden, Karperien et al. 2003). Together, VEGF and MMP-9 are the key players for the events of the terminal stage of endochondral bone formation, and their subsequent replacement by bone.

Although hypertrophic chondrocytes are active, they do not survive the process of vascular invasion from metaphyseal bone below, and the ultimate fate of these cells is death (Provot and Schipani 2005). Hypertrophic chondrocytes undergo apoptosis in order for vascularization and bone formation to occur. However, the transition from hypertrophic to apoptotic chondrocytes is currently under review. It is known that apoptosis is regulated by the expression of B-cell leukemia-2 protein (Bcl-2, an anti-

apoptotic molecule) and Bcl-2-associated X protein (Bax, a pro-apoptotic molecule) (Mocetti, Silvestrini et al. 2001), and cellular susceptibility to apoptosis seems to depend on the expression ratio and dimerisation of these two proteins (Mocetti, Silvestrini et al. 2001). In growth plate chondrocytes, parathyroid hormone-related peptide (PTHrP) upregulates Bcl-2 expression to control the rate of chondrocyte turnover (Orth 1999). However, the detailed biology involved in chondrocyte apoptosis needs to be further clarified

1.2.3 Metaphyseal bone formation

The metaphysis begins at the end of each cartilaginous cell column of the hypertrophic zone. Major functions of the metaphysis include the removal of the mineralized cartilaginous matrix of the hypertrophic zone, formation of bone and remodeling of the trabeculae (Bianco, Cancedda et al. 1998). Metaphyseal bone formation begins as blood vessels invade the mineralised hypertrophic cartilage which brings in two cell types (osteoblasts and osteoclasts) that replace the cartilage matrix with bones (Parfitt 2002). Osteoblasts penetrate the invading calcified cartilage and replace it with spongy bone. At the same time, osteoclasts resorb and remodel the calcified cartilage and the temporary spongy bone to allow longitudinal bone growth and form bone marrow. The cartilage in the epiphyses continues to grow and replaced by bone, so the developing bone lengthens.

1.3 Bone cells involved in bone modeling and remodeling

Bone is a dynamic tissue with its structure and shape changing continuously to provide a mechanically sound structural framework. During childhood and adolescence, bones are sculpted by modeling, which permits bone formation at one site and removal

of old bones at another site within the same bone. This process allows each bone to grow in size and change in shape (Biewener, Swartz et al. 1986). The remodeling process becomes dominant after peak bone mass is reached. This process involves the replacement of aged bone tissue by new bone tissue in order to maintain bone mass (Seeman 2008). Bone remodeling continues through adult life with a balanced rate between bone resorption and bone formation for maintaining its structural and mineral integrity. Bone remodeling occurs in 4 stages (Michaud and Goodin 2006):

1. **Resorption** Bone remodeling begins with bone resorption when osteocytes (osteoblasts that have become embedded within the bone matrix) sense strain or microfracture, and send signals to bone surface lining cells to activate osteoclasts for bone resorption.
2. **Reversal** During resorption, growth factors and proteins are released from osteoblast precursors to stimulate the differentiation and proliferation of osteoblasts into the remodeling site.
3. **Formation** Osteoblasts form a matrix and deposit new bone at the site of resorption.
4. **Resting** Osteoblasts are then converted to osteocytes until a new remodeling cycle begins.

1.3.1 Osteoblasts and osteocytes

Osteoblasts arise when osteoprogenitor cells (or mesenchymal cells) located near bony surfaces and within the bone marrow differentiate under the influence of growth factors, such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β) and bone morphogenetic proteins

(BMPs) (Nakashima and de Crombrughe 2003). In both intramembranous and endochondral ossification, osteoblasts play an important role in ECM production and matrix mineralisation, which gives strength to skeleton. Osteoblasts also play a pivotal role in calcium homeostasis by controlling calcium deposition in the blood (Nakashima and de Crombrughe 2003). Osteoblasts take one of the three routes after bone formation. Firstly, they can remain on the bone surface, decrease their synthetic activity and become bone-lining cells. Secondly, they can become osteocytes by surrounding themselves with the matrix they secrete. Osteocytes are mature osteoblasts that are responsible for maintaining bone matrix. Thirdly, osteoblasts can be lost from the cell surface by apoptosis (Manolagas 2000).

1.3.2 Osteoclast differentiation and regulation

Osteoclasts are large multinucleated cells that arise from haemopoietic cells of the monocyte/macrophage lineage. The most remarkable feature of osteoclast morphology is the “ruffled border”, with the function to mediate resorption of the calcified cartilage or bone matrix (Nordahl, Andersson et al. 1998). The ruffled border is surrounded by the “clear site”, where the surface membrane of the osteoclast lies directly against the calcified surface (Nordahl, Andersson et al. 1998). The clear zone has the ability to seal off the distinct area between the calcified surface and osteoclast and allows the formation of a microenvironment suitable for resorption (Manolagas 2000).

1.3.2.1 Osteoclast differentiation

Osteoclasts are differentiated from hematopoietic cells. The osteoclast differentiation involves several major stages (**Figure 1.3**). The hematopoietic stem cells (HSC) give rise to circulating mononuclear cells, termed colony forming unit-granulocyte/macrophage (CFU-GM), or osteoclast precursors. CFU-GM then proliferates under the stimulation of macrophage/monocyte-colony forming factor (M-CSF), followed by differentiation with the stimulation of M-CSF and Receptor activator of nuclear factor kappa B ligand (RANKL) to become pre-fusion osteoclasts (pre-osteoclasts), which express tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor (CTR). Pre-osteoclasts will then fuse to become multinucleated cells under the stimulation of M-CSF and RANKL. However, the multinucleated osteoclasts are not functional due to lack of ruffled membrane for bone resorption, until further stimulation of RANKL to stimulate ruffled border formation (Suda, Takahashi et al. 1999; Feng 2005).

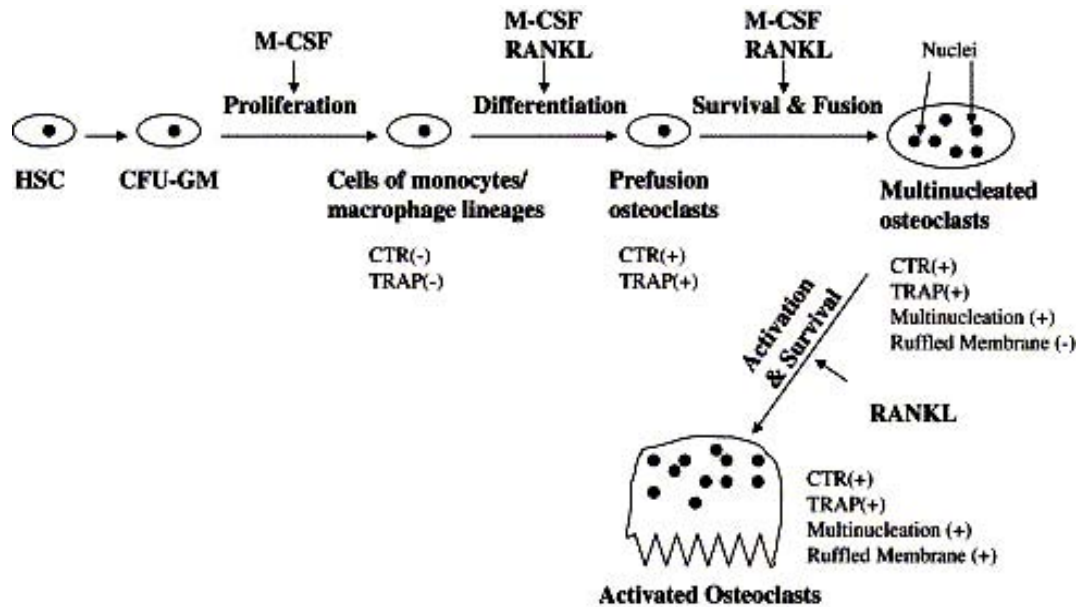


Figure 1.3 Osteoclast differentiation pathway.

The differentiation pathway of osteoclast progenitors into functionally active osteoclasts and the cytokines required for each step of the pathway. Pathway adapted from Feng Xu (2005), *Gene* (350: 1-13)

1.3.2.2 Osteoclast and osteoblast communication

Cells in osteoclast and osteoblast lineages communicate with each other through cell-cell interaction, which occurs in a basic multicellular unit (BMU) (**Figure 1.4**). Osteoclast–osteoblast interactions occur at various stages of differentiation (Matsuo and Irie 2008). The initiation of osteoclastogenesis largely depends on the interaction between osteoclast precursors and cells of the osteoblast lineage. Osteoblasts produce M-CSF, which is required for survival of cells in the macrophage–osteoclast lineage for the initial stage of osteoclast differentiation. Osteoblast/stromal cells also express RANKL, which binds to their receptor RANK on osteoclast precursors to activate a signal transduction cascade that leads to osteoclast differentiation (**Figure 1.3**). In mature osteoclasts, RANKL continues to mediate osteoclast activation and survival (Feng 2005). In addition, osteoblasts/stromal cells also produce osteoprotegerin (OPG), which is decoy receptor for RANKL. OPG is a secreted protein with homology to members of the TNF receptor family, which functions as a soluble decoy receptor to RANKL and competes with RANK for RANKL binding (Jones, Kong et al. 2002). Consequently, OPG is an effective inhibitor of osteoclast maturation and activation.

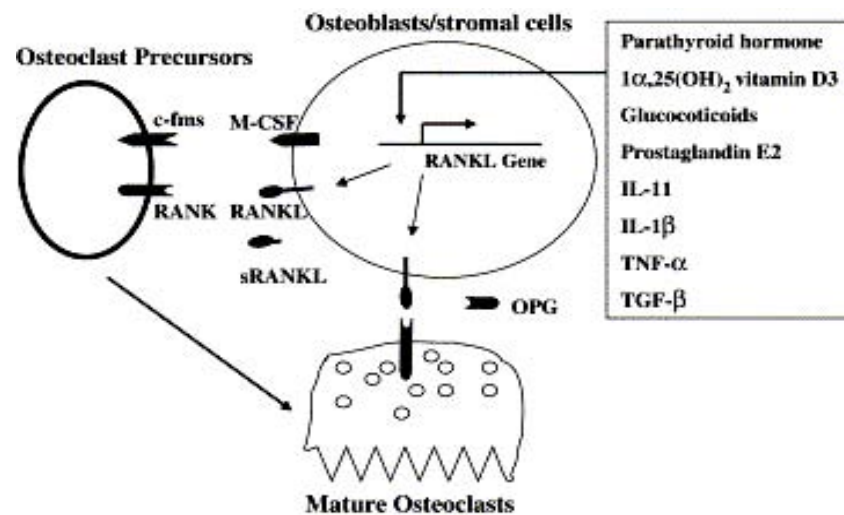


Figure 1.4 Osteoclast-osteoblast communications for osteoclastogenesis.

Osteoblasts/stromal cells support osteoclast differentiation by serving as a source of RANKL and M-CSF, which then bind to their respective receptors c-fms and RANK on osteoclast precursors to stimulate osteoclast formation. In addition, other osteotropic hormones and cytokines such as IL-1 β , TNF α , prostaglandin E2, IL-11 and parathyroid hormone have also been shown to stimulate RANKL gene expression in osteoblasts and stromal cells, hence enhance osteoclastogenesis. Pathway adapted from Feng Xu (2005), *Gene* (350: 1-13)

1.3.2.3 Cytokines involved in osteoclast regulation

Osteoclast development is restricted to the bone microenvironment, suggesting that other factors may exist to act in concert with RANKL/RANK. Several soluble factors can enhance osteoclastogenesis through RANKL induction in osteoblasts, these factors include PTHrP, TNF α , IL-1 β , IL-11, thyroid hormone, 1,25-(OH) $_2$, vitamin D and prostaglandin E $_2$ (Matsuo and Irie 2008). In contrast, TGF- β suppresses RANKL gene expression (**Figure 1.4**) (Jones, Kong et al. 2002; Matsuo and Irie 2008).

TNFs were discovered to be important in bone with the characterization of a major role of RANKL in osteoclast differentiation. Many of the TNF-family receptors activate NF- κ B and are reported to replace or augment RANK signalling, particularly TNF- α receptors (Blair, Robinson et al. 2005). TNF- α is an important member of the TNF family that modulates osteoclast formation, which exerts its function via receptors TNFR1 and TNFR2 (Feng 2005).

IL-1 is a member of the Toll-like receptor family, which is involved in scavenger activity. IL-1 can be produced by cells such as monocytes/macrophages, osteoblasts and osteoclasts. IL-1 acts directly on pre-osteoclast during osteoclastogenesis, and exerts its effect on RANKL-stimulated osteoclast formation by increasing the fusion rate of mononuclear osteoclast precursor cells (Roux and Orcel 2000; Lee, Gardner et al. 2006). Other cytokines such as IL-6 and IL-11 have short intracellular domains, and can be activated by their co-receptor GP-130. IL-6 and IL-11 support osteoclast generation through a secondary pathway, and is not required for osteoclast formation *in vivo* (Blair, Robinson et al. 2005).

TGF- β is abundant in bone matrix and can be released and activated by both osteoblasts and osteoclasts which stimulates both bone formation and bone resorption (Fuller, Lean et al. 2000), suggesting such effects may depend on factors present, such as stromal cells or osteoclast precursor population. Local injections of TGF- β in mice have been shown to act on osteoblasts and stimulate the cellular processes underlying osteogenesis (Mackie and Trechsel 1990). *In vitro* studies also revealed that TGF- β can induce extracellular matrix secretion, enhance osteoprogenitor cell proliferation and differentiation (Bonewald and Dallas 1994). In addition, *in vitro* data has also shown that TGF- β can suppress RANKL expression (Quinn, Itoh et al. 2001), and stimulates production of OPG (Takai, Kanematsu et al. 1998). In contrast to the inhibitory actions of TGF- β on osteoclastogenesis, TGF- β has also been reported to increase osteoclast differentiation in marrow haematopoietic cells in the absence of osteoblasts, by acting on macrophage-like osteoclast precursors (Quinn, Itoh et al. 2001). The balance between the suppressing and enhancing actions of TGF- β is not predictable, but may depend on the numbers of osteoblast and osteoclast precursors present.

1.4 Regulation of bone growth

Skeletal growth throughout life is characterized by three phases (van der Eerden, Karperien et al. 2003): (1) Rapid growth from fetal life to three years of age; (2) Slow growth during the early age of childhood up to puberty age; and (3) Increased rate of longitudinal bone growth during puberty until the peak height for an individual is reached. The process of longitudinal bone growth can be regulated by both genetic and environmental factors, which may influence the final height and bone mass of an individual.

1.4.1 Genetic factors affecting bone growth

Major genetic factors involved in regulating longitudinal bone growth include hormones, vitamins, growth factors and transcriptional factors (van der Eerden, Karperien et al. 2003). Among these, insulin-like growth factors (IGFs) play important roles in regulating cartilage and bone development. While IGF-II is essential for prenatal development, IGF-I continues to function throughout postnatal development (Lupu, Terwilliger et al. 2001). Recent studies showed that IGF-I not only promotes growth plate chondrocyte proliferation (Fisher, Meyer et al. 2005), it also promotes bone formation by stimulating osteoblast proliferation, differentiation and matrix synthesis (Nakashima and de Crombrughe 2003; Niu and Rosen 2005). After birth, growth hormone (GH) is the most important modulator for longitudinal bone growth (Lupu, Terwilliger et al. 2001; Xian 2007), and has a dual (IGF-I independent and IGF-I dependent) role in promoting growth plate chondrocyte proliferation, bone formation and bone remodeling (Werther, Haynes et al. 1993; Wang, Zhou et al. 2004; Mrak, Villa et al. 2007). In addition, sex steroids have also been shown to exert both direct and indirect effects on longitudinal bone growth, especially during puberty (Murphy, Khaw et al. 1993). Currently, it is known that sex steroids can suppress the formation of bone-resorptive cells osteoclasts, possibly by down regulating production of cytokines involved in bone resorption, such as IL-1, TNF- α and IL-6 (Riggs 2000; Zallone 2006), and up-regulating production of osteoclastogenesis inhibitory factor OPG by osteoblasts (Chen, Kaji et al. 2004; Zallone 2006). Apart from IGFs, GH and steroid hormones, bone cells also synthesize a variety of other growth factors such as PDGF, FGF, transforming growth factor β (TGF- β) and BMP (Mohan and Baylink 1991). All these factors have important roles in the maintenance of skeletal cell number, in modulation of fracture healing/repair, and regulation of bone remodeling (Canalis, McCarthy et al.

1988).

1.4.2 Environmental factors affecting bone growth

Besides genetic control, many lifestyle/environmental factors including exercise, nutrition and medical treatments also play important roles in regulating bone growth/remodeling. Adequate physical exercise and loading are important for influencing bone growth, bone mass accumulation and bone strength (Khan, McKay et al. 2000).

1.4.2.1 Radiation therapy

Radiation therapy plays an important role in the treatment of childhood malignancies, which can negatively impact the growth plate function, causing skeletal abnormalities and disturbance in skeletal development within the irradiated field (De Smet, Kuhns et al. 1976). In children undertaking radiation treatment, bone growth may be impaired, resulting in limb shortening (Probert and Parker 1975; Marinovic, Dorgeret et al. 2005). Several animal studies examined the changes in the growth plate following irradiation, and reported that growth plate of irradiated mice is characterized by disorganised columnar structure, loss of proliferative zone and chondrocyte apoptosis (Pateder, Eliseev et al. 2001; Horton, Margulies et al. 2006) in the initial stage. This is confirmed by immunohistochemistry showing elevated expression of proapoptotic indicator caspase-3, and decreased expression of chondrocyte differentiation and proliferation molecules, such as PTHrP, FGF and TGF- β (Damron, Mathur et al. 2004). The disruption of endochondral bone formation by irradiation therefore results in growth plate dysfunction and skeletal abnormality (Damron, Spadaro et al. 2000; Pateder, Eliseev et al. 2001).

Despite growth plate dysfunction following radiation exposure, the ability for recovery of growth was observed after radiation ceases. In the metaphysis, no impaired osteoblast function was observed; however, the presence of thickened trabeculae beneath the growth plate and of cartilagenous islands within cortical shafts of long bones indicated that bone remodeling was deficient (Anderson, Colyer et al. 1979). Osteoclast counts also demonstrated marrow aplasia followed by progressive decline of osteoclast formation, suggesting the impaired remodeling probably resulted from the radiation injury to osteoclast precursors (Anderson, Colyer et al. 1979).

1.4.2.2 Malnutrition

Adequate nutritional intake is also essential for optimal skeletal growth in children, as dietary intake can directly or indirectly influence bone structure and metabolism. For example, high protein intake increases acid production and renal acid excretion, which has been claimed to directly increase bone resorption and calcium excretion (Rizzoli 2008). For indirect effects of nutrients, protein intake can stimulate the IGF-I production in liver, hence stimulate osteoblast proliferation (Ammann, Bourrin et al. 2000). Calcium and vitamin D are also necessary for normal skeletal development. Inadequate intake of calcium and vitamin D has been shown to reduce calcium absorption and increased bone loss (Bueno and Czepielewski 2008). Calcium is known as a fundamental nutrient for bone mineralization, formation and maintenance of the structure and rigidity of the skeleton (Bueno and Czepielewski 2008). Studies have shown that long-term calcium supplement intake in children or adolescents enhances bone mineral acquisition rate, and is associated with a higher peak bone mass (Weaver 2000). The positive effect of calcium has been explained by the reduction in bone remodeling (Rizzoli 2008), as one earlier study showed the plasma level of osteocalcin

(biomarker for bone remodeling) was significantly reduced in calcium supplemented children (Johnston, Miller et al. 1992). It has also been suggested that calcium may not only affect bone remodeling but also modeling of skeleton (Bonjour, Carrie et al. 1997). Vitamin D (1, 25-(OH)₂D₃) is known to regulate calcium and phosphorous metabolism, and maintain serum calcium and phosphorus levels in a normal state for metabolic functions, such as bone mineralization (Bueno and Czepielewski 2008). The positive association between calcium and vitamin D has been well documented (Bischoff-Ferrari, Dietrich et al. 2004; Jackson, LaCroix et al. 2006). Research has shown that vitamin D is essential for skeleton growth during childhood, and vitamin D deficiency during growth caused delayed growth, bone abnormalities, and increased fracture risks in adulthood (Holick 2007). Vitamin D is known to protect against fracture by decreasing PTH and increase bone mass (Dawson-Hughes and Bischoff-Ferrari 2007; Brewer, Williams et al. 2011). Despite the inconsistency of clinical trials, it appears that additional benefits and positive effects can be achieved by higher vitamin D levels.

1.4.2.3 Chemotherapy

The primary cause of cancer-treatment-induced bone loss includes radiation therapy, hormone therapy and chemotherapy. In paediatric population, common types of cancer include acute lymphoblastic leukemia (ALL), brain tumors, non-Hodgkin's lymphoma (NHL) and Hodgkin's disease (HD) (von der Weid 2008). Several agents used in chemotherapy have been shown to have direct effects on bone metabolism, including glucocorticoids, methotrexate (MTX), cyclophosphamide, ifosfamide and 5-fluorouracil (Michaud and Goodin 2006). Cyclophosphamide has been shown to inhibit both bone formation and bone resorption directly by arresting cell division of preosteoblasts and osteoclasts (Wang and Shih 1986). In patients receiving ifosfamide

or ifosfamide/cisplatin chemotherapy, a decrease of serum osteocalcin and phosphate levels were observed, indicating low osteoblast activity, which may eventually lead to defective bone mineralization and low bone formation (Kother, Schindler et al. 1992). In animal studies, 5-fluorouracil (commonly used for treating solid tumors in both adult and children) was found to induce apoptosis of chondrocytes, as well as among osteoblasts and preosteoblasts (Xian, Cool et al. 2006), resulting in reduced bone volume. Apart from the in vivo analysis, results from in vitro studies also supports that chemotherapeutic agents can inhibit osteoblast proliferation and differentiation (Glackin, Murray et al. 1992), therefore reduces rate of bone formation.

Since bone mass accumulation occurs mainly during childhood and adolescence, chemotherapy during this period may influence bone mass accumulation and leads to lower peak bone mass. Many studies of childhood cancer treatment have reported different skeletal problems in children during and after chemotherapy (Crofton, Ahmed et al. 2000; Athanassiadou F 2005). MTX is the most commonly used anti-folate antimetabolite in the treatment of paediatric cancers, particularly ALL (Crofton, Ahmed et al. 2000), as well as inflammatory diseases such as rheumatoid arthritis (Suzuki, Nakagawa et al. 1997). The drug MTX acts to inhibit the reduction of tetrahydrofolate by dihydrofolate reductase, thus inhibiting DNA and RNA synthesis, which in term inhibits cancer cell proliferation (**Figure 1.5**) (Minaur, Kounali et al. 2002; Chabner and Roberts 2005). MTX at high dose of 100-1000 mg/m² is used for the treatment of ALL (Matherly and Taub 1999; Carey, Hockenberry et al. 2007). High-dose MTX treatment has been shown to cause osteopenia and increased fracture risks in children (van der Sluis, van den Heuvel-Eibrink et al. 2000; Baim, Binkley et al. 2008).

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This figure is included on page 24 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.5 The action mechanism of MTX.

MTX firstly enters the cell cycle through reduced folate carrier (a) using folate receptor-activated endocytic pathway (b). Once MTX enters the cell, it becomes polyglutamated (Glu) by the enzyme folypolyglutamate synthase (c). MTX(Glu)_n then inhibit the enzyme dihydrofolate reductase for the conversion of dihydrofolate (FH₂) to tetrahydrofolate (FH₄) (d). The depletion of FH₄ cause a reduction in thymidylate (TMP) synthesis (e), which in term inhibits DNA synthesis, affecting both thymidine and purine biosynthesis (f), which are essential for RNA production. Pathway obtained from Chabner, B. A. (2005), *Nature Reviews Cancer* (5: 65-72)

The following manuscript (Section 1.5) focuses on reviewing the clinical issues of cancer chemotherapy-induced skeletal abnormalities, the pathobiology for chemotherapy-induced bone damages, current investigations for treatments preventing chemotherapy-induced bone damages. The second manuscript (Section 1.6) focuses on the current known mechanisms underlying MTX-induced skeletal defects with animal models.

**1.5 PATHOBIOLOGY AND PREVENTION OF CANCER CHEMOTHERAPY-
INDUCED BONE GROWTH ARREST, BONE LOSS, AND
OSTEONECROSIS (published review article)**

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**1.6 METHOTREXATE TOXICITY IN GROWING LONG BONES OF YOUNG
RATS: A MODEL FOR STUDYING CANCER CHEMOTHERAPY-
INDUCED BONE GROWTH DEFECTS IN CHILDREN**

(published review article)

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Methotrexate toxicity in growing long bones of young rats: a model for studying cancer chemotherapy-induced bone growth defects in children

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Abstract

The advancement and intensive use of chemotherapy in treating childhood cancers has led to a growing population of young cancer survivors who face increased bone health risks. However, the underlying mechanisms for chemotherapy-induced skeletal defects remain largely unclear. Methotrexate (MTX), the most commonly used anti-metabolite in paediatric cancer treatment, is known to cause bone growth defects in children undergoing chemotherapy. Animal studies have not only confirmed the clinical observations, but have increased our understanding of the mechanisms underlying chemotherapy-induced skeletal damage. These models revealed that high-dose MTX can cause growth plate dysfunction, damage osteoprogenitor cells, suppress bone formation, increase bone resorption and marrow adipogenesis, resulting in overall bone loss. While recent rat studies have shown that antidote folinic acid can reduce MTX damage in the growth plate and bone, future studies should investigate potential adjuvant treatments to reduce chemotherapy-induced skeletal toxicities.

Introduction

During childhood and adolescence, bone continues to grow until a peak height and peak bone mass are achieved. It is during these periods that children are most vulnerable to interference to skeletal growth, and disturbance to the growing skeleton results from disruption to the processes of endochondral ossification and/or bone remodeling, which may predispose children to earlier onset of skeletal defects. Due to significant advancements and higher survival rate, cancer chemotherapy has been gaining popularity in treatment of paediatric cancers, and has become an important risk factor for bone growth defects in paediatric cancer patients. Intensive chemotherapy for childhood cancers has been shown to cause bone growth defects (bone loss, osteopenia and fractures).

Methotrexate is the most commonly used anti-metabolite in childhood oncology, and both clinical and experimental studies have demonstrated methotrexate-induced bone growth impairment. This article reviews previous studies in which rat models of methotrexate chemotherapy have been used to investigate chemotherapy-induced bone defects, mechanisms of bone growth arrest and bone loss, and recovery potential.

Bone growth and regulation

Bone growth is the process involving fascinating changes in morphology and biochemistry during development and growth, which gradually ceases until adolescence ends. During bone growth in childhood and adolescence, lengthening of long bones depends on the process of endochondral ossification, in which the growth plate cartilage continues to produce calcified cartilage which serves as a template for formation of primary trabecular bone [1]. Growth

plate is situated at both ends of long bones, which is composed of three distinct zones: the resting, proliferative and hypertrophic zones. Bone growth begins as progenitor cells at resting zone are activated and enter the cell cycle at the proliferative zone [2], and produce extracellular matrix rich in collagen-II and aggrecan [1]. The hypertrophic chondrocytes secrete matrix rich in collagen-X and direct mineralisation of their surrounding matrix while undergoing apoptosis [3]. Metaphyseal primary bone formation begins as blood vessels invade the mineralised hypertrophic cartilage, which brings in two cell types (osteoblasts and osteoclasts) that remodel the mineralized cartilage to primary woven bone [1]. While osteoclasts resorb the calcified cartilage, osteoblasts penetrate the invaded calcified cartilage and replace it with spongy bone [1]. Bone lengthens as growth plate cartilage continues to grow and is replaced by bone. Longitudinal bone growth is mainly regulated by genetic and hormonal factors such as growth hormone (GH), insulin-like growth factors (IGFs) [4, 5], thyroid hormone and glucocorticoids, sex steroids [6-8], fibroblast growth factors (FGF), epidermal growth factor and related ligands [9] transforming growth factor β (TGF- β) and bone morphogenic protein (BMP) [10, 11]. However, environmental factors such as nutrition [12-14] and medical treatments including chemotherapy have also been shown to be important determinants for bone growth in children, influencing the final height and bone mass of an individual.

The clinical issue of methotrexate-induced skeletal defects

Cancer chemotherapy has been achieving better success in treatment of paediatric cancers, with a survival rate over 80% in treating acute lymphoblastic leukaemia (ALL) which is the major childhood cancer. Consequently, childhood chemotherapy has become an important risk factor for bone growth defects in paediatric cancer patients. Methotrexate (MTX) is the

most widely used anti-metabolite in the treatment of childhood cancers, and is critical for treating acute lymphoblastic leukemia (ALL) [15]. MTX is a folate antagonist, commonly used at high-doses for the treatment of malignancies (100-1,000 mg/m²) [16, 17], and at lower doses (5-25 mg/week) for the treatment of inflammatory diseases such as rheumatoid arthritis (RA) [17, 18]. Frequently reported adverse effects associated with high-dose MTX include nausea, abdominal distress, intestinal mucositis [19], leukopenia and bone marrow suppression [20]. High-dose MTX has also been shown to have significant damaging effects on bone growth in children [21].

Clinical studies have highlighted osteopenia as a complication for childhood malignancies, characterised by reduced BMD and increased fracture risks [21, 22]. During intensive chemotherapy, children treated with high-dose MTX in combination with corticosteroids showed depressed bone formation and enhanced bone resorption [23, 24]. On the other hand, some studies reported no significant BMD reduction in survivors of ALL [25]. Since ALL treatment regimens are multiple drug combination therapies with or without cranial irradiation, it is difficult to determine the effects of high-dose MTX alone on bone growth. However, high-doses of MTX and corticosteroid together were found to be associated with longitudinal growth arrest [26, 27], a high risk of low bone mass and failure for BMD recovery even after discontinuation of treatment [25]. Overall, the use of high-dose MTX in treating paediatric ALL is encouraged, however early onset of skeletal complications must be monitored.

Mechanistic studies on MTX skeletal defects with animal models

The mechanism of how childhood cancer chemotherapy affects bone growth remains largely unknown. Since all ALL treatment protocols vary not only in dosage, but also route of administration and use of cranial irradiation, it is difficult to distinguish the effects of individual chemotherapeutic agents on bone growth. Hence, many laboratory studies have been conducted which have enabled investigations into the effects and underlying mechanisms of different chemotherapeutic agents alone on bone growth. For example, in a rat chemotherapy model, doxorubicin, an anthracycline antibiotic and cytotoxic (anti-neoplastic) agent commonly used against various cancers, caused thinning of growth plate, disturbance of chondrocyte columnar arrangement, increased number of fat cells but decreased hematopoietic cellularity in the bone marrow [28]. Similarly, *in vitro* studies using human bone marrow cells have shown that corticosteroids (commonly used for treating ALL) can significantly suppress osteoblastic activity, resulting in decreased bone formation [29]. Corticosteroid-induced osteopenia/osteoporosis has been confirmed in animal models [30], and has been shown to reduce bone mineral density and be associated with increased fracture risks in children [31]. In this section, animal studies investigating the mechanisms for MTX-induced skeletal damage are reviewed.

Methotrexate chemotherapy-induced growth plate dysfunction

As bone lengthening is the result of endochondral ossification at the growth plate, chemotherapy-induced growth plate damage may impact on bone lengthening. An earlier *in vitro* study examining effects of chemotherapeutic agents on chondrocyte proliferation observed no effects of MTX on proliferating chondrocytes [32]. In a rat study, MTX at 60 mg/m² body surface area (injections once weekly given for 8 weeks) was shown to have no effects on proliferating chondrocytes, but to cause an increase in hypertrophic zone thickness

and number of hypertrophic chondrocytes [33]. More recent animal studies revealed that while long-term low-dose MTX treatment caused no damage to the growth plate, two cycles of high-dose MTX (at 0.75mg/kg, 5 days on/9 days off/5 days on) caused a significant decrease in growth plate height [34] (Fig-1), which was due to the reduction of chondrocyte proliferation (Fig-1) and collagen-II production, as well as the induction of chondrocyte apoptosis possibly through the Fas/FasL death receptor pathway [35] (Fig-1). Due to the growth plate dysfunction, a significant reduction in the thickness of newly formed primary spongiosa bone was also found in the adjacent metaphyseal bone, mirroring the thinning of the growth plate [35, 36]. These studies suggest that the effect of MTX on growth plate structure and function is largely dependent on the treatment dose and regimen.

Damaging effects of methotrexate chemotherapy on osteoblasts, osteoprogenitors and stem cells

High-dose MTX (100-1000 mg/m²) acts by reversibly inhibiting the enzyme dihydrofolate-reductase (DHFR). DHFR is essential for the synthesis of purine and thymidylate, thus inhibition of DHFR can ultimately inhibit DNA synthesis and therefore cell proliferation [17]. Osteoblast number and function are important indicators of bone formation and bone mass. Earlier studies which analysed the effects of MTX chemotherapy on bone metabolism revealed that MTX has an inhibitory effect on osteoblast function without altering osteoblast numbers *in vivo* [37]. Another study demonstrated that MTX-induced osteoblastic damage led to a diminished mineralizing surface, mineral apposition rate and bone formation rate [38]. Consistently, an *in vitro* study using mouse osteoblasts in culture showed that MTX can decrease osteoblastic cell function in a dose-dependent manner, as indicated by a reduction of matrix calcification and supernatant osteocalcin levels [39]. Although earlier studies found no

obvious changes in osteoblast density after MTX treatment in rats, more recent studies reported that both acute high-dose and chronic low-dose MTX treatment in rats can reduce osteoblast density on trabecular bone surface [34, 36].

Osteoblasts are derived from stromal progenitor cells or mesenchymal stem cells (MSCs), which can differentiate into osteogenic, chondrogenic or adipogenic cells [40] (Fig-2). The osteogenic ability of these cells makes it possible to study the toxicity of chemotherapy alone on bone marrow osteoprogenitor cell population and their osteogenic potential. Previously, high-dose chemotherapy (in absence of irradiation) was found to have a direct effect on the stromal population in patients undergoing chemotherapy and cause depletion of bone marrow progenitor cells [41], and resulted in decreased osteoblast differentiation and bone formation [41, 42]. Recently, rat models of MTX chemotherapy also demonstrated a reduction in bone marrow osteoprogenitor cells and suppressed stromal progenitor cell proliferation [34, 36]. These *in vivo* studies suggest that chemotherapy with several drugs in combination or with MTX alone impairs the osteogenic commitment of the bone marrow progenitor cell population, the severity and recovery of which are major determinants of the extent of bone loss and recovery potential following chemotherapy.

In vitro studies found that human bone marrow MSCs appear more resistant to MTX, cyclophosphamide and busulphan than peripheral blood mononuclear cells [43], and that isolated MSCs from normal or chemotherapy-exposed patients remain unaffected by the presence of cytotoxic agents in culture when assessed for differentiation or proliferative potential [44]. These studies suggest that while the already committed osteoprogenitor cells can be more easily affected by chemotherapy drugs, the quiescent MSCs appear to have the

capacity to maintain their number and their stem cell character both *in vivo* and *in vitro* in response to some chemotherapeutic agents [44]. This perhaps explains why the bone and bone marrow stroma can regenerate in the MTX acute treatment models in rats [34-36].

Methotrexate chemotherapy-induced marrow adiposity

In recent rat studies of MTX 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 [35, 36] (Fig-2). Consistently, one early *in vitro* study demonstrated that the presence of MTX can significantly increase the number of fat-containing cells in bone marrow culture [45]. These studies suggest that MTX chemotherapy can cause a reciprocal switch in bone vs. fat volume in the bone marrow microenvironment. Since adipocytes and osteoblasts share a common precursor (bone marrow MSC), it has been proposed that bone loss may result from a switch in favour of adipocyte differentiation over osteoblast commitment. Since the Wnt signalling pathway stimulates osteoblast lineage commitment and inhibits adipocyte formation [46, 47], it is of interest to examine whether deregulation of Wnt signaling may be involved in this bone/fat reciprocal relationship following MTX chemotherapy.

Effects of Methotrexate on haematopoietic cells and osteoclast formation

In addition to the damage to osteoblasts, bone marrow stromal cells (discussed above), haematopoietic stem cells (HSCs) and haematopoiesis [48], another possible mechanism for chemotherapy-induced decrease in bone mass in children is the increased formation of bone resorptive cells (osteoclasts) and the alteration to the bone remodeling balance in favour of

bone resorption. Clinically, children undertaking high-dose MTX treatment have lower bone mass, with increased urinary and faecal calcium excretion, suggesting increased bone resorption [24, 49]. Results from both short- and long-term rat studies revealed that MTX can cause an increase in osteoclast density on trabecular bone surface [34, 38, 50] (Fig-3). Similarly, an increase in the number of empty Howship lacunae on the trabecular surface [51] and excretion of hydroxyproline [50] following MTX administration are evident in animal studies which further support the argument of increased bone resorption. A recent *ex vivo* study using bone marrow cells obtained from rats treated with MTX showed an increase in the osteoclast precursor cell pool which express surface marker CD11b⁺ and an increase in *ex vivo* osteoclast formation [34] (Fig-3). Mac-1 (CD11b/CD18) has been shown to play a role in facilitating the differentiation of osteoclast precursors into mature osteoclasts when stimulated by the key osteoclastogenic cytokine RANKL [52]. Collectively, this indicates that MTX chemotherapy affects osteoclastogenesis at the precursor level.

Some clinical data revealed an increased serum level of pro-inflammatory cytokine TNF- α in patients undergoing chemotherapy [53], suggests a potential role for pro-inflammatory cytokines in chemotherapy-induced osteoclastogenesis. It is known that, apart from RANKL, osteoclast differentiation and activity can be enhanced by pro-inflammatory mediators such as IL-1, IL-6 and TNF- α [54]. Whilst increased precursor and mature osteoclast presence within the bone strongly suggests increased resorptive activity, no animal studies have directly investigated this link in chemotherapy model. Future studies are required to investigate the potential role of pro-inflammatory cytokines in osteoclastogenesis, as well as the mechanisms by which MTX chemotherapy may induce an inflammatory response within the bone marrow microenvironment.

Conclusions and future perspectives

As longitudinal bone growth occurs during childhood and adolescence, altered bone metabolism during this period may interfere with bone growth and bone mass accrual, which may result in lower peak bone mass, potentially leading to premature onset of osteopenia and increased fracture risk [55]. The advancement and success of chemotherapy in treating childhood cancers (particularly ALL) and thus its increasing use in paediatric oncology have resulted in a growing population of young cancer survivors with increased bone health risks (reduced bone growth and lower peak bone mass). Although the mechanism for chemotherapy-induced bone damage is multifactorial, recent research has revealed that chemotherapeutic agents can directly impair bone growth. In particular, rat studies have confirmed that MTX can directly disrupt the growth plate structure and function by inducing chondrocyte apoptosis, reducing chondrocyte proliferation and cartilage protein synthesis. Dysfunction of the growth plate therefore reduces formation of primary woven bone. Direct damage to osteoblasts by decreasing osteoblast activity/formation (possibly through inducing the switch in the bone marrow stromal cells towards adipogenic differentiation at the expense of osteogenesis) and bone marrow osteoprogenitor cells also contributes to reduced bone formation. In addition, MTX chemotherapy has also been shown to increase osteoclast formation and cause aggravated bone resorption, contributing to the associated bone loss.

Given the increased rates of fractures and early onset of osteopenia in childhood survivors of ALL, future studies should investigate strategies to reduce skeletal toxicities and improve quality of life of chemotherapy patients. Currently, recommendations and therapeutic strategies for reducing childhood bone loss during chemotherapy are limited, and there have

been few studies investigating potential adjuvant treatments to reduce chemotherapy-induced skeletal toxicities. In this context, the rat models of MTX chemotherapy have also been shown to be useful in demonstrating that folinic acid, an antidote used clinically to reduce toxicity to soft tissues such as gut and bone marrow haematopoietic cells, is also efficacious to reduce or prevent MTX chemotherapy-induced bone growth defects [34].

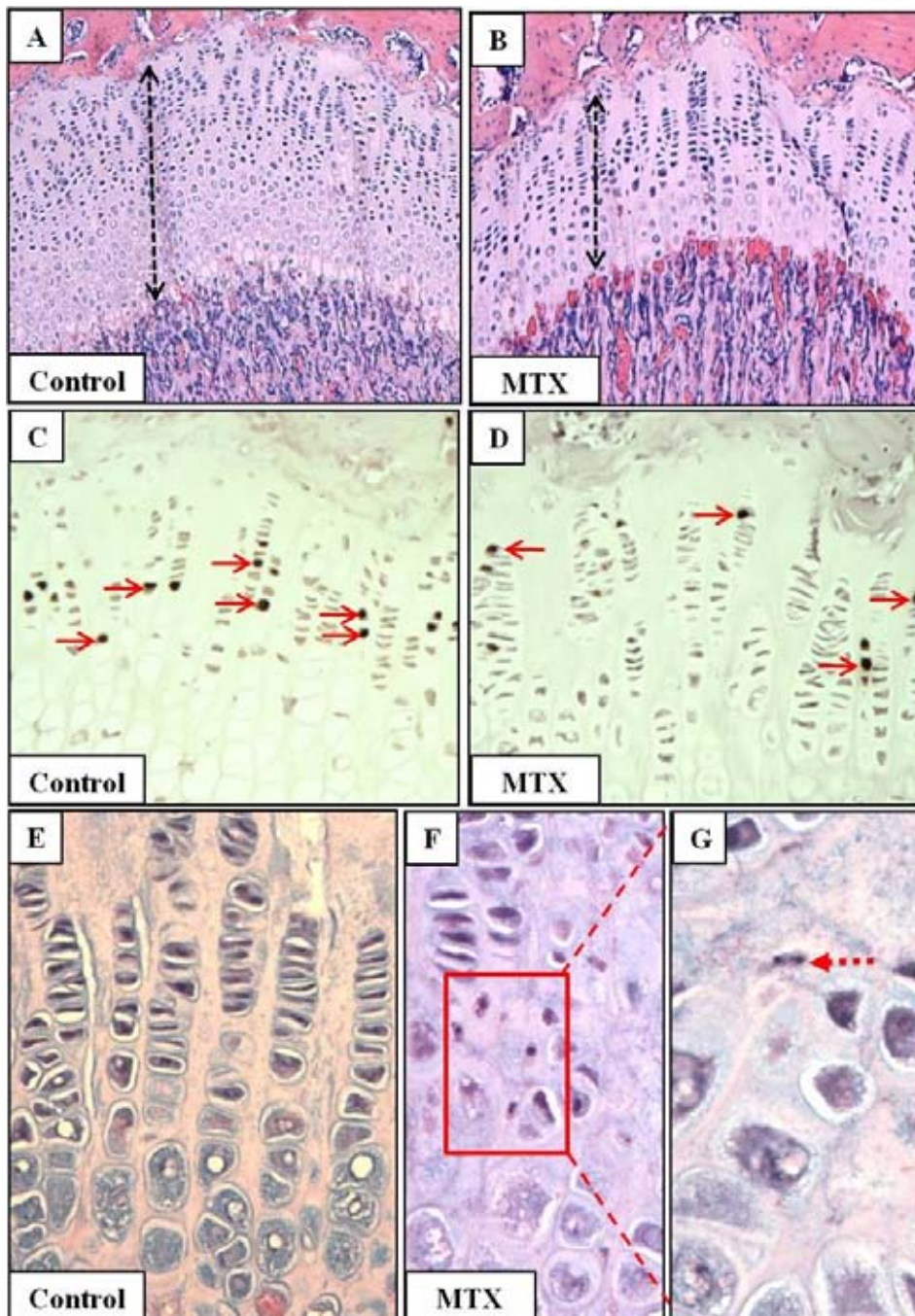
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Figure 1. Effect of acute high-dose MTX chemotherapy on growth plate structure and cellular changes in young rats. H&E stained section of a normal rat tibial growth plate (A) and a MTX-treated rat growth plate (B). Dashed line represents total heights of growth plates. BrdU labeling showing proliferative chondrocytes in a normal rat (C) and a MTX-treated rat (D), with arrows pointing to proliferating chondrocytes. Normal proliferative/hypertrophic

chondrocytes of a normal rat (E) showing no apoptosis; MTX-treated rats with apoptotic chondrocytes in lower proliferative/upper hypertrophic zone (F), and a magnified view of apoptotic chondrocyte (G). (Images are from the authors' own lab and have not been published previously.)

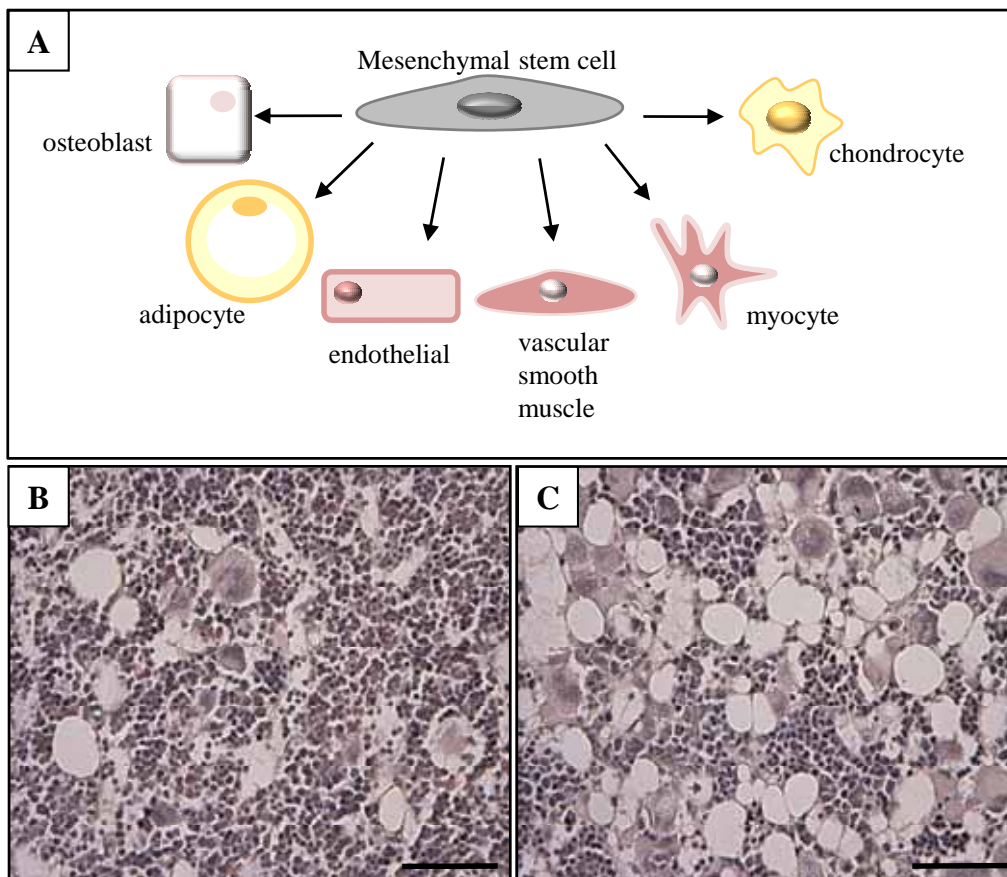


Figure 2: Mesenchymal stem cell commitment and effects of MTX chemotherapy in bone marrow adiposity. Multipotency of the mesenchymal stem cell (A), illustrated by the capacity to differentiate down a number of cell lineages. H&E stained bone marrow section taken from a control rat (B) and from an acute high-dose MTX-treated rat (C) showing adipocyte-rich bone marrow. (Images are from the authors' own lab and have not been published previously.)

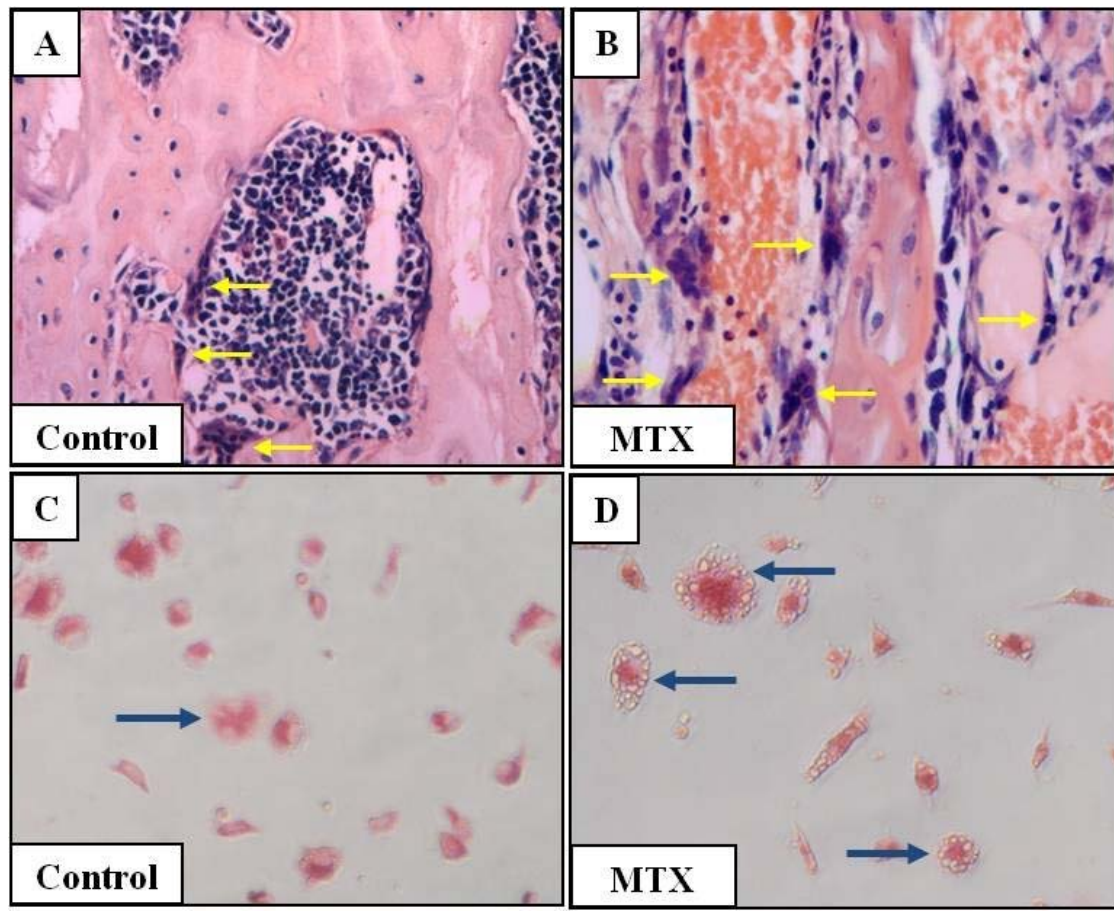


Figure 3. Effect of MTX chemotherapy on osteoclast density in young rats. H&E stained sections showing osteoclasts along trabecular bone surface in a control rat (A) and a MTX-treated rat (B), with arrows pointing to multinucleated osteoclasts. TRAP-stained osteoclasts formed *ex vivo* from bone marrow cells of a control rat (C) and a MTX-treated rat (D), with arrows pointing to multinucleated TRAP⁺ osteoclasts. (Images are from the authors' own lab and have not been published previously.)

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1.7 Project rationale, aims and hypothesis

Chemotherapy is an important part of cancer treatment. Methotrexate, an inhibitor of dihydrofolate reductase and DNA synthesis, is a commonly used chemotherapeutic agent in childhood oncology when used in combination with other chemotherapy agents, and has shown to induce bone growth defects in pediatric cancer patients and in experimental young rats. Using animal models, some previous studies have shown the short-term damaging effects of skeleton by methotrexate, including both growth plate structural damages and metaphyseal bone loss (Xian, Cool et al. 2007; Xian, Cool et al. 2008). Several *in vitro* studies have also shown the damaging effects of methotrexate to bone cells (Davies, Evans et al. 2002; Minaur, Kounali et al. 2002). However, limited studies are found on the effects of chronic methotrexate treatment on skeletal damages. Despite many clinical and some previous animal studies reporting changes in bone density, structure and bone cells, mechanistic studies that have investigated the underlying mechanisms for these histological and cellular damages are limited. Furthermore, methotrexate-induced bone growth defects may pre-dispose to osteopenia and causes bone fragility and an increased susceptibility to fractures; therefore, development of strategies for early prevention and treatment of chemotherapy-induced bone damages is essential to decrease the risk of bone fracture. Folinic acid (or Leucovorin) supplementation has been clinically used to lower the toxicity level of folate antagonist in body soft tissues (Ortiz, Shea et al. 1998; Harten 2005), such as methotrexate, as the adverse effects of high-dose methotrexate are thought to be mediated via folate antagonism (Whittle and Hughes 2004). Previous preliminary experiments from our lab has shown promising results that folinic acid treatment during short-term methotrexate chemotherapy can significantly preserve the growth plate structure (by preserving the proliferating cells and reducing methotrexate-induced

apoptosis) and trabecular bone volume from methotrexate-induced damages (Xian, Cool et al. 2008). However, the long-term benefits of folinic acid in reducing toxicity of long bones remain to be investigated. Therefore, using chronic methotrexate chemotherapy trial in young rats, the aims of this project were:

- (1) To examine the damaging effects of chronic low-dose methotrexate treatment on the skeleton of young rats and bone cell precursors, as well as the potential protective effects of folinic acid against chronic methotrexate-induced bone damages.
- (2) To examine the structural damages of chronic high-dose methotrexate treatment in the growth plate of young rats, underlying mechanisms for the damages, as well as the prevention of growth plate damages with folinic acid supplementation in rats receiving chronic high-dose methotrexate.
- (3) To examine the structural damages in the metaphysis of young rats receiving chronic high-dose methotrexate treatment, the underlying mechanisms for the damages, as well as the prevention of metaphyseal damages with folinic acid supplementation.

It is hypothesized that chronic methotrexate treatment in young rats may cause structural damages of both growth plate and metaphysis, resulting in severe bone growth defects through cellular and molecular damages. The structural, cellular and molecular damages of methotrexate may be dose-dependent, with possible full, partial or no recovery following methotrexate treatment, depending on the dose and length of treatment. However, supplementary treatments with folinic acid may reduce the toxicity of methotrexate and preserves the growth plate and metaphysis structures by reducing

and/or preventing the cellular and molecular damages during chronic methotrexate chemotherapy.

CHAPTER 2

**DAMAGING EFFECTS OF CHRONIC LOW-DOSE
METHOTREXATE USAGE ON PRIMARY BONE
FORMATION IN YOUNG RATS AND POTENTIAL
PROTECTIVE EFFECTS OF FOLINIC ACID
SUPPLEMENTARY TREATMENT**

**DAMAGING EFFECTS OF CHRONIC LOW-DOSE METHOTREXATE
USAGE ON PRIMARY BONE FORMATION IN YOUNG RATS AND
POTENTIAL PROTECTIVE EFFECTS OF FOLINIC ACID
SUPPLEMENTARY TREATMENT**

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CHAPTER THREE

PREVENTION OF GROWTH PLATE DAMAGE WITH FOLINIC ACID SUPPLEMENTATION IN YOUNG RATS RECEIVING LONG-TERM METHOTREXATE

Abstract:

Methotrexate (MTX) is a commonly used anti-metabolite for paediatric cancers and MTX chemotherapy is known to cause bone growth arrest in paediatric patients and in rodents. As growth plate functions to produce a template for longitudinal bone lengthening, this study investigated the pathophysiological mechanisms behind long-term MTX chemotherapy-induced growth plate damage in young rats, and the potential protective effects of supplementary antidote folinic acid (FA). The current study demonstrated that long-term high-dose MTX treatment induced chondrocyte apoptosis, reduced total numbers of chondrocytes, disrupted their columnar arrangement, suppressed major matrix protein collagen-II expression, but did not affect overall growth plate thickness. Long-term MTX treatment also induced chondroclast recruitment at the site of vascular invasion at the cartilage/bone transitional zone. Long-term FA supplementation prevented MTX-induced growth plate damage not only morphologically, but also by preventing MTX-induced chondrocyte apoptosis, preserving chondrocyte numbers, and suppressing MTX-induced over-recruitment of chondroclasts. FA supplementation may potentially be useful for protecting bone growth in paediatric patients receiving long-term high dose MTX chemotherapy.

Introduction:

During childhood, bone lengthens through the process of endochondral ossification which takes place at the epiphyseal growth plate at the ends of long bones. Endochondral ossification is a finely balanced process of cartilage growth, matrix formation and cartilage calcification that acts as a scaffold for bone formation (Hochberg 2002). The sequence of cellular events that constitutes endochondral ossification involves chondrocyte proliferation, maturation, hypertrophy and apoptosis (Hochberg 2002), resulting in cartilage calcification and the recruitment of different bone cells (Iannotti 1990). The replacement of calcified cartilage by bone involves mineralization of the hypertrophic cartilage, apoptosis of hypertrophic chondrocytes, blood vessel invasion, the recruitment of cartilage-resorbing cells chondroclasts and bone-forming cells osteoblasts (Gerber, Vu et al. 1999; Farquharson and Jefferies 2000; van der Eerden, Karperien et al. 2003).

The production of calcified cartilage scaffold for bone deposition relies on the regulation of growth plate chondrocyte proliferation, differentiation, apoptosis and cartilage resorption. Any disruption of this carefully controlled process will result in bone growth defects. It is now clear that apart from genetic factors, environmental and life style factors such as medical treatments can significantly affect endochondral ossification and bone lengthening. With the development of more successful chemotherapy and increasing childhood cancer survivor rates, particularly for leukemias that account for almost one third of childhood cancers (Marinovic, Dorgeret et al. 2005), a variety of health problems have also arisen from cancer itself or its treatment, and have become clinically significant with ageing (Haddy, Mosher et al. 2001). One such effect is the chemotherapy-associated bone growth abnormalities,

including bone growth arrest, fractures, osteopenia and osteoporosis in survivors of paediatric cancers (Crofton, Ahmed et al. 2000). Bone metabolism in children with acute lymphoblastic leukemia ALL is known to be disturbed after chemotherapy, resulting in reduced bone lengthening and bone loss compared to healthy age-matched controls. Since bone defects during childhood may predispose to osteopenia and osteoporosis, it is important to understand the mechanisms of chemotherapy-induced bone damage and develop strategies for prevention of such damages.

Methotrexate (MTX) is prescribed widely for the treatment of both cancers (particularly for acute lymphoblastic leukemia (ALL) being the most common childhood cancer) and rheumatoid arthritis (RA). MTX at low-dose of 5-25mg/week is frequently used to treat RA, while MTX at high-dose of 100-12000 mg/m² is commonly used for the treatment of paediatric malignancies. High-dose MTX acts by inhibiting the enzyme dihydrofolate-reductase, which blocks thymidylate and purine synthesis that are necessary for DNA replication of cancer cells, and ultimately leads to cell death (Minaur, Kounali et al. 2002). Many studies have reported the effects of long-term low-dose MTX on bone metabolism, with majority of studies showing no significant adverse effects on bone mineral density (BMD) (Kita and Sierakowski 2002; Borchers, Keen et al. 2004). However, long-term intensive neoadjuvant chemotherapy with MTX has been shown to cause serious damage to bone development, especially during the period of bone accumulation in paediatric patients (Mandel, Atkinson et al. 2004; Jarfelt, Fors et al. 2006).

Due to high success rates and the intensified use of chemotherapy in children, it is important to develop potential strategies for protecting bone growth during MTX

chemotherapy. Folinic acid (FA), an antidote that has been clinically used to reduce toxicity of high dose MTX in soft tissues, was recently shown to have protective properties against MTX chemotherapy in the skeleton of young rats in an acute study (Xian, Cool et al. 2008). Despite some previous studies showing that chemotherapy can suppress skeletal growth by disrupting growth plate structure and functioning of bone cells (van Leeuwen, Hartel et al. 2003; Xian, Cool et al. 2007; Xian, Cool et al. 2008), no long-term studies with high-dose MTX chemotherapy have examined chemotherapy-induced growth plate cellular damage, its contribution to the failure of bone deposition, and potential protective effects of FA. The current study examined the effect of long-term high-dose MTX treatment in the growth plate of young rats, the cellular and molecular mechanisms for MTX-induced growth plate damage, as well as the potential protective effects of FA supplementary treatment.

Materials and Methods:

Animal Trials and specimen collection

Groups of young male Sprague-Dawley rats weighing between 130-150g (approximately 5 weeks old, in their rapid growing phase) were randomly allocated into three treatment groups receiving saline, MTX, or MTX plus FA (MTX+FA) (n=10 per treatment group). During the induction phase, rats were subcutaneously injected with MTX daily at 0.65mg/kg for 5 consecutive days, followed by 9 days of rest (5 days on/9 days off) (**Figure 3.1**). FA was injected intraperitoneally 6 hours after MTX at 0.87mg/kg. During the maintenance phase (day 15 to week 6), rats received MTX at 1.3mg/kg with or without FA at 1.3 mg/kg twice weekly (**Figure 3.1**). By the end of week 6, rats were sacrificed using carbon dioxide overdose. Ninety minutes prior to sacrifice, rats were injected with 5'-bromo-2'-deoxyuridine (BrdU) (Sigma, NSW,

Australia) at 50mg/kg to label S-phase nuclei for cell proliferation study. The above protocols followed the Australian NHMRC Code of Practice for the Care and Use of Animals and were approved by Women's and Children's Hospital Animal Ethics Committee (South Australia). Left tibias were dissected free of soft tissue, fixed in 10% formalin, decalcified then embedded in paraffin. Paraffin sections of 4µm thick were cut and mounted on positively charged SuperFrost PlusTM-coated glass slides for histological and immunohistochemical analysis. From the right tibia, growth plate cartilage were collected and stored at -80°C for RNA extraction.



Figure 3.1:

Dosing schedules for the induction and maintenance phases of long-term methotrexate (MTX) chemotherapy with folinic acid (FA) supplementary treatment. ★ indicates injection of MTX, FA and Saline.

Histological analysis of growth plate thickness and chondrocyte number

Paraffin sections mounted on glass slides were dewaxed and stained with 0.3% Alcian blue in 3% acetic acid followed by haematoxylin and eosin (H&E) staining. Stained sections were used for morphometric measurements of growth plate zonal heights and columnar chondrocyte counts. Briefly, growth plate zonal heights were obtained by measuring the height of each individual growth plate zone: resting, proliferative and hypertrophic zone. The numbers of proliferative and hypertrophic chondrocytes were measured per column (cells/mm length).

BrdU labeling, in situ TUNEL labeling of growth plate chondrocytes

To examine the effects of MTX treatment alone or with FA (MTX+FA) on proliferation of chondrocytes, paraffin sections were used for BrdU labeling as described (Xian, Cool et al. 2006) with BrdU antibody (Dako, Carpinteria, CA, USA). In the growth plate, numbers of BrdU⁺ cells were counted within the proliferative zone and expressed as per unit area of growth plate proliferative zone (cells/mm²). To examine the treatment effects on chondrocyte apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche Applied Science, NSW, Australia) was performed on sections, which fluorescently labels nuclear DNA fragmentation by the enzyme terminal deoxynucleotidyl transferase (TdT) as described (Xian, Cool et al. 2006). Apoptotic cells were detected and quantified by fluorescence microscopy, with characteristic of condensed/fragmented nuclei. Total number of apoptotic cells and total area of growth plate (excluding cartilage/bone transitional zone) were used to calculate the apoptotic cell density (cells/mm²).

TRAP staining and chondroclast measurement

To examine the effects of MTX and MTX+FA treatments on recruitment of cartilage resorbing cells chondroclasts, paraffin sections were stained with tartrate-resistant acid phosphatase (TRAP), and then counterstained with haematoxylin. Sections were photographed and TRAP⁺ cells (stained ruby red in colour and containing multiple nuclei) along the growth plate hypertrophic cartilage – metaphysis bone transitional zone were identified as chondroclasts, and counted across the entire growth plate width (cells/mm).

Quantitative RT-PCR analysis of gene expression

To examine treatment effects on expression of growth plate regulatory genes, relative mRNA expression levels were measured by real time RT-PCR for cartilage protein (collagen-IIa), molecules involved in controlling apoptosis (Bcl-2, Bax, Fas, and Fas-L), and angiogenic protein vascular endothelial growth factor (VEGF). Total RNA from the growth plate was extracted with TRI reagent (Sigma, St. Louis, U.S.) then DNase treated with TURBO DNA-freeTM Kit (Ambion, Austin, U.S.) for the removal of contaminating DNA from RNA samples. Samples with an A260/A280 ratio of 1.8 or above were used to synthesize single stranded cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to manufacturer's instructions. SYBR Green PCR assays (Applied Biosystems) for each target molecule and reference gene Cyclophilin A (Cyc-A) were performed using primers (**Table 3.1**) (Xian, Howarth et al. 2004; Zhou, Foster et al. 2004; Xian, Cool et al. 2006) in triplicate on cDNA samples. PCR assays were run on a 7500 Fast Real-Time PCR System (Applied Biosystems). Analysis of gene expression was done using the comparative Ct ($2^{-\Delta\text{CT}}$) method (Zhou, Foster et al. 2004).

Gene	Forward Primer	Reverse Primer
Cyclophilin A	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG
Collagen IIa	GGGCTCCCAGAACATCAGCTACCA	TCGGCCCTCATCTCCACATGATTG
Bcl-2	CAGCATGCGACCTCTGTTTG	TCTGCTGACCTCACTTGTGG
Bax	CGAGAGGTCTTCTTCCGTGT	GAGCACCAGTTTGCTAGCAA
Fas	AAGATCGATGAGATCGAGCACA	AAGCTTGACACGCACCAGTCT
Fas-L	GAGCTGTGGCTACCGGTGATAT	ACTCACGGAGTTCTGCCAGTTC
VEGF	ATCTTCAAGCCGTCCTGTGTG	TGAGGTTTGATCCGCATGATC

Table 3. 1: Primer sequences used in this study

Statistics

Data are presented as means \pm SEM and analysed by one-way analysis of variance (ANOVA) with GraphPad Prism 6 Software. When the significance levels ($P < 0.05$) were achieved, a post hoc analysis of groups were performed using a Tukey's test.

Results:

Throughout this long-term trial, weights of animals were monitored daily during the induction phase, and twice weekly during the maintenance phase. Rats received high-dose MTX showed approximately 10%-15% of body weight reduction after the first cycle of 5 once-daily injections (body weight drop during day 5 to day 9) (data not shown). MTX-treated rats also showed signs of diarrhea and appeared less active. During the induction phase, one rat died due to MTX toxicity, and three more rats were culled during the maintenance phase due to the severe MTX toxicity. By the end of week 6, rats received MTX alone showed approximately 10% body weight reduction and 5% tibial length reduction when compared to control rats ($P>0.05$). Rats treated with FA following MTX administration showed no signs of MTX toxicity, with body weights and bone lengths similar to control rats throughout the trial. These results indicate FA supplementation can preserve the body weight gains and bone lengthening during long-term MTX treatment and FA supplementation appears to protect the animals from growth retardation, with no signs of toxicity observed throughout both induction and maintenance phase.

Treatment effects on growth plate thickness and structural changes

By histomorphometrical measurements, the proximal tibial growth plate was approximately 350 μ m thick in normal rats, comprising of approximately 40 μ m of resting zone, 80 μ m of proliferative zone and 230 μ m of hypertrophic zone (**Figure 3.2A**). Although there were no significant changes in total growth plate thickness (**Figure 3.2A-C**), counting of chondrocytes per column at the growth plate proliferative and hypertrophic zones revealed significantly reduced chondrocyte number per column after the long-term MTX treatment when compared to saline-treated control rats

($P < 0.01$ vs. control) (**Figure 3.2D-F**). However, supplementary treatment with FA partially preserved the chondrocyte numbers ($P < 0.05$ vs. control) (**Figure 3.2F**). Apart from the significant changes in chondrocyte numbers observed, chondrocytes of MTX-treated rats appeared to be more chaotic with disrupted columnar structure when compared to control or FA-supplemented rats.

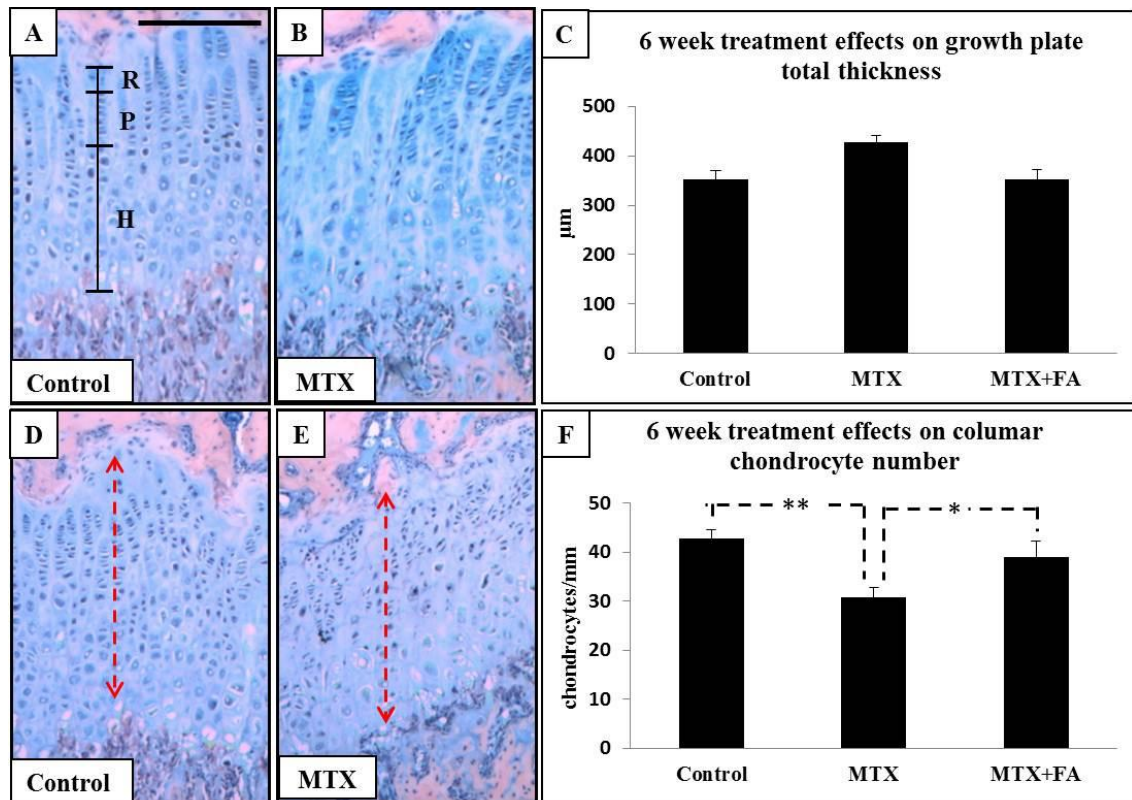


Figure 3.2:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folinic acid (FA) on total growth plate thickness and columnar chondrocyte number. H&E staining of a control rat growth plate (A): R= resting zone, P= proliferative zone and H= hypertrophic zone. H&E staining of a MTX-treated rat growth plate (B). H&E staining of a control rat growth plate with chondrocytes in columnar arrangement (D), and a MTX-treated rat growth plate with chondrocytes in disrupted columnar arrangement (E). Chondrocytes were counted along the red dotted line. Treatment effects were shown for growth plate thickness (C) and columnar chondrocyte number (F). n=6 per group, scale bar on panel A=250μl which also applies to others.

Treatment effects on chondrocyte proliferation and collagen-II gene expression

Chondrocyte proliferation in the proliferative zone, as revealed by BrdU labeling and expressed as BrdU⁺ cells/mm² (**Figure 3.3A** showing normal growth plate), was not significantly affected by 6 weeks' high-dose MTX treatment or with supplementary FA treatment (**Figure 3.3B**). Similarly, mRNA expression of the major cartilage protein collagen-II was also not affected by MTX treatment alone or with FA supplementation (**Figure 3.3C**).

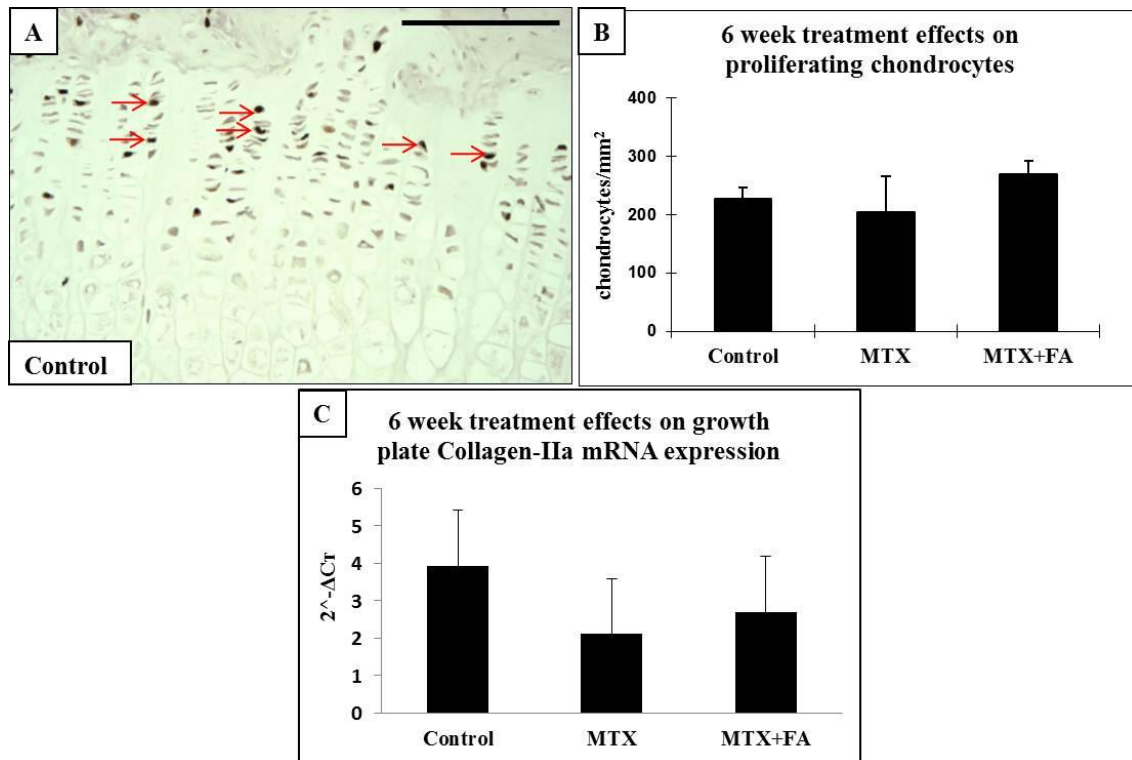


Figure 3.3:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folic acid (FA) on proliferation of chondrocytes and collagen-II mRNA expression in growth plate. BrdU labeling (arrows) of chondrocytes at proliferative zone of growth plate (A). Treatment effects on growth plate chondrocyte proliferation (B). Treatment effects on mRNA expression of collagen-IIa (C). n=6 per group, scale bar on panel A=200 μ l.

Treatment effects on chondrocyte apoptosis and expression of apoptosis-regulatory genes

The above analyses have shown that long-term high-dose MTX treatment causes reduction in chondrocyte numbers without affecting growth plate heights and has minimal effects on growth plate proliferation. To further examine treatment effects on growth plate chondrocytes, their apoptosis was analysed by in-situ TUNEL labeling. Apoptotic cells are normally rarely identified within the growth plate proliferative and hypertrophic zones (**Figure 3.4A**), except at the cartilage-bone transitional region. After 6 weeks of high-dose MTX treatment, significant induction of chondrocyte apoptosis was observed within the growth plate lower proliferative and upper hypertrophic zones as shown by green fluorescent TUNEL positive staining with nuclei fragmentation) and density quantification (cells/mm²) (P<0.001) (**Figure 3. 4B & 3.4C**). On the other hand, FA supplementary treatment significantly suppressed the induction of chondrocyte apoptosis when compared to MTX-treated rats (P<0.001) (**Figure 3.4D**).

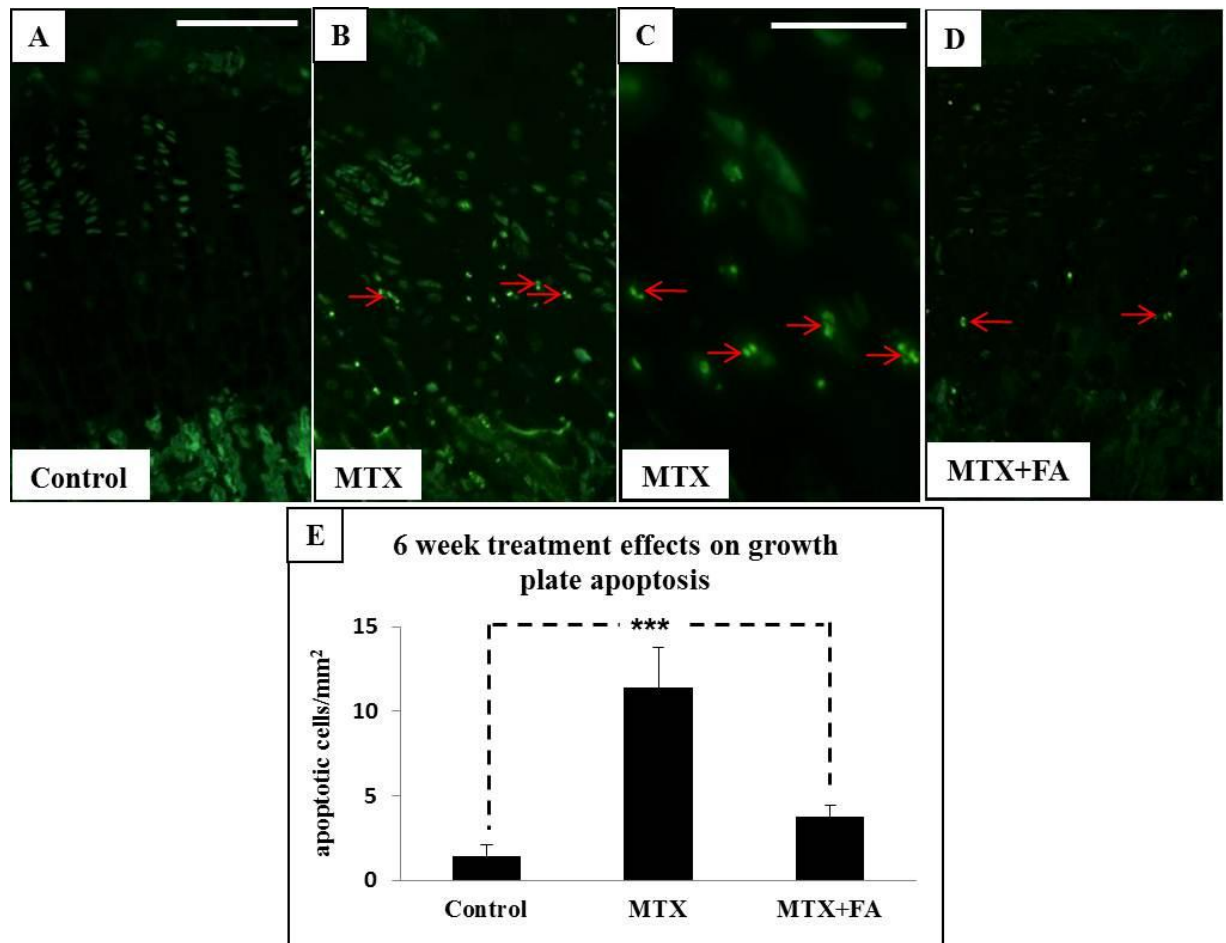


Figure 3.4:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folinic acid (FA) on growth plate chondrocyte apoptosis.

TUNEL staining of a control rat growth plate (A), MTX-treated rat growth plate (B, C), and MTX+FA treated rat growth plate (D), with arrows pointing to positive TUNEL labeling for apoptotic chondrocytes. Treatment effects on growth plate chondrocyte apoptosis (E). n=6 per group, scale bars on panel A, B & D= 200 μ l, C= 100 μ l.

Since treatment effects on chondrocyte apoptosis was significantly affected from histological observation, quantitative analysis by real time RT-PCR was carried out to further examine the expression of apoptosis regulatory genes involved in both mitochondrial pathway (Bcl-2 and Bax) and death receptor pathway (Fas and Fas-L) (Green and Kroemer 2004; Shankar and Srivastava 2004). Interestingly, mRNA expression of both anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax were not affected by treatments (**Figure 3.5A & 3.5B**). Expression of both death receptor Fas and its ligand (Fas-L) also showed no significant differences between all treatment groups, but a trend of up-regulation in MTX-treated rats (**Figure 3.5C & 3.5D**), while this trend is not seen in rats receiving supplementary FA treatment (**Figure 3.5C & 3.5D**). These results suggest that the death receptor pathway may possibly contribute more towards MTX-induced chondrocyte apoptosis, which may be prevented by long-term FA supplementation.

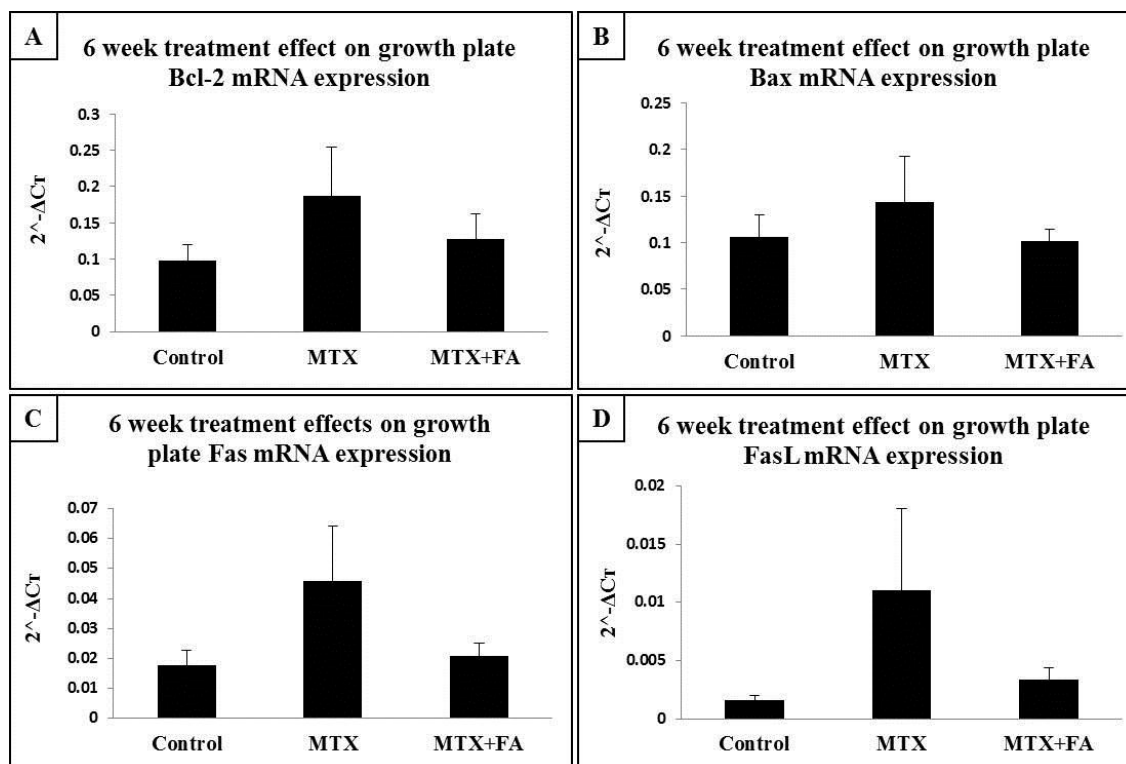


Figure 3.5:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folic acid (FA) on mRNA expression of apoptotic genes in growth plate. Treatment effects on mRNA expression of apoptotic genes for mitochondrial pathway Bcl-2 (A) and Bax (B), and apoptotic genes for death receptor pathway Fas (C) and Fas-L (D).

Treatment effects on chondroclast recruitment and VEGF gene expression

Chondroclasts are large multinucleated cells that resorb growth plate mineralized hypertrophic cartilage during endochondral ossification and are present at the cartilage-metaphysis bone border. In this study, density measurements show that long-term high-dose MTX treatment non-significantly induced chondroclast recruitment (shown by multinucleated TRAP⁺ cells adhering to the transitional zone) **(Figure 3.6A & 3. 6C)**, and that long-term FA supplementation was able to significantly suppress the MTX-induced chondroclast recruitment ($P<0.05$) **(Figure 3.6B & 3.6C)**.

Metaphyseal vascular invasion is known to regulate by several factors, including VEGF produced by hypertrophic chondrocytes (Karsenty and Wagner 2002). VEGF is known as the key stimulator for vascular invasion, which brings in chondroclasts for the removal of cartilage matrix, and osteoblasts for osteogenesis (Karsenty and Wagner 2002), and VEGF is known to be important for the recruitment and/or differentiation of chondroclasts (Gerber, Vu et al. 1999). However, RT-PCR analysis revealed no changes of VEGF mRNA expression in all treatment groups **(Figure 3.6D)**.

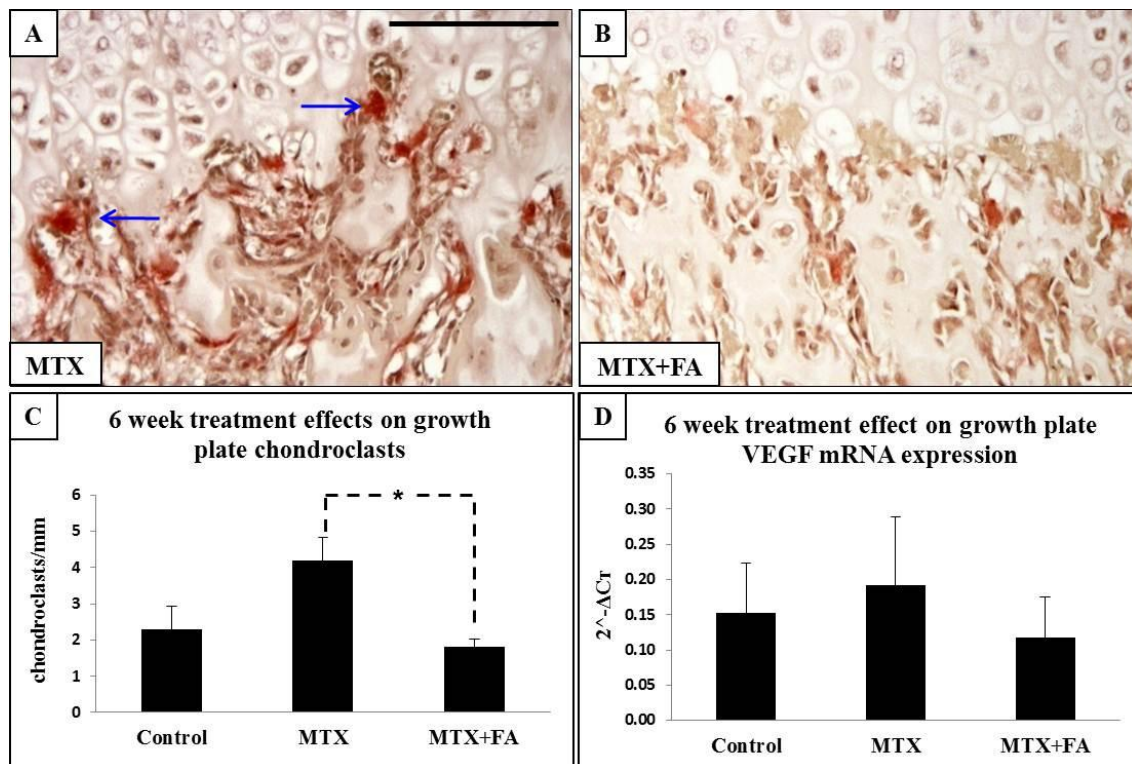


Figure 3.6:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folinic acid (FA) on chondroclast numbers and VEGF expression.

TRAP staining of the growth plate/metaphysis transitional zone of a MTX-treated rat (A) and a MTX+FA treated rat (B), with arrows pointing to TRAP⁺ multinucleated chondroclasts. Treatment effects on growth plate chondroclast count (C) and mRNA expression of angiogenic factor VEGF in the growth plate (D). n=6 per group, scale bars on panel A & B= 100μl.

Discussion:

MTX, a commonly used anti-metabolite for paediatric oncology, has been found in association with serious bone damage in paediatric patients both during and after chemotherapy, especially during the period of bone accumulation in paediatric patients (Armstrong, Chow et al. 2009; Haddy, Mosher et al. 2009). Although several animal studies have reported that high-dose MTX can suppress bone growth, affecting chondrocyte proliferation and apoptosis (van Leeuwen, Kamps et al. 2000; Xian, Cool et al. 2007), no detailed molecular studies have been carried out to investigate mechanisms causing these cellular changes. In the current *in vivo* study, we have investigated potential underlying mechanisms of MTX-induced damages at the growth plate- the main region responsible for longitudinal bone growth, as well as the potential protective effects and action mechanisms of supplementary FA in young rats receiving long-term MTX chemotherapy.

Growth plate chondrocytes and their division rate are the principal determinants of longitudinal bone growth, thus measurement of chondrocyte number, chondrocyte proliferation and apoptosis directly correlate to the longitudinal growth rate (Farquharson and Jefferies 2000). The current study showed long-term high-dose MTX chemotherapy caused slight but non-significant increase of growth plate thickness, which is consistent with the finding of one previous study in rats receiving high-dose MTX (van Leeuwen, Hartel et al. 2003). Previous short-term study also reported minimal distortion of chondrocyte columnar arrangement despite disappearance of some cell columns after 5 days high-dose MTX treatment in rats (Xian, Cool et al. 2007). In the current study, however, apart from a significant loss of cells, the remaining chondrocytes appeared more chaotic in arrangement. Thus, distortion in chondrocyte

columniation was much more severely affected in the current chronic model in comparison to previous short-term treatment, suggesting disruption of chondrocyte organization by MTX is dependent on the duration of treatment. As chondrocytes are normally highly organized into columns, which represent the functional units for longitudinal bone growth (Farquharson and Jefferies 2000), disturbance of chondrocyte structure and number may affect the rate of longitudinal bone growth. However, FA supplementary treatment in this study significantly preserved chondrocyte numbers and columnar structure during the long-term MTX chemotherapy, suggesting FA can diminish the damaging effects of MTX on growth plate.

The number of proliferating chondrocytes and their division rates are the key factors in controlling longitudinal bone growth. This current study revealed that long-term high-dose MTX treatment in rats caused no significant changes in chondrocyte proliferation, a finding which is in contrast to our previous studies, which reported that short-term high-dose MTX treatment in rats can suppress chondrocyte proliferation (Xian, Cool et al. 2007; Xian, Cool et al. 2008), with damage being able to be reversed by FA supplementary treatment (Xian, Cool et al. 2008). However, a few earlier studies revealed similar results to the current study, that MTX alone had no effects on proliferative response of chondrocyte both *in vivo* and *in vitro* (Robson, Anderson et al. 1998; van Leeuwen, Hartel et al. 2003). These results suggest that perhaps chondrocyte proliferation is more severely affected after the acute 5 consecutive once daily MTX treatment in comparison to chronic twice weekly treatment, which could be related to differences in the cumulative drug effects with time.

Collagen-II accounts for 30% to 60% of the extracellular matrix (ECM), which is the most abundant collagen in growth plate cartilage (Yang, Li et al. 1997), serving as a ligand of chondrocyte integrins that mediate cell migration, proliferation and differentiation (Hynes 1992; van der Eerden, Karperien et al. 2003). Mutations in collagen-II have been associated with disturbance of the cartilage matrix resulting in spondyloepiphyseal dysplasia and hypochondriasis, both are associated with short stature (Spranger, Winterpacht et al. 1994; Muragaki, Mariman et al. 1996). Mice with inactivated collagen-II gene have also been shown not able to support the survival of chondrocytes (Yang, Li et al. 1997). In the case of Kniest dysplasia, impaired collagen-II can result in the formation of imperfect collagen fibrils, leading to disruption of chondrocyte organization (Poole, Pidoux et al. 1988). These suggest that the reduced collagen-II synthesis after long-term MTX chemotherapy could play a role in the suppressed bone lengthening, as well as disruption of chondrocyte columnar arrangement. However, the current study revealed that collagen-II expression in the growth plate was not significantly suppressed in rats receiving long-term MTX chemotherapy, hence no rescue was seen from chronic FA supplementary treatment.

Production of calcified cartilage scaffold for trabecular bone deposition relies not only on chondrocyte differentiation, proliferation and survival, but also on the apoptosis of chondrocytes. In a normal rat growth plate, chondrocytes undergo apoptosis only at the growth plate/metaphysis transitional region. In the current study, long-term high-dose MTX treatment has profound effects in inducing chondrocyte apoptosis at lower proliferative and upper hypertrophic zones, where apoptosis is normally not observed. This finding is consistent with previous animal studies, which showed different chemotherapeutic agents are able to induce apoptosis in different cell

populations, and at different regions of the bone (Xian, Cool et al. 2006; Xian, Cool et al. 2007; Xian, Cool et al. 2007). Two major pathways are known to be involved in chemotherapy-induced apoptosis, including the death receptor pathway and mitochondrial dysfunction pathway (Kaufmann and Earnshaw 2000). Death receptor pathway involves the ligation of death receptor by a ligand (e.g. Fas by FasL), which results in binding of the adaptor protein FADD, receptor trimerization, recruitment of pro-caspase-8 and activation of caspase-8 (Kaufmann and Earnshaw 2000), whereas the mitochondrial pathway involves binding of pro-apoptotic Bcl-2 family members Bak and Bax, release of cytochrome c, phosphorylation of pro-caspase-9 and activation of caspase-9 (Kaufmann and Earnshaw 2000). Results from the current study showed that long-term high-dose MTX treatment caused more notable induction of Fas and Fas-L expression in comparison to Bcl-2 and Bax, which is consistent with findings in the previous study involving acute high-dose MTX treatment (Xian, Cool et al. 2007). 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, Silvestrini et al. 2001), suggesting different chemotherapeutic agents may induce apoptosis by different apoptotic pathways. FA supplementary treatment in the current study was found to significantly reduce MTX-induced chondrocyte apoptosis, which is consistent with its ability in suppressing the MTX-induced Fas and Fas-L up-regulated expression. Results from this study suggest long-term FA supplementary treatment can prevent MTX-induced chondrocyte apoptosis via the suppression of pro-apoptotic molecules involved in the death receptor pathway, and preserve chondrocyte numbers.

The conversion of growth plate calcified cartilage template into bone is not simply a switch of cell phenotypes. This switch involves the invasion of blood vessels

into the ECM surrounding hypertrophic chondrocytes, which brings in haematopoietic-derived chondroclasts that progressively erode the cartilaginous matrix (Goldring, Tsuchimochi et al. 2006). Chondroclasts express less TRAP activity and have less developed ruffled-border membrane than osteoclasts *in vivo* (Nordahl, Andersson et al. 1998). Along with vascular invasion, osteoblast progenitors are recruited, with osteoblasts eventually replacing the eroded cartilaginous ECM with trabecular bone (Goldring, Tsuchimochi et al. 2006). Studies which focus on the sequential events from vascular invasion to osteogenesis have highlighted the importance of certain growth factors and proteases, with metalloproteinase 9 (MMP-9) and VEGF being most extensively studied. VEGF-mediated vascular invasion is the essential coordinator of chondrocyte death, chondroclast recruitment/function, angiogenesis and cartilage remodeling at the end of growth plate (Kuznetsova, Kushlinskii et al. 2003; Goldring, Tsuchimochi et al. 2006). Previous studies have reported that blocking of VEGF can result in delaying of chondrocyte death and chondroclast recruitment (Gerber, Vu et al. 1999), suggesting VEGF couples neovascularization with the recruitment of chondroclasts. An earlier study has suggested that impaired chondroclast differentiation may lead to reduced resorption of mineralized cartilage, which, in turn, resulted in an increase of bone volume (Turner, Evans et al. 1994). Results from the current study revealed long-term high-dose MTX treatment in rats did not significantly induce chondroclast recruitment/formation, nor affected VEGF mRNA expression in the growth plate, suggesting that MTX-induced non-significant chondroclast recruitment may not be related to VEGF expression. On the other hand, FA supplementary treatment in this study was shown to suppress MTX-induced chondroclast recruitment. Chondroclast recruitment/formation plays an important role in the resorption of growth plate mineralized cartilage, leading to lower delivery of growth plate cartilage feeding

into the metaphysis for bone conversion, which may potentially affect bone volume (as discussed in chapter 4). Results of chondroclast numbers from this chapter suggest that FA supplementary treatment can prevent MTX-induced over-resorption of mineralized cartilage at the vascular invasion zone, and hence preserve cartilage-bone conversion and bone lengthening. Further studies are required to study the biological implications and mechanisms of MTX-induced chondroclast recruitment and to investigate potential other factors involved in the process of hypertrophic cartilage remodeling and bone deposition.

Summary

Using a rat chemotherapy model, the current study revealed that chronic high-dose MTX administration in young rats can cause growth plate structural damages, including reduction of chondrocyte numbers and disruption of chondrocyte columnar arrangement. Cellular and molecular analysis revealed that the MTX-induced growth plate damages are mainly due to the induction of chondrocyte apoptosis (possibly via death receptor pathway) and enhanced recruitment of chondroclasts at the cartilage/bone transitional zone. Furthermore, the current study demonstrated that long-term FA supplementary treatment along with high-dose MTX was able to preserve the overall growth plate structure and function by preserving chondrocyte number and preventing chondrocytes from undergoing apoptosis and suppressing chondroclast recruitment/formation induced by MTX chemotherapy. Since growth plate serves as a template for endochondral bone formation, there is no doubt that dysfunction of the growth plate can subsequently contribute to the reduction of bone deposition and bone lengthening. The protective properties of FA at the growth plate might potentially be useful in protecting bone growth in childhood oncology involving MTX chemotherapy. Future studies are required to investigate the long-term protective effects of FA against MTX chemotherapy-induced bone damage.

CHAPTER FOUR

PREVENTION OF METAPHYSEAL BONE DAMAGE WITH FOLINIC ACID SUPPLEMENTATION IN YOUNG RATS RECEIVING LONG-TERM METHOTREXATE

Abstract:

Methotrexate (MTX), a potent inhibitor of dihydrofolate reductase frequently used in the treatment of acute lymphoblastic leukemia in paediatric patients and a number of other malignancies, is known to cause significant bone growth defects. The previous chapter has described the structural and functional damages in the growth plate caused by long-term MTX chemotherapy and the protective effects of folinic acid (FA) supplementation. The current chapter will describe the treatment effects on the metaphyseal bone of long-term MTX chemotherapy with or without FA supplementation in young rats. The current study revealed that chronic MTX treatment at a high dose caused a significant reduction in the primary spongiosa height and a reduction in trabecular bone volume, which was probably due to the combination effects of increased bone resorption by osteoclasts and reduced bone formation due to the apoptosis of osteoblasts. While gene expression studies did not reveal significant changes in mRNA levels locally in the bone of key molecules known to regulate osteoclastogenesis, ELISA assays revealed significantly increased protein concentrations of osteoclastogenic cytokine IL-1 β in blood plasma obtained from MTX-treated rats. Consistently, plasma from MTX-treated rats, in comparison with control rats, can induce significantly more osteoclasts formed from bone marrow cells isolated from normal rats, suggesting increased bone resorption observed after long-term MTX chemotherapy was induced probably more by systemic factors rather than factors locally produced at the metaphysis. Moreover, this study revealed an increase in marrow adiposity after chronic MTX treatment, suggesting a greater adipocyte differentiation potential over osteoblast differentiation of bone marrow stromal cells in young rats following 6 weeks of MTX treatment. On the other hand, FA supplementation following MTX administration was shown to protect against MTX-

induced metaphyseal damages by suppressing IL-1 β protein level within the blood plasma, preventing induction of osteoblast apoptosis and reducing the degree of marrow adiposity. These findings indicate that FA supplementation can prevent metaphyseal damages from chronic MTX administration, and may potentially be useful in paediatric patients who are at risk of skeletal growth suppression as a result of chronic MTX chemotherapy.

Introduction

Bone is a dynamic tissue with its structure and shape changing continuously to provide a structural framework. During childhood and adolescence, bones are sculpted by modeling, which permits bone formation at one site and removal of old bones at another site within the same bone (Seeman 2008). The remodeling process becomes dominant after peak bone mass is reached. This process involves the replacement of aged bone tissue by new bone tissue in order to maintain bone mass. Bone remodeling begins at a quiescent bone surface with the recruitment of osteoclasts which activates bone resorption. After the resorption stops, osteoblasts fill the resorptive site for bone deposition and mineralization (Seeman 2008). The interactions between osteoblast and osteoclasts must be carefully balanced; any disruption to this organized network may lead to metabolic bone diseases, such as osteoporosis (Alliston and Derynck 2002).

Osteoclasts (responsible for bone resorption) are derived from osteoclast precursors originated from haematopoietic stem cells (HSCs) within the bone marrow (Takahashi, Udagawa et al. 1999). Osteoclast development involves multiple steps: proliferation, differentiation, fusion and maturation of osteoclasts, with the involvement of osteoblasts via cell to cell contact (Alliston and Derynck 2002). Osteoclast

precursors express receptor activator of NF- κ B (RANK), which binds RANK ligand (RANKL) through cell-to-cell interaction with osteoblasts, with the interaction between RANK and RANKL stimulating osteoclast precursors to differentiate into mature, bone resorbing osteoclasts (Alliston and Derynck 2002). Osteoprotegerin (OPG), also produced by osteoblasts, acts as a decoy receptor in the RANK-RANKL signaling system, binds to RANKL and prevents it from binding to RANK, which effectively inhibits osteoclast maturation. Hence, the balance between osteoclast promoting RANKL and osteoclast inhibiting OPG (RANKL/OPG ratio) is important in regulating osteoclast numbers and activity. Apart from local factors, cytokines generated in the bone microenvironment and in circulation may also alter the normal bone remodeling process, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α). It has been reported that these cytokines generated in the bone microenvironment may be responsible for stimulating osteoclastic bone resorption. In the case of rheumatoid arthritis (RA), several studies have pointed out the roles of TNF α and IL-1 in joint destruction (Zwerina, Redlich et al. 2007). Studies have also shown the increase in the mRNA expression of RANKL in synovial tissues from RA patients (Roux and Orcel 2000), suggesting the production of these cytokines may contribute to osteoclast formation and bone destruction by inducing RANKL expression by osteoblasts/stromal cells (Boyle, Simonet et al. 2003).

Osteoblasts are derived from mesenchymal stem cells (MSCs) at periosteum and within the bone marrow, which also give rise to chondrocytes, myoblasts and adipocytes (Georgiou, Foster et al. 2010) as driven by different transcription factors (Harada and Rodan 2003). Osteoblast differentiation is well known to be regulated by 2 transcriptional factors: runt-related transcriptional factor 2 (RUNX-2) and Osterix (Osx)

(Harada and Rodan 2003), and mature osteoblasts normally express bone proteins such as osteocalcin (OCN). Similarly, marrow adipocytes develop under the influence of peroxisome proliferator-activated receptor gamma (PPAR γ), which regulates the lineage commitment of marrow MSCs towards adipocytes and away from osteoblasts (Lecka-Czernik and Suva 2006; Georgiou, Foster et al. 2010).

Bone modeling and remodeling and the associated osteoblast/osteoclast formation and activities are also influenced by environmental factors such as medical treatments. It is known that methotrexate (MTX), a commonly used anti-metabolite in childhood cancers, particularly for the treatment of acute lymphoblastic leukemia (ALL), has been reported to cause bone growth defects in both paediatric cancer patients and experimental animals. Clinically, MTX osteopathy has also been reported in survivors of ALL (Mandel, Atkinson et al. 2004) and osteosarcoma (Ecklund, Laor et al. 1997), which is characterized by osteopenia, dense zones of provisional calcification, transverse metaphyseal bands, multiple fractures and bone pain (Ecklund, Laor et al. 1997). Both short- and long-term animal studies have also reported that MTX at high-dose can result in the reduction of bone volume, with reduced recruitment of osteoblasts, and/or increased formation of osteoclasts (van Leeuwen, Kamps et al. 2000; van Leeuwen, Hartel et al. 2003). On the other hand, long-term low-dose MTX did not seem to cause much damage to both the growth plate and metaphysis of rats (Chapter 2), or affects bone mineral density in rheumatoid arthritis patients (Minaur, Kounali et al. 2002), suggesting that MTX-induced bone growth damage or bone defects is dose dependent.

Despite existing clinical and animal studies showing long-term high-dose MTX treatment in animals can cause an overall reduction of bone volume, the mechanisms underlying MTX-induced bone loss remain poorly understood, and currently there is a lack of treatments that can prevent bone loss during chemotherapy. The previous chapter (Chapter 3) has described the effects on the structure and function of the growth plate of high-dose long-term MTX treatment, with possible prevention by supplementary FA treatment in young rats. This chapter describes effects on the metaphyseal bone of the above long-term MTX with or without FA supplementary treatments of the same animal trial by characterizing structural, cellular and molecular changes in the metaphysis of the treated rats.

Materials/Methods

Animal Trials and specimen collection

The long-term MTX trial with and without FA supplementary has been described in Chapter 3 (Methods and Materials). In this trial, cardiac puncture was performed to obtain peripheral blood in lithium-heparin collection tubes, from which plasma was obtained and at -80°C until use in analysis. Left tibiae were dissected free of soft tissues, fixed in 10% formalin for 24 hours, decalcified in Immunocal (Decal Corp, Tallman, New York) then embedded in paraffin, from which paraffin sections of 4µm thick were cut and mounted on positively charged SuperFrost PlusTM-coated glass slides for histological and immunohistochemical analysis. Left femurs were also dissected free of soft tissues, wrapped in saline-soaked gauze and stored at -80°C until used for micro-computed tomography (µCT) analysis (see below). From the right tibia, metaphyseal bones of approximate 1cm long were collected and stored at -80°C for RNA extraction.

Histomorphometric analysis of metaphyseal bone: Primary spongiosa heights, osteoblast density and adipocyte density

Paraffin sections were de-waxed and stained with haematoxylin and eosin (H&E). H&E stained sections were used for basic histomorphometric measurements including primary spongiosa heights, osteoblast and adipocyte density. Briefly, primary spongiosa heights were obtained by measuring the heights between the end of growth plate and the top of secondary spongiosa, where trabecular bones are larger in size, fewer in number, and more distantly apart in spacing. Osteoblast density was obtained by counting cuboidal mononuclear cells along the trabecular surface in 6 sequential images along primary spongiosa with 200x magnification, and expressed as osteoblasts number per mm² trabecular area. Adipocytes within bone marrow area were counted using image analysis software in 4 random images within secondary spongiosa, and expressed as adipocyte number per mm² marrow area.

TRAP staining and osteoclast density measurements

Osteoclasts are multinucleated cells found along the trabecular surface responsible for bone resorption. Histochemical staining of tartrate-resistant acid phosphatase (TRAP, a marker of osteoclasts) was performed for identifying osteoclasts. Briefly, paraffin sections were de-waxed and rehydrated, incubated with acetate buffer pH 5.0 for 30 minutes, and stained with acetate buffer containing naphthol AS-B1 phosphate sodium salt (Sigma) and pararosanilin (1g/ml basic fuchsin) for 40 minutes at 37°C, then counterstained with haematoxylin. TRAP positive (TRAP⁺) mature osteoclasts containing 3 or more nuclei were counted along the trabecular surface within primary spongiosa, and expressed as osteoclasts number per mm² trabecular bone area (osteoclasts/ mm²).

In situ DNA nick translation (ISNT) for labeling apoptosis of bone cells

To determine treatment effects on the apoptosis of bone cells, *in situ* nick translation (ISNT) was conducted to label the presence of fragmented DNA by direct incorporation of labeled nucleotide in free 3'-OH termini resulting from both single stranded and double stranded DNA breaks. Briefly, paraffin sections were de-waxed and treated with 3% hydrogen peroxide in 50% methanol for 10 minutes to block endogenous peroxidase activity, rinsed with 0.2% glycine for 20 minutes, followed by digestion with proteinase K (20ug/ml, Roche, NSW, Australia) in a humidified chamber at 37°C for 20 minutes. Sections were then incubated at 37°C for an hour with nick translation mixture, which consists of 0.1mM digoxigenin-11-dUTP (DIG-11-dUTP), 1.9mM dTTP, 2mM of dATP, dCTP and dGTP, 50mM Tris-HCl, 5mM MgCl₂ and 0.1mM DTT, 0.25U/100ul Kornberg polymerase (all reagents obtained from Roche, NSW, Australia). The incorporated nucleotides for DNA strand breaks were then detected by incubation with anti-DIG-peroxidase (Roche) for an hour (Noble, Stevens et al. 1997) followed by an enzyme reaction with DAB substrate (DAKO, CA, USA) for colour development. Once brown colour was developed, reaction was stopped by dipping the slides in 2% sodium acetate, and then counterstained with haematoxylin. Osteoblast apoptosis was quantified within both primary and secondary spongiosa along the surface of trabecular bone, and expressed as apoptotic osteoblasts number per mm² trabecular bone area (apoptotic osteoblasts/mm²).

Ex-vivo micro-computed tomography (μ CT) and analysis of bone parameters

Treatment effects on overall trabecular bone volume and trabecular bone structures were examined by an *in vivo* μ -CT scanner (Skyscan 1076: Skyscan, Antwerp, Belgium). Briefly, femurs wrapped in saline-soaked gauze were thawed for an hour prior scanning. The femurs wrapped in gauze were then secured within a polystyrene foam cylinder and attached tightly to the animal/sample bed with masking tape, surrounded with X-ray source and rotating camera. The machine is equipped with a 20-100 kV X-ray source with a spot size of $<5 \mu\text{m}$. From the Skyscan software, a 1.0mm aluminium filter was selected for scanning rat bones. Other settings were made in the scan dialog box with details of scan width of 35mm, resolution of $9\mu\text{m}$ pixel, rotation step size 0.8 and averaging of 4 for noise reduction in order to achieve better imaging. After completion of scanning, 2 dimensional cross sections (transverse plane) were reconstructed with Skyscan's volumetric reconstruction software *NRecon* through the bone. The settings for image reconstruction parameters were set identical for all samples (**Table 1**). The reconstructed bone images can be viewed in Skyscan's "*DataViewer*" software program, and saved as coronal images for bone parameter analysis. These coronal images were then analysed by the program "*CT-Analyzer (CTan)*" for complete quantitative analysis of reconstructed images. An average of 200 slices of coronal images from the centre of each bone were selected, with region of interest (top of metaphysis excluding periosteum) traced for analysis of various bone parameters. Region of interest selected for analysis were defined by the histogram tool, where bone index was set at 90 for all animals. From the 3D analysis menu, additional analysis can be selected for measurement. In this study, the following factors were

measured: bone volume (BV), total volume (TV), bone volume/total volume ratio (BV/TV %), trabecular bone thickness, trabecular number and trabecular separation.

Settings of NRecon software for image reconstruction	
Position for reconstruction	970
Top position for reconstruction	2050
Bottom position for reconstruction	50
Beam hardening	50%
Number of steps	5
Ring artifact reduction	12
Dynamic image range	0.00-0.06

Table 4.1: NRecon software settings for reconstruction of transverse images.

Ex-vivo plasma-induced osteoclast formation assay

To determine whether circulating factors may play a role in osteoclastogenesis after long-term MTX treatments, *ex-vivo* osteoclast formation assay was performed with plasma derived from treated rats and bone marrow cells from normal rats. For collection of normal bone marrow cells from control rats, both ends of the right tibia and right femurs were removed, placed in 1.5ml eppendorf tubes and centrifuged for one minute to collect bone marrow. Bone marrow was re-suspended in basal minimal essential medium (α -MEM) (JRH Biosciences, KS, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 50 μ l/ml Pen/Strep (Invitrogen), 15mM HEPES (Sigma, NSW, Australia) and 130 μ M L-ascorbate, and dissociated through 18-

gauge and 21-gauge needles, followed by filtration through a 70 μ m nylon cell filter strainer (BD Biosciences, NSW, Australia) for removal of bone debris. In order to obtain high-concentration of bone marrow mononuclear cells (BM-MNCs), a commercial medium LymphoprepTM was added to the suspension in 1:1 ratio with physiological saline to obtain maximum yield of BM-MNCs. As the majority of BM-MNCs have densities below 1.077 g/ml, these cells can therefore be isolated by high speed centrifugation for 20 minutes with LymphoprepTM medium (which has a density of 1.077 m/ml), which allows BM-MNCs to retain at the sample/medium interface. The low density BM-MNCs interface was then removed, centrifuged, washed with PBS, with cell pellet re-suspended in basal α -MEM (as described earlier) and cultured overnight at 37°C with 5% CO₂. The non-adherent hematopoietic cells were collected the following day and plated in 96-well tray at the density of 3x10⁵ cells/well in triplicate, and cultured overnight in α -MEM media containing 50 μ g/ml Pen/Strep, 15mM HEPES, 10ng/ml M-CSF (Peprotech, Rocky Hill, NJ) and 10% rat plasma. Cells were re-fed the next day with similar plasma-containing media excluding M-CSF, with cell morphology checked under microscope daily until mature osteoclast formation occurred (normally 5 days), in which time cell culture was then ended and fixed with 4% formaldehyde (day 5). TRAP staining was performed (as described earlier) to identify osteoclasts in culture. To aid identifying osteoclasts, cells were counterstained with Hoechst 33258 fluorescent dye (Invitrogen), which fluorescently labels DNA for visualization of nuclei. Cells were then photographed and TRAP⁺ cells containing three or more nuclei were identified as osteoclasts. Results of *ex vivo* osteoclasts formation assay were expressed as percentage osteoclasts over the total number of TRAP⁺ cells (osteoclasts/total TRAP⁺ cells %).

Measurement of pro-inflammatory cytokines in plasma by ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed to analyse and identify potential cytokines responsible for plasma-induced osteoclastogenesis *ex vivo*. Sandwich ELISA method was employed for the quantitative determination of TNF- α , RANKL and IL-1 β in plasma samples obtained from treated rats according to manufacturer's instructions of ELISA kits for TNF- α (BD Biosciences, San Diego, CA, USA), RANKL (R&D, Minneapolis, USA) and IL-1 β (R&D, Minneapolis, USA). Briefly, microtitre plate wells were coated with working concentrations of monoclonal anti-rat antibodies (as suggested by manufacturer's instructions) overnight in 4°C fridge to allow complete binding. Wells were then washed and blocked with blocking buffer (1% BSA/PBS) to ensure protein binding sites were saturated. Then 100 μ l of rat plasma and standards (with 7 standard concentrations of 0.625, 1.25, 2.5, 5, 10, 20, and 40 pg/ml) in blocking buffer were then added to wells in triplicates, covered in adhesive strip and incubated for 2 hours at room temperature. This was followed by labeling of detection antibody, Streptavidin-HRP and substrate solution 3,3',5,5'-Tetramethylbenzidine (Sigma, St. Louis, USA) for colour development. Reaction was stopped by adding 50 μ l of 2M H₂SO₄, and absorbance was read immediately by using a microplate reader (VICTOR³™ V Multilabel Counter model 1420) set at target wavelengths (450nm and 570nm). Subtraction of optical density readings at 570nm from readings at 450nm will correct the optical imperfections in the plate. To obtain standard linear graph, mean absorbance for each standard was plotted on the y-axis against the concentration on the x-axis on logarithmic scale, with equation of best fit line determined by regression analysis. For diluted plasma samples, the concentration read from the best fit line was multiplied by the dilution factor.

Quantitative RT-PCR analysis of gene expression

Real time RT-PCR assay was performed for molecular analysis of treatment effects on mRNA expression of key molecules involved in osteoblast differentiation (Osterix, Osteocalcin), osteoclast differentiation (RANK, RANKL, OPG, TNF α and For RNA extraction, metaphysis samples were first crushed by mortar and pestle in the presence of liquid nitrogen (LN₂), transferred into clean 10ml tubes in small amount of LN₂. Once LN₂ has evaporated from the tube, TRI reagent (Sigma, St. Louis, USA) was then added to metaphysis powder for extracting RNA according to manufacturer's instructions. Extracted RNA was then DNase treated with TURBO DNA-free™ Kit (Ambion, Austin, USA.) for the removal of contaminating DNA. Samples with an A260/A280 ratio of 1.8 or above were used to synthesize single stranded cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA) according to manufacturer's instructions. SYBR Green PCR assays (Applied Biosystems, CA, USA) for each target molecule and reference gene Cyclophilin A (Cyc-A) were performed using PCR primers (**Table 2**) (Zhou, Foster et al. 2004; Xian, Cool et al. 2007) in triplicate on cDNA samples. PCR assays were run on a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative gene expression was calculated using the comparative Ct ($2^{-\Delta CT}$) method, with Cyclophilin A (Cyc A) serves as the endogenous control (Zhou, Foster et al. 2004).

Gene	Forward Primer	Reverse Primer
Cyclophilin A	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG
RANK	GGGAAAACGCTGACAGCTAATC	GGTCCCCTGAGGACTCCTTATT
RANKL	CCGTGCAAAGGGAATTACAAC	GAGCCACGAACCTTCCATCA
OPG	CACAGCTCGCAAGAGCAAAC	ATATGCCGTTGCACACTGCTT
TNF α	ATGGCCCAGACCCTCACACTCAGA	CTCCGCTTGGTGGTTTGCTACGAC
OSCAR	CTGGTCATCAGTTCCGAAGG	CTATGATGCCCAAGCAGATG
PPAR γ	AACGTGAAGCCCATCGAGGACATC	CTTGCGAACAGCTGGGAGGAC

Table 4.2: Primer sequences used in this study

Statistics

Data are presented as means \pm SEM and analysed by one-way analysis of variance (ANOVA) with GraphPad Prism 6 Software. When the significance levels ($P < 0.05$) were achieved, a post hoc analysis of groups was performed using a Tukey's test.

Results

Treatment effects on overall structural changes in metaphysis

In previous chapter, we have reported that 6 weeks treatments with MTX caused no significant changes in total thickness of the growth plate. However, histomorphometric measurements revealed that 6 weeks of high-dose MTX treatment was able to significantly reduce primary spongiosa heights when compared to control rats (**Figure 4.1A , 4.1B & 4.1D**) ($P<0.05$), while supplementary treatment with FA was able to prevent the MTX-induced primary spongiosa height reduction (**Figure 4.1B, 4.1C & 4.1D**) ($P<0.05$ compared to MTX alone group).

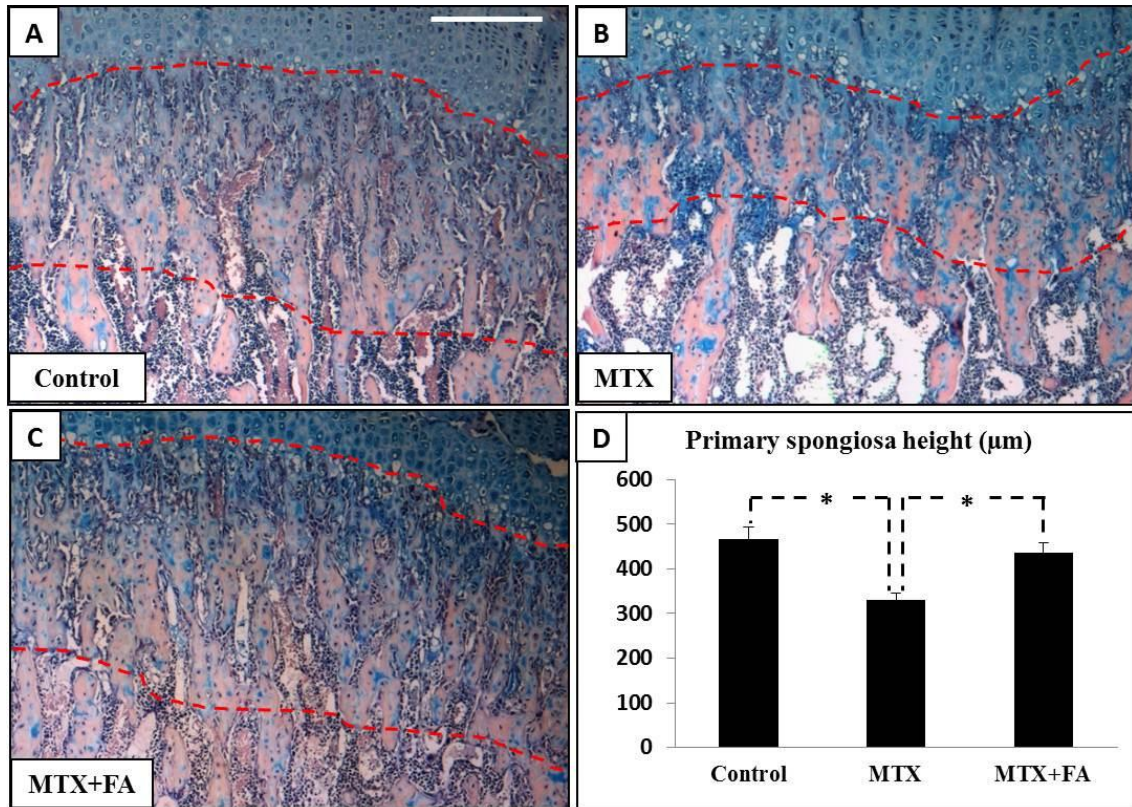
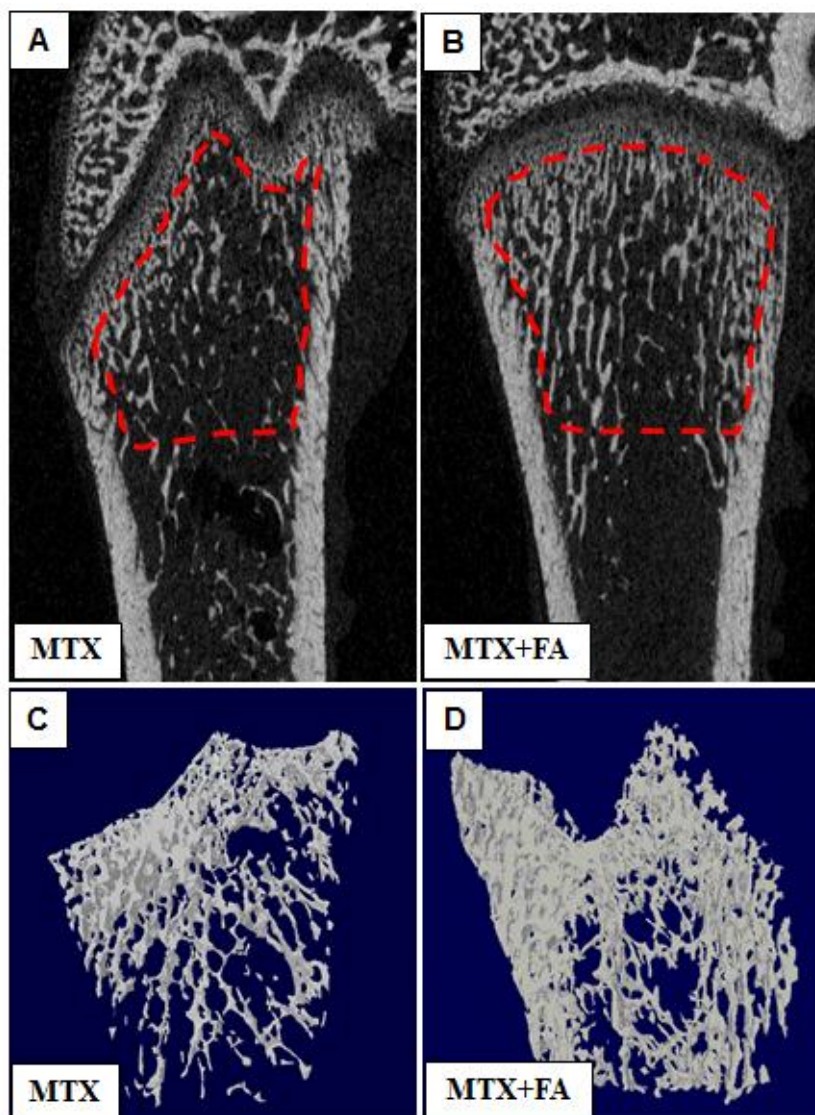


Figure 4.1:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folinic acid (FA) on primary spongiosa thickness. H&E alcian staining of metaphyseal bone in a control rat (A), MTX-treated rat (B), and a MTX+FA treated rat (C), with dotted lines marking the primary spongiosa zone, and treatment effects on primary spongiosa thickness (D). n=6, scale bar on panel A=250μl which also applies to other photos.

To further examine the treatment effects on the overall structural changes of metaphysis, μ CT evaluation of the proximal ends of rat femurs was conducted. By μ CT, long-term high-dose MTX treatment caused a 19% bone volume reduction (BV/TV%) in the metaphysis when compared to control rats of the same age ($20.17 \pm 4.2\%$ vs. $24.92 \pm 2.1\%$) ($P > 0.05$) (**Figure 4.2A, 4.2C & 4.2E**), while supplementary treatment with FA was able to partially prevent the reduction in the bone volume caused by MTX treatment ($27.65 \pm 4.1\%$ for MTX+FA group vs. $20.17 \pm 4.2\%$ for MTX alone group) ($P > 0.05$) (**Figure 4.2B, 4.2D & 4.2E**). Trabecular structural analysis by μ CT also revealed that, when compared to control rats, long-term high-dose MTX treatment caused reduction in average trabecular number (2.49/mm vs. 3.15/mm), slight increase of trabecular thickness (0.081mm vs. 0.071mm) and trabecular separation (0.26mm vs. 0.25mm) ($P > 0.05$) (**Figure 4.2E**). When compare to MTX-treated rats, supplementary treatment with FA resulted in greater numbers of trabeculae (3.73/mm vs. 2.49/mm) and reduction of trabecular spacing (0.2mm vs. 0.26mm) (**Figure 4.2E**), with similar trabecular thickness compared to control rats (**Figure 4.2E**). These μ CT bone volume and trabecular bone structure data were confirmed with a quantitative histomorphometric imaging method using H&E stained bone sections (results not shown).



E	Control	MTX	MTX+FA
Bone volume/Tissue volume (%)	24.92 (+/-2.1)	20.17 (+/-4.2)	27.65 (+/-4.1)
Trabecular thickness (mm)	0.077	0.081	0.075
Trabecular number/mm	3.15	2.49	3.73
Trabecular separation (mm)	0.25	0.26	0.2

Figure 4.2:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folic acid (FA) on trabecular bone volume and bone structure.

Longitudinal cross-sections of femur from a MTX-treated rat (A) and a MTX+FA treated rat (B), with area traced in red dotted lines being used for measurement of bone volume and bone structures. Three-dimensional images showing trabecular bone structures of a MTX-treated rat (C) and a MTX+FA treated rat (D). Treatment effects on trabecular bone volume and structural changes (E).

Treatment effects on osteoblast density, osteoblastic gene expression, and osteoblast apoptosis

Trabecular bone structure is directly influenced by numbers and activity of osteoblasts. Counting of osteoblasts on H&E stained sections revealed that high-dose MTX treatment significantly reduced osteoblast density in the primary spongiosa when compared to control rats ($P<0.01$) (**Figure 4.3A & 4.3C**), while FA supplementary treatment can preserve the osteoblast numbers ($P<0.01$ compared to MTX alone group) (**Figure 4.3B & 4.3C**). Molecular analysis of treatment effects on osteoblast differentiation was carried out by assessing the mRNA expression of bone matrix protein osteocalcin and osteogenic transcription factor osterix in metaphyseal bone. Interestingly, MTX treatment caused a slight but statistically non-significant increase in osteocalcin expression (**Figure 4.3D**) but no changes in the osterix levels when compared to control rats (**Figure 4.3E**). FA supplementary treatment caused an obvious but statistically non-significant reduction in osteocalcin expression (**Figure 4.3D**) and no changes in osterix expression when compared to both control and MTX-treated rats (**Figure 4.3E**).

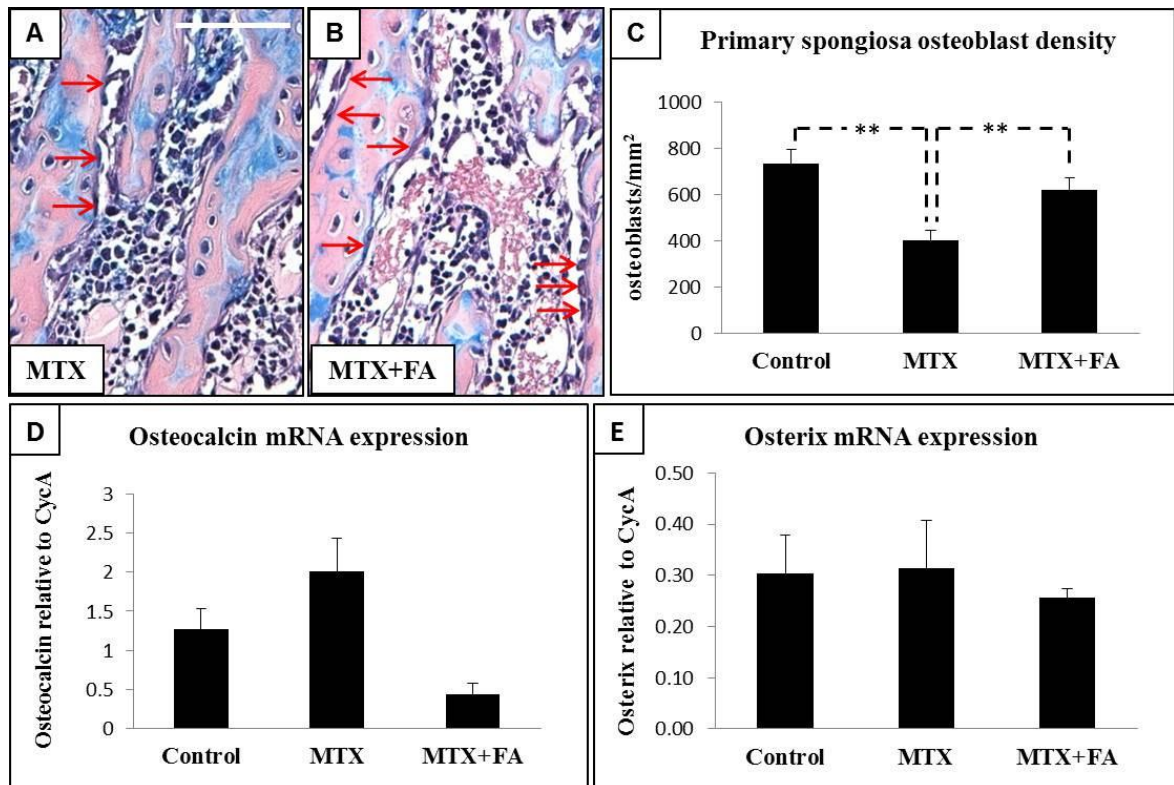


Figure 4.3:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folic acid (FA) on osteoblast density and mRNA expression of genes involved in osteoblast differentiation. H&E stained sections of a tibia from a MTX-treated rat (A) and a MTX+FA treated rat (B), with arrows pointing to osteoblasts. Treatment effects on osteoblast density in primary spongiosa (C). Treatment effects on metaphyseal-mRNA gene expression of Osteocalcin (D) and Osterix (E) relative to Cyclophilin-A. n=6, scale bar on panel A & B=100 μ l.

Since long-term MTX treatment caused a significant reduction in osteoblast density without affecting mRNA expression of osteocalcin and osterix, and BrdU labeling analysis revealed no significant changes in the proliferation of osteoblasts (data not shown), treatment effects on osteoblast apoptosis were assessed. Apoptosis analysis by *in situ* Nick Translation labeling revealed that long-term, high-dose MTX treatment caused an induction of apoptosis among osteoblasts in metaphysis particularly in the secondary spongiosa ($P > 0.05$ when compared to control rats) (**Figure 4.4A, 4.4C & 4.4D**). Interestingly, 6 weeks of FA supplementary treatment appeared to prevent the MTX-induced osteoblast apoptosis in both primary and secondary spongiosa (**Figure 4.4B, 4.4C & 4.4D**).

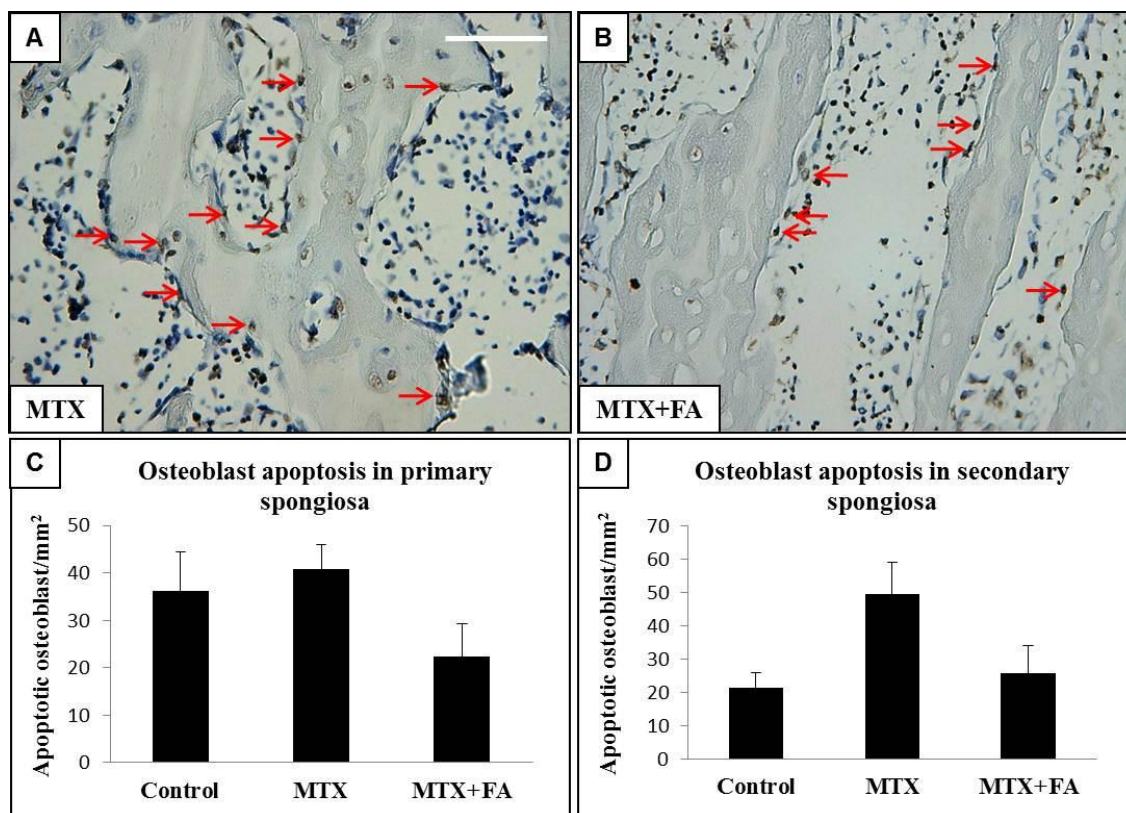


Figure 4.4:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folinic acid (FA) on osteoblast apoptosis. Secondary spongiosa of a MTX-treated rat (A) and a MTX+FA treated rat (B), with In Situ Nick Translation detecting apoptotic osteoblasts (brown in colour, pointed by red arrows). Treatment effects on osteoblast apoptosis in primary spongiosa (C) and secondary spongiosa (D). n=6, scale bar on panels A & B= 250 μ l.

Treatment effects on osteoclast density and expression of genes regulating osteoclastogenesis

To examine whether long-term MTX treatment can influence the density of bone resorptive cells osteoclasts, TRAP staining was performed. Counting of TRAP⁺ cells adhering on trabecular surface revealed that MTX treatment significantly increased osteoclast density in the primary spongiosa when compared to control rats ($P < 0.01$) (**Figure 4.5A & 4.5C**), while supplementary FA treatment significantly suppressed MTX-induced increased osteoclast density ($P < 0.05$ compared to MTX alone group) (**Figure 4.5B & 4.5C**). However, RT-PCR gene expression analysis revealed no significant changes in metaphyseal bone in mRNA expression of RANK, TNF α , OSCAR (Osteoclast-associated receptor, a newly identified osteoclast-specific receptor which is important in the process of osteoclast co-stimulation) and RANKL/OPG expression ratio between all treatment groups (**Figure 4.5D-4.5G**), a finding which is not consistent with the increased osteoclast density observed from histology analysis.

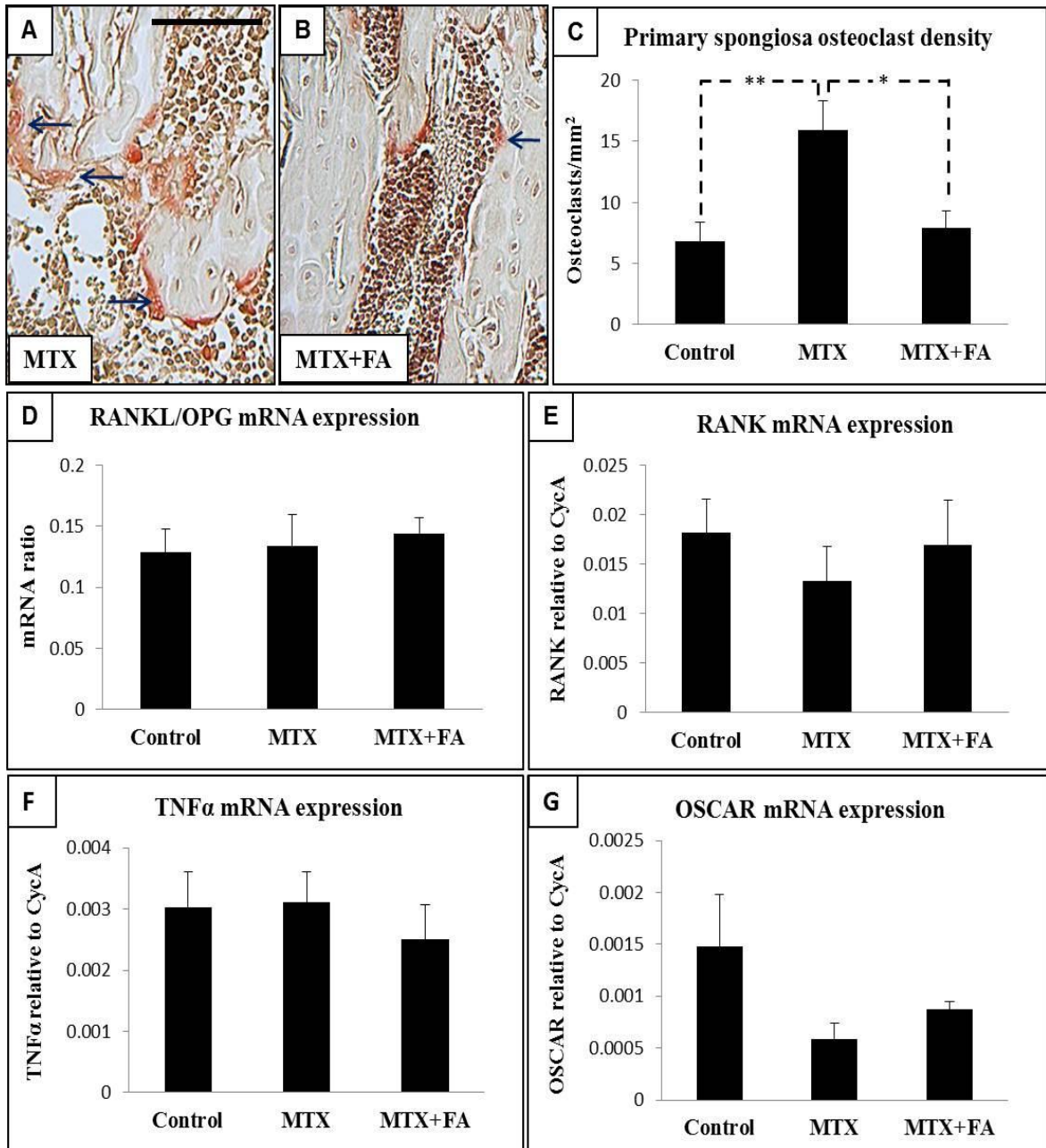


Figure 4.5:

Effects of treatments for 6 weeks with methotrexate (MTX) alone or with supplementary folic acid (FA) on osteoclast density and mRNA expression of genes involved in osteoclastogenesis. TRAP stained sections of tibia from a MTX-treated rat (A) and MTX+FA treated rat (B), with arrows pointing to multinucleated TRAP⁺ osteoclasts. Treatment effects on osteoclast density in primary spongiosa (C). Treatment effects on metaphyseal RANKL/OPG mRNA expression ratio (D), metaphyseal mRNA gene expression of RANK (E), TNF α (F) and OSCAR (G) relative to Cyclophilin-A. n=6, scale bar on panels A & B= 200 μ l.

Since RT-PCR revealed no significant changes in the mRNA expression of osteoclastogenic molecules locally in the metaphyseal bone, systemic treatment effects were investigated by examining the osteoclast-forming potential of bone marrow cells from normal rats as supported by the plasma from treated rats in comparison to control rats. *Ex-vivo* osteoclast formation assay without exogenous RANKL added but supported by plasma from treated rats revealed that plasma obtained from MTX-treated rats induced formation of more osteoclasts when compared to plasma of control rats ($P>0.05$) (**Figure 4.6A & 4.6C**), while plasma obtained from FA supplementary treated rats was able to suppress the osteoclast formation induced by the plasma obtained from MTX-treated rats ($P>0.05$) (**Figure 4.6B & 4.6C**).

ELISA was conducted with plasma samples for identifying potential circulating osteoclastogenic factors within the blood plasma. Sandwich ELISA revealed that both TNF α and RANKL protein concentrations were not significantly altered in the plasma obtained from MTX-treated rats compared to control rats or rats treated with FA supplementation ($P>0.05$) (**Figure 4.6D & 4.6E**). However, IL-1 β protein concentration in the plasma was significantly increased following 6 weeks MTX administration when compared to normal rat plasma ($P<0.01$) (**Figure 4.6F**), and rats received FA supplementary treatment were found to express similar levels of plasma TNF α and RANKL when compared to plasma of MTX-treated rats (**Figure 4.6D & 4.6E**), but with an approximately 18% reduction of IL-1 β protein concentration when compared to plasma of MTX-treated rats ($P>0.05$) (**Figure 4.6F**).

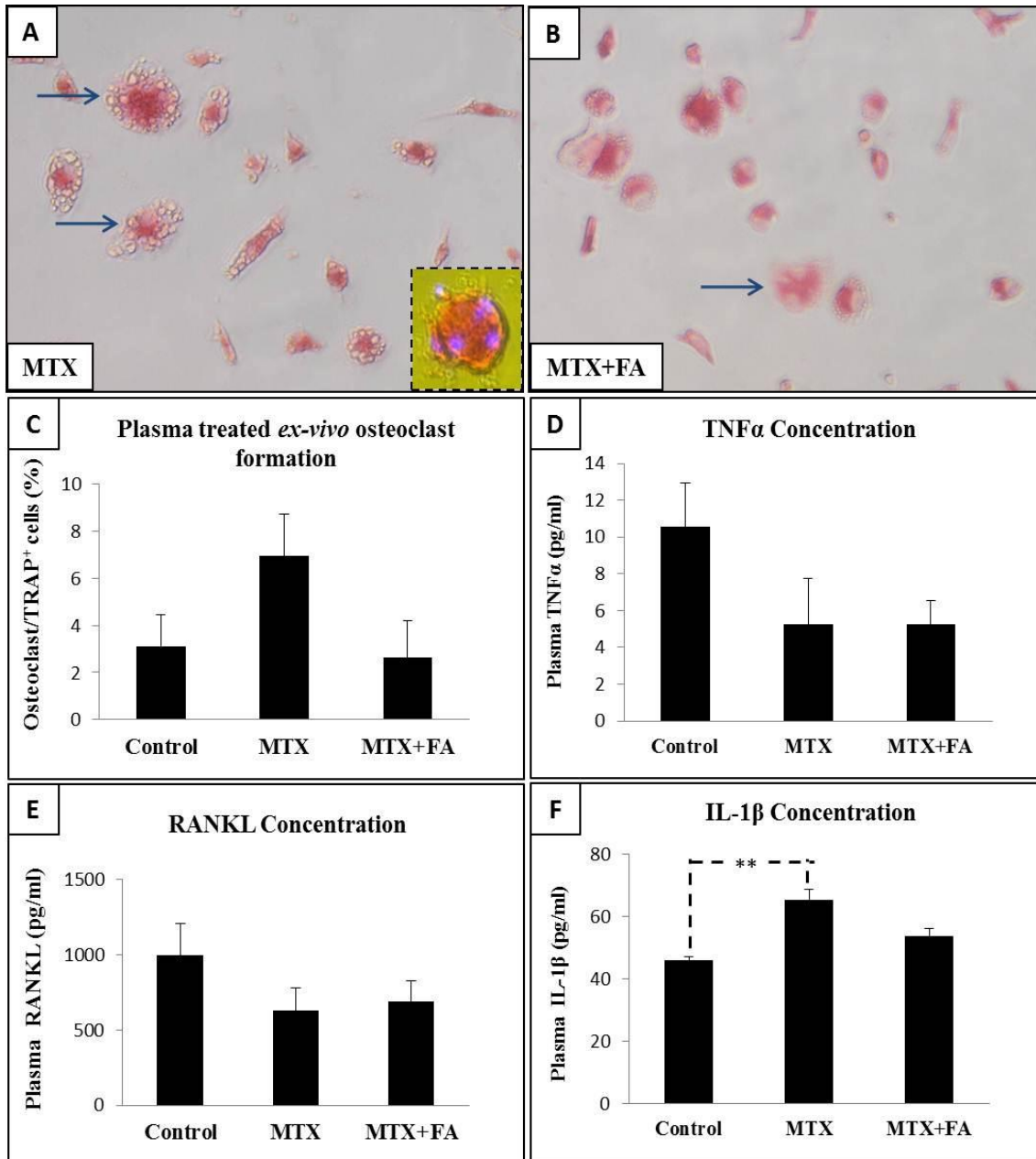


Figure 4.6:

Effects on plasma-induced ex-vivo osteoclast formation. In vitro osteoclast formation from normal rat bone marrow cells as induced by plasma obtained from rats treated for 6 weeks with MTX alone (A) or with folic acid (FA) combination (B). TRAP stained positive cells, with arrows pointing to multinucleated TRAP⁺ osteoclasts. Hoechst stain was performed to aid visualising nuclei of TRAP⁺ cells (in dotted square). Comparison of the extents of effects on plasma-induced ex-vivo osteoclast formation with plasma from rats of different treatment groups (C). Plasma concentrations of TNF α (D), RANKL (E) and IL-1 β (F). n=6 for all assays.

Treatment effects on bone marrow adipocyte density

To investigate whether bone loss following long-term MTX treatment is associated with an increase of adipocyte formation, density of adipocytes was quantified within the bone marrow from H&E stained tibia sections. This analysis revealed that 6-week MTX treatment caused a significant increase in adipocyte density within the bone marrow of secondary spongiosa when compared to the control normal metaphysis ($P < 0.001$) (**Figure 4.7A & 4.7C**). FA supplementary treatment was able to significantly suppress the MTX-induced increased adipocyte density ($P < 0.001$) (**Figure 4.7B & 4.7C**). However, RT-PCR analysis revealed no significant changes in the expression of adipogenic regulatory gene PPAR γ between all treatment groups ($P > 0.05$) (**Figure 4.7D**).

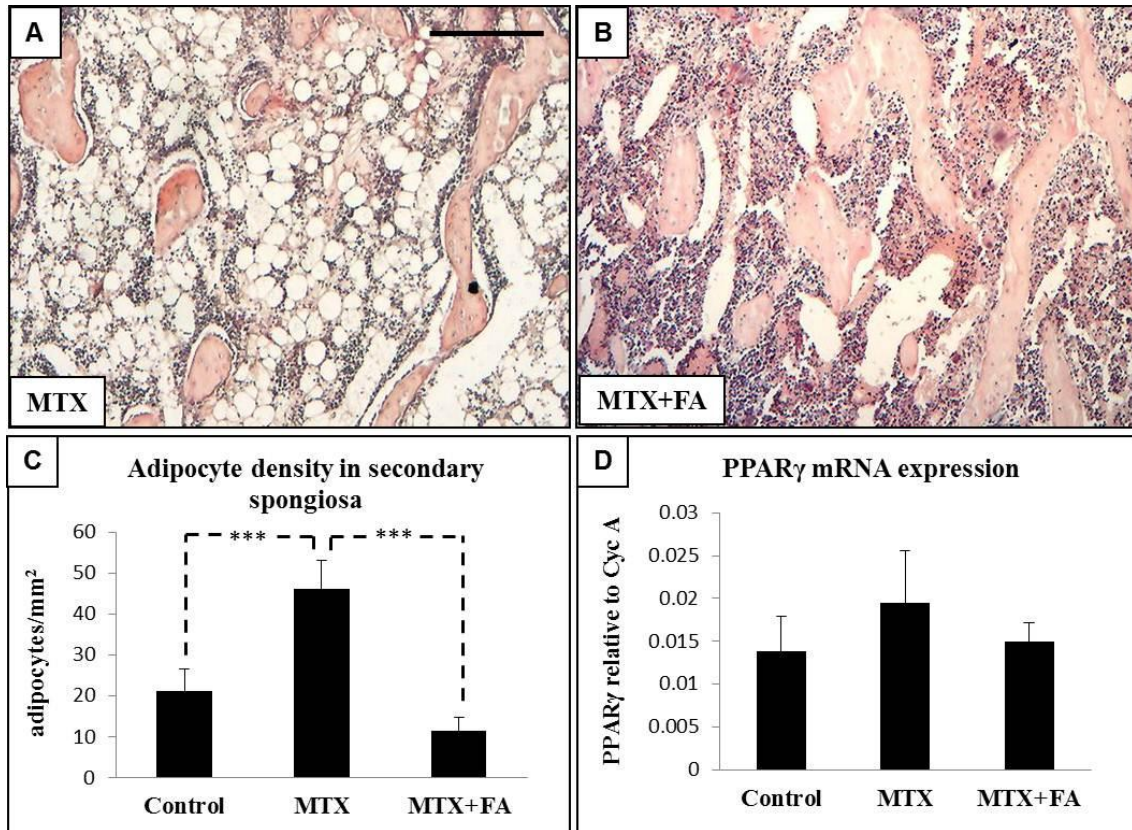


Figure 4.7:

Effects of treatment for 6 weeks with methotrexate (MTX) alone or with supplementary folinic acid (FA) on adipocyte density and mRNA expression of genes involved in adipogenesis. H&E stained sections of tibia from a MTX-treated rat (A) and MTX+FA treated rat (B). Treatment effects on adipocyte density within bone marrow area of secondary spongiosa (C). mRNA gene expression of PPAR γ relative to CyclophilinA (D). n=6, scale bar on panels A & B= 250 μ l

Discussion

Cancer patients are at an increased risk for developing skeletal complications as a result of chemotherapy. Many commonly-used chemotherapeutic agents have been shown to cause bone loss by inducing bone resorption and bone turnover, such as glucocorticoids, cyclophosphamide and 5-fluorouracil (Guise 2006). Clinically, use of high dose MTX (a commonly used anti-metabolite in combination chemotherapy for paediatric acute lymphoblastic leukaemia) has been shown associated with skeletal morbidities characterised by bone pain, fractures and osteopenia (Brennan, Rahim et al. 1999; Warner, Evans et al. 1999), with reduced peak bone mass in adult survivors (Brennan, Rahim et al. 1999). As trabecular bone deposition is reliant on production of calcified cartilage scaffold, it is not surprising that damages to growth plate chondrocytes may lead to failure in bone deposition and result in bone loss. Using a rat model, the previous chapter (Chapter 3) has investigated potential mechanisms for chronic high-dose MTX-induced growth plate damages, as well as protective effects of folinic acid (FA) supplementation in the growth plate. Although some studies have reported bone loss after acute or chronic MTX administration in animals (Wheeler, Vander Griend et al. 1995; van Leeuwen, Kamps et al. 2000; Xian, Cool et al. 2007), and proposed this may be due to decreased bone formation and increased bone resorption, the mechanisms for chronic MTX-induced metaphyseal damages remain poorly understood, and it is unknown whether FA could protect metaphyseal bone during long-term high dose MTX chemotherapy. Using a chronic chemotherapy model in young rats, this current study observed significant effects of high-dose MTX chemotherapy and protective effects of FA supplementation on overall structure, function and gene expression in the metaphyseal bone. In addition, as demonstrated by *ex-vivo* osteoclast formation assay and ELISA using blood plasma obtained from

treated rats, systemic effects of chronic treatments were shown as a potential contribution to the bone resorption.

The current study further revealed a reduction in the thickness of primary spongiosa, reflecting reduced bone lengthening following chronic MTX chemotherapy. However, μ CT analysis from this study revealed no significant changes in bone volume or trabecular structures, which is inconsistent with previous findings that reported acute high-dose MTX treatment can cause reduction in bone volume, with fewer numbers but increased size of bony trabeculae (Xian, Cool et al. 2007).

The cellular activities on the trabecular bone surface determine and modify the size and shape of trabecular bone, influencing bone volume and bone mass. Clinically, levels of type-I collagen and alkaline phosphatase (markers for bone formation) were found suppressed in serum of patients immediately after the administration of chemotherapeutic agents, suggesting chemotherapy has adverse effects on osteoblasts *in vivo* (Crofton, Ahmed et al. 2000). This was supported by *in vitro* studies using human osteoblast-like cells (hOB), in which MTX at clinically relevant concentrations was able to reduce numbers of hOBs in culture due to depletion of their precursors (Davies, Evans et al. 2002; Davies, Evans et al. 2002). In an acute rat study, short-term MTX administration was able to reduce osteoblast density by suppressing osteoblast/preosteoblast proliferation *in vivo* (Xian, Cool et al. 2007). Results from the current study showed that chronic high-dose MTX treatment was also able to decrease osteoblast density, which was probably due to the induction of osteoblast apoptosis, rather than affecting osteoblast proliferation, contributing to the decline in bone formation. Although our histological observations showed a reduced trabecular bone

volume with decreased osteoblast density, quantitative RT-PCR analysis revealed a non-significant increase in gene expression of osteogenic transcription factor *Osx* and major osteoblast protein OCN in metaphyseal bone, suggesting an increase in osteogenic potential following chronic high-dose MTX-treatment. Consistently, while a recent study with rats treated with low-dose MTX for a long-term observed a reduced osteoblast density, the size of osteoprogenitor pool within bone marrow of the treated rats as examined by *ex vivo* cell culture analysis was increased non-significantly (chapter 2), which again suggested a greater osteoblast differentiation potential within the bone marrow after long-term MTX chemotherapy. The increase in osteogenic potential during maintenance MTX chemotherapy (of a lower intensity) perhaps indicates initiation of a recovery mechanism in order to compensate for the damaged bone environment caused during the intense induction treatment phase.

Since the process of bone formation and bone resorption are closely linked and regulated and imbalance of the two processes can cause skeletal morbidity, the current study also examined the treatment effects on bone resorptive cells osteoclasts. Clinically, long-term glucocorticoid treatment is known to induce nuclear factor kappa-B (NF- κ B) ligand (RANKL) production by osteoblasts, resulting in bone resorption with net loss of bone over time (Olney 2009). This is consistent with *in vitro* studies which demonstrated that Dexamethasone at a low concentration ($<10^{-8}$ M) was able to enhance RANKL-induced osteoclast formation at the early stage of osteoclast differentiation (Takuma, Kaneda et al. 2003; Hozumi, Osaki et al. 2009). A recent *ex vivo* study using bone marrow haematopoietic cells obtained from MTX-treated rats revealed an increase in osteoclast precursor cell pool which express preosteoclast surface marker CD11b⁺ (chapter 2), which is consistent with the observed increased

osteoclast density on bone surface *in vivo* (chapter 2). In the current study, the osteoclast density on trabecular surface was also significantly increased following long-term high-dose MTX treatment in young rats. However, RT-PCR mRNA expression analysis revealed a lack of significant changes in expression of RANKL/OPG ratio and levels of IL-6, TNF and IL-1 in metaphysis, indicating local expression of osteoclastogenic signals was not affected during maintenance MTX chemotherapy phase. Therefore, although the current study has revealed significant morphological damages during maintenance MTX chemotherapy, there were no significant molecular changes in expression revealed for key genes involved in both osteoblastogenesis and osteoclastogenesis. This observation perhaps indicates that bone loss was mainly resulting from the severe cellular and molecular damages during the intensive induction chemotherapy phase prior to the maintenance phase (Friedlaender, Tross et al. 1984; Wheeler, Vander Griend et al. 1995; Xian, Cool et al. 2007), in which the structural damaging effects resulting from the initial intensive induction treatment phase may have persisted into longer-term maintenance phase while molecular recovery starts to take place in order to compensate for the damaged bone environment.

However, although expression of osteoclastogenic signals was not affected locally in the metaphysis, blood plasma obtained from MTX-treated rats was shown able to induce more osteoclast formation *ex vivo* in bone marrow cells from normal rats, with protein levels of IL-1 β , but not TNF nor RANKL being shown to be significantly elevated when compared to normal rat plasma. IL-1 is a pro-inflammatory cytokine that can induce bone destruction in diseases including osteoporosis and rheumatoid arthritis (Findlay and Haynes 2005; Rusinska and Chlebna-Sokol 2005). Both isoforms (IL-1 α and IL-1 β) can bind to IL-1 receptors and activate downstream signalling pathways

(Dinarello 1996). A previous animal study has demonstrated that injections of IL-1 α or IL-1 β can stimulate bone resorption with elevated plasma calcium concentrations *in vivo* (Boyce, Aufdemorte et al. 1989), consistent with the results from our study that systemic IL-1 levels were higher in MTX-treated rats where a higher osteoclast density and bone loss were observed. However, although both isoforms had no effects on osteoclast precursors *in vitro* (Trebec-Reynolds, Voronov et al. 2010), they were able to increase the numbers of large osteoclasts in culture (Nishihara, Takahashi et al. 1994; Trebec-Reynolds, Voronov et al. 2010). In addition, IL-1 β was also found to change the morphology of large osteoclasts by inducing large pit formation (Trebec-Reynolds, Voronov et al. 2010). In addition, studies have also reported that IL-1 alone is insufficient to induce osteoclast differentiation from precursors (Jimi, Nakamura et al. 1998), and IL-6 and/or TNF α may synergize with IL-1 to enhance osteoclast bone resorption (Devlin, Reddy et al. 1998; Kwan Tat, Padrines et al. 2004). However, ELISA analysis from the current study revealed reduced TNF α and RANKL plasma levels in rats treated with MTX, suggesting perhaps long-term MTX chemotherapy-induced bone resorption is mediated by systemic IL-1 alone on the later stage of osteoclastogenesis, independent of TNF α or RANKL/RANK interaction. While further studies are required to test this possibility, one recent study has shown that IL-1 has the potential to induce osteoclast differentiation if sufficient levels of IL-1RI (IL-1 receptor 1) were expressed in osteoclast precursors (Kim, Jin et al. 2009). In addition, while IL-6 has been shown to enhance the effects of cytokines on bone resorption both *in vitro* and *in vivo* (Kurihara, Bertolini et al. 1990; Devlin, Reddy et al. 1998), it remains to be investigated whether circulating IL-1 mediated bone resorption can be enhanced by the possibility of upregulated production of IL-6 in MTX-treated rats.

In the current study, although bone volume wasn't significantly reduced following long-term high-dose MTX administration, adipocyte density was found significantly increased within the metaphysis of MTX-treated rats, despite the insignificant increase in expression of adipogenic master transcription factor PPAR γ (Lecka-Czernik 2010) in the metaphysis. Previously, PPAR γ has been identified to be a key transcriptional factor for adipocyte differentiation that regulates the lineage commitment of both marrow MSCs towards adipocytes and away from osteoblasts, and of HSCs towards osteoclasts (Lecka-Czernik 2010). Similar to our observations, previous clinical studies have reported osteopenia and excess adiposity following the treatment of childhood ALL with or without cranial irradiation (Tillmann, Darlington et al. 2002; Davies, Evans et al. 2004). Glucocorticoid administration has also been reported to increase adiposity clinically, which was confirmed by animal studies reporting leptin insensitivity in rats receiving glucocorticoid, which contributes to the predisposition of both osteopenia and obesity (Zakrzewska, Cusin et al. 1997; Campbell, Peckett et al. 2011). The phenomenon for reduced bone volume with increased marrow adiposity is also well described in ageing-related osteoporosis (Verma, Rajaratnam et al. 2002). With ageing, there is increase adiposity due to the predominant differentiation of MSCs into adipocytes at the expense of osteoblastogenesis (Gimble and Nuttall 2004), and the increased marrow fat is followed by bone loss (Verma, Rajaratnam et al. 2002). Hence, it may be possible that adipocyte infiltration into the bone marrow occurs during the induction chemotherapy phase, in which the loss of bone and bone marrow cells is replaced by more space-filling adipose tissue, and these bone/fat volume changes may have persisted into the maintenance phase after chronic chemotherapy treatment. Moreover, the inverse relationship between osteoblastic and adipocyte differentiation in bone marrow stromal cell has been

shown by several *in vitro* studies (Beresford, Bennett et al. 1992; Locklin, Williamson et al. 1995). The current study has attempted to investigate treatment effects on osteogenesis and adipogenesis potential of bone marrow stromal cells isolated from treated rats; however, the attempt was unfortunately not successful as the bone marrow samples from this long-term study were accidentally lost. However, a recent published study from our lab has demonstrated that acute intensive MTX treatment (equivalent to the induction phase of the current study) can cause a significant switch from osteogenesis to adipogenesis in bone and bone marrow (Georgiou, Scherer et al. 2011), with osteogenic transcription factors Runx2 and Osterix being decreased but adipogenic genes PPAR γ and FABP4 been up-regulated in the stromal population. In the current study, although it is not known whether MTX treatment has or not changed the differentiation potential of bone marrow MSCs more towards adipocyte differentiation over osteoblast commitment, the increased adipocyte density, reduced osteoblast density with unchanged adipogenic and osteogenic gene expression may be explained by the possibility of increased adipogenesis at the expense of osteoblastogenesis within the bone marrow during the early intensive induction phase of MTX treatment. Perhaps gene expression changes, which had occurred at an earlier stage during the more intensive induction phase, predisposed the histological changes that persisted for a long term. In addition, studies have reported that differentiated adipogenic cell lines express RANKL and OPG which regulate osteoclast differentiation (Kelly, Tanaka et al. 1998; Hozumi, Osaki et al. 2009). The mechanism underlying MTX treatment-induced potential bone/fat switch and whether this has contributed to the increased osteoclast density remain to be investigated.

Current treatment programs for paediatric cancers provide more than an 80% cure rate. However, skeletal morbidity such as osteopenia, short stature and bone fragility has been reported in survivors of childhood malignancies during and after chemotherapy treatments (van der Sluis, van den Heuvel-Eibrink et al. 2002; Marinovic, Dorgeret et al. 2005). While some children have demonstrated osteopenia at the time of diagnosis, skeletal morbidities continue to develop as they progress through chemotherapy (Davies, Evans et al. 2005). Due to the high success rates in cancer treatment, MTX remains to be an important component of chemotherapy for treating paediatric malignancies. Since MTX chemotherapy has been clinically associated with bone pain, osteopenia and increased fracture risks (Brennan, Rahim et al. 1999), it is necessary to develop potential strategies to protect bone growth during chemotherapy. Since folates are essential for cell proliferation and survival (Matherly 2001; Jhaveri, Rait et al. 2004), folate deficiency caused by repeated use of MTX could be one of the possible cause of skeletal defects. Folinic acid (FA), an anti-dote that has been clinically used for supplementing MTX therapy to reduce hepatotoxicity and gastrointestinal side effects without lowering the efficacy of MTX (Hoekstra, van Ede et al. 2003), was found to protect against MTX-induced bone damage in short-term animal study (Xian, Cool et al. 2008), as well as preserving the growth plate structure and functions during long-term maintenance MTX chemotherapy (as reported in Chapter 3). This chapter further revealed that supplementary FA treatment during the long-term MTX chemotherapy preserved not only primary spongiosa height but overall trabecular bone volume by maintaining trabecular number and trabecular spacing. Interestingly, FA treatment was found to preserve metaphyseal osteoblast numbers by preventing osteoblasts from MTX-induced apoptosis, without significantly affecting osteoblast differentiation as revealed by mRNA expression of *Osx* and *osteocalcin*. Previously, a

short-term study has revealed FA supplementation during acute intensive MTX chemotherapy can preserve osteoblast numbers and bone marrow stromal cell pool in rats (Xian, Cool et al. 2008). Furthermore, FA supplementary treatment in the current study was also found to significantly suppress long-term MTX treatment-induced osteoclast density *in vivo*. The ability of FA in reducing MTX-induced osteoclast formation *in vivo* and *ex vivo* was also discussed in recent short-term animal studies (Chapter 2). While the current study revealed no significant changes in metaphyseal mRNA expression of genes involved in osteoclastogenesis from FA supplementation, plasma obtained from the FA+MTX-treated rats was found to suppress osteoclast formation *ex vivo* by suppressing IL-1 β protein expression (when compared to plasma of MTX alone-treated rats). These results indicate that FA supplementary treatment can suppress MTX-induced osteoclast formation and bone resorption systemically via suppression of circulating osteoclastogenic cytokines particularly IL-1. Moreover, the current study revealed that FA supplementary treatment can significantly reduce bone marrow adiposity from MTX chemotherapy, indicating FA supplementary treatment may potentially preserve bone volume by preserving osteoblastic differentiation and preventing over adipogenic differentiation.

Summary

Using a chronic rat chemotherapy model, the current study revealed high-dose MTX treatment can damage the metaphysis of young rats by causing a significant reduction of primary spongiosa height, which in turn reflects reduced bone lengthening. Cellular and histology analyses further revealed decreased osteoblast density, increased osteoclast number on trabecular surface and increased adiposity within the bone marrow. Furthermore, MTX-induced osteoblastic damage was found probably mainly due to the induction of apoptosis, rather than suppression of proliferation. The increased osteoclastic number was induced probably systemically by factors including IL-1 within circulating plasma, rather than osteoclastogenic factors locally produced at the metaphysis. Furthermore, histological observations from this study also revealed the presence of an inverse relationship between bone and fat volume in bone after long-term MTX chemotherapy. Interestingly, molecular analysis with specimens collected at the end of long-term maintenance treatment phase revealed no significant changes in the mRNA expression of genes involved in osteogenesis, osteoclastogenesis and adipocyte differentiation. Overall, histological, cellular and molecular analyses of this study suggest that the differentiation potentials of osteoblasts, osteoclasts and adipocytes were not severely affected during maintenance chemotherapy phase, and that perhaps bone loss seen morphologically could be due to the intense daily MTX treatment during the induction chemotherapy phase, in which the damaging effects may persist into the long-term maintenance treatment phase. On the other hand, FA supplementary treatment during this long-term MTX chemotherapy appeared to reverse the MTX-induced bone loss and preserve the trabecular bone structures by lowering levels of osteoclastogenic cytokine IL-1 within the circulating plasma and osteoclast density on bone surface, preventing osteoblasts from undergoing apoptosis and

reducing bone marrow adiposity. Based on these observations, paediatric patients who are at risk of skeletal growth suppression and low bone mass from chronic MTX chemotherapy could benefit from supplementation with FA during the long-term MTX chemotherapy.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSIONS, and FUTURE DIRECTIONS

5.1 General Discussion

The “endochondral” longitudinal bone growth can be influenced by many genetic and environmental factors through affecting progenitor supply, proliferation, maturation, activity and survival of growth plate chondrocytes and bone-forming osteoblasts and calcified tissue-resorptive osteoclasts or chondroclasts (Xian 2007). Due to the high success rate and intensifying use of chemotherapy in paediatric cancers, chemotherapy-induced bone growth defects, osteoporosis, osteonecrosis, and fractures are becoming major long-term adverse effects in young cancer patients and survivors. Apart from many clinical reports (Brennan, Rahim et al. 1999; Davies, Evans et al. 2005), the adverse effects have been shown by several animal studies, which reported significant bone loss after administration of single or combination of chemotherapeutic agents, including 5-fluorouracil, methotrexate, cisplatin, doxorubicin, cyclophosphamide and etoposide (van Leeuwen, Hartel et al. 2003; Xian, Cool et al. 2006; Xian, Cool et al. 2007; Xian, Cool et al. 2007). Although these animal *in vivo* studies have shown osteotoxic effects of chemotherapy drugs generally by causing growth plate thinning and/or bone volume reduction, and *in vitro* cell culture studies have reported that chemotherapeutic agents have direct effects on chondrocytes and osteoblasts (Robson, Anderson et al. 1998; Davies, Evans et al. 2002), knowledge on chemotherapy-induced bone damages at cellular/molecular level is unclear. In addition, there is a lack of preventative treatments that can protect bone growth and prevent bone loss during childhood cancer chemotherapy. Therefore, this PhD project, using both acute and chronic rat chemotherapy models using the most commonly used anti-metabolite methotrexate (MTX), firstly examined and compared the effects on the overall structural changes of growth plate and metaphyseal bone following short- and long-term MTX treatments at both low- and high-doses. Cellular/molecular

mechanisms for MTX-induced growth plate and bone damages were also investigated by immunostaining, real time RT-PCR analysis, and analyses on pools of osteoprogenitor cells, osteoclast precursors and osteoclast formation *ex vivo*. Apart from examining the effects locally at the bone/bone marrow, using blood plasma obtained from treated rats, this project further examined potential contribution of systemic regulation on bone loss by examining serum potential of inducing osteoclast formation and levels of osteoclastogenic cytokines by sandwich ELISA technique. In addition, this study also investigated the efficacy in prevention of MTX-induced bone growth defects with folic acid (FA) an antidote clinically used following MTX chemotherapy to ameliorate MTX-associated side effects and toxicities in soft tissues (Ortiz, Shea et al. 1998; Whittle and Hughes 2004). Finally, as limited studies have examined the protective properties of FA in MTX-induced bone growth damages (Iqbal, Ahmed et al. 2003; Xian, Cool et al. 2008; Fan, Cool et al. 2009), the current PhD project also investigated how FA supplementary treatment prevents damages from MTX treatment on the cellular/molecular/systemic levels.

Methotrexate (MTX), an anti-metabolite inhibiting the enzyme dihydrofolate reductase, is commonly used for the treatment of malignancies at high doses (100-1000 mg/m²); while low-dose MTX (5-25 mg/week) is commonly used for the treatment of rheumatoid arthritis (RA) (Minaur, Kounali et al. 2002), used as one of the first-line disease-modifying anti-rheumatic drugs (DMARDs) in both paediatric and adult RA patients (Nagashima, Matsuoka et al. 2006). However, how high vs. low doses of MTX affect bone growth remains unclear. Here, we initially examined whether dose and length of drug exposure may influence MTX toxicity in growing long bones. This study revealed that low-dose MTX treatment in young rats caused no obvious changes in

body weights and bone lengths when compared to control rats (chapter 2). However, in another chronic animal trial from the later high-dose study (chapter 3 and 4), 6-week MTX treatment at high dose caused some body weight and tibial length reduction. This is consistent with our previous publication which reported short-term high-dose MTX administration resulted in shorter tibial bone (Xian, Cool et al. 2007). Histomorphometric analysis revealed that high-dose MTX treatment both acute (0.75mg/kg, 5 days on/9 days off/5 days on) and chronic (0.65mg/kg for 5 days on/9 days off for the induction phase, followed by 1.3mg/kg twice per week for 4 weeks for the maintenance phase) caused severe growth plate and metaphyseal damages. However, chronic low-dose MTX administration (0.4mg/kg for 5 days on/9 days off/5 days on for induction phase, 0.2mg/kg (once weekly from day 20 to week 14 for maintenance phase) resulted in no damaging effects in the growth plate and no significant suppression in primary spongiosa bone heights at the metaphysis. Our results are in consistency with most clinical studies that reported no negative effects on bone density in RA patients receiving low-dose MTX (Salaffi, Carotti et al. 1995; Minaur, Kounali et al. 2002), although a few studies have reported the occurrence of osteopenia as a result of low-dose MTX treatment (Preston, Diamond et al. 1993; Rudler, Pouchot et al. 2003). These results suggest that MTX-induced skeletal toxicity in growing long bones is both dose- and time- dependent.

Despite many clinical studies and some previous animal studies reporting changes in bone density, structure and bone cells, mechanistic studies that have investigated the underlying mechanisms for these histological and cellular damages have been limited. Since bone lengthens through the process of endochondral ossification (Farquharson and Jefferies 2000), in which the production of calcified

cartilage scaffold for bone deposition relies on the regulation of growth plate chondrocyte activities (Hochberg 2002), any disruption of this carefully controlled process will result in bone growth defects. Therefore, to investigate the contribution of chemotherapy-induced growth plate damages to overall skeletal damages and underlying mechanisms, a chronic high-dose MTX chemotherapy trial was conducted for examining the cellular and molecular mechanisms of MTX-induced damages at the growth plate- the main region responsible for longitudinal bone growth. This trial was based on the clinical chemotherapy regime (with intense induction phase of daily treatment, followed by twice weekly administration at a much higher dose) (Powles, Sirohi et al. 2002; Carey, Hockenberry et al. 2007).

Results from our chronic MTX chemotherapy trial revealed significant loss of chondrocytes and the remaining growth plate chondrocytes appeared chaotic in arrangement. Since chondrocytes act as functional units for the growth plate (Farquharson and Jefferies 2000), distortion of chondrocyte columniation and disappearance of chondrocyte as a result of chronic MTX chemotherapy may affect the rate of longitudinal bone growth. To characterize the cellular mechanisms for MTX-chemotherapy-induced chondrocyte damages, BrdU labeling and *in situ* TUNEL labeling were employed for examining chondrocyte proliferation and apoptosis respectively. BrdU labeling revealed no significant changes in chondrocyte proliferation, while *in situ* TUNEL labeling revealed significant induction of chondrocyte apoptosis at lower proliferative and upper hypertrophic zones, where chondrocyte apoptosis is normally not observed. By further examining possible apoptotic pathways involved in MTX-induced chondrocyte apoptosis, quantitative real time RT-PCR was used to examine the mRNA expression of genes involved in death

receptor pathway (Fas, FasL) and mitochondrial dysfunction pathway (Bcl-2, Bax) (Kaufmann and Earnshaw 2000). Molecular analysis revealed more notable induction of Fas and Fas-L expression in comparison to Bcl-2 and Bax during maintenance MTX chemotherapy phase, which is consistent with the findings of one previous acute MTX study (Xian, Cool et al. 2007). However, an induction of anti-apoptotic gene Bcl-2 was also observed in both the current and previous studies (Xian, Cool et al. 2007), suggesting that Bcl-2 is possibly involved in minimizing apoptotic induction during MTX chemotherapy. In addition, as collagen II (major growth plate cartilage protein) serves as a ligand of chondrocyte integrins that mediate chondrocyte proliferation, differentiation and survival, and its level reflects growth plate synthetic activity (Yang, Li et al. 1997; van Leeuwen, Hartel et al. 2003), suppression of collagen-II mRNA expression in this study further support the distortion of chondrocyte columniation and chondrocyte apoptosis as well as a reduced growth plate function as a result of chronic MTX chemotherapy.

Endochondral ossification also involves chondrocyte hypertrophy and apoptosis, followed by remodeling of calcified cartilage into bone (Karsenty and Wagner 2002). This remodeling process involves chondroclasts, osteoblasts and osteoclasts with various cytokines and growth factors (Karsenty and Wagner 2002; Ai-Aql, Alagl et al. 2008). In order to examine whether maintenance MTX chemotherapy can affect the conversion of growth plate calcified cartilage template into bone, TRAP staining was performed for measurement of chondroclasts (cartilage resorptive cells) along the cartilage-bone transitional zone, and histological and μ CT analyses were carried out in metaphysis bone. Chondroclasts were found to be greatly induced during maintenance MTX chemotherapy. In addition, H&E staining and *ex vivo* μ CT analysis revealed

reduction of both primary spongiosa heights and metaphyseal bone volume during maintenance MTX chemotherapy. Together with the growth plate results in this study, data from this study indicate that chronic MTX chemotherapy can induce chondroclast recruitment/formation and resulted in the increased resorption of mineralized cartilage, leading to lower production or delivery of calcified growth plate cartilage feeding into the metaphysis for bone conversion, hence potentially negatively affecting overall bone volume.

To further examine the underlying mechanisms for the reduced bone volume during maintenance MTX chemotherapy, histological and biochemical staining was used to investigate MTX-induced cellular changes. Using H&E staining and *in situ* DNA nick translation (ISNT) technique, it was found that chronic MTX treatment can significantly reduce osteoblast density due to induction of osteoblast apoptosis. However, gene expression examination for MTX-induced cellular damages using quantitative Real Time RT-PCR revealed no changes in expression of osteogenic transcription factor osterix and major osteoblast protein osteocalcin during the maintenance MTX chemotherapy. The unchanged expression in osteogenic genes perhaps indicates that osteogenic potential was not affected during the maintenance phase, but at the intensive early stage of chemotherapy. To further examine whether these cellular damages seen morphologically are related to the treatment effects at their precursor cell levels or at early stages of osteoblastogenesis, an *ex vivo* CFU-f assay with alkaline phosphatase (ALP) was performed with stromal cells isolated from MTX treated rats. This study revealed non-significant changes in the bone marrow osteoprogenitor pool after chronic low-dose MTX treatment. However, a recent study from our lab has demonstrated significant reduction of osteoprogenitor pool after acute

high-dose MTX treatment, in which the damage was recovered by day 14 when histological changes are normalized (Georgiou K and Xian CJ, University of South Australia; manuscript under review by Journal of Cellular Physiology). These findings are consistent with findings from clinical studies which showed that toxicity to stromal progenitor cells caused by chemotherapy (5-FU, epidoxorubicin and cyclophosphamide) is dose-dependent, with possible irreversibly depletion of CFU-f population in some patients receiving high-dose chemotherapy (Banfi A 2001; Davies, Evans et al. 2002). Hence, results from our *in vivo* and *ex vivo* analyses together with previous clinical findings suggest that MTX-induced damages in osteogenic differentiation potential is both dose- and time- dependent, with possible partial, full or no recovery of progenitor/precursor cells depending on the toxicity accumulation/clearance after cessation of MTX administration.

As osteoblasts and adipocytes share a common precursor, one possibility that could be potentially involved in MTX-induced bone loss is the increased marrow adiposity. The inverse relationship between bone volume and fat volume has been shown by several studies, including both age-related osteoporosis (Verma, Rajaratnam et al. 2002) and glucocorticoid treatment (Campbell, Peckett et al. 2011). This study revealed significant increase of bone marrow adipocyte density after chronic MTX treatment, with increased metaphyseal mRNA expression of adipogenic transcription factor PPAR γ . These preliminary findings suggest the possibility that chronic MTX treatment may potentially drive bone marrow MSCs more towards adipocyte differentiation in the expense of osteoblast commitment, a possibility that is yet to be investigated in future studies.

In addition to the damages in stromal cells and osteoblasts, this study also revealed increase in osteoclast density after MTX treatment in metaphyseal bone in a dose-dependent manner. Our results support previous studies that reported chemotherapy-induced bone loss may be due to both decreased bone formation and increased bone resorption (Wheeler, Vander Griend et al. 1995; Michaud and Goodin 2006). *Ex vivo* analysis on osteoclast precursors (OCPs) with CD11b/c monoclonal antibody (a marker for monocytic osteoclast precursors) and osteoclast formation assay using bone marrow cells isolated from MTX-treated rats further support our histological findings. We showed that MTX chemotherapy can cause an increase in bone marrow OCP pool, leading to increased osteoclast formation and hence increased bone resorption, which contributes to MTX-induced bone loss. Similar results were observed clinically for glucocorticoid-induced bone loss, in which bone resorption is caused by RANKL-induced osteoclast formation at the early stage of osteoclast differentiation (Takuma, Kaneda et al. 2003; Hozumi, Osaki et al. 2009). However, in our current study, quantitative Real Time RT-PCR revealed lack of significant changes in RANKL/OPG mRNA expression ratio and expressions of osteoclastogenic cytokines IL-6, TNF and IL-1 in metaphysis, suggesting osteoclastogenesis was affected by signals whose gene expression were not significantly changed locally at the metaphysis during maintenance MTX chemotherapy.

Apart from gene expression examination of osteoclastogenesis-regulatory molecules locally expressed in bone and bone marrow, using sandwich ELISA technique, this study also examined levels of some cytokines present systemically in peripheral blood. We demonstrated significant elevation of IL-1 β , but reduced TNF α and RANKL levels in blood plasma obtained from chronic high-dose MTX-treated rats.

Plasma analysis from this study raises the possibility that chronic MTX chemotherapy-induced bone resorption can be mediated by systemic IL-1 and some other unidentified factors, independent of TNF α or RANKL induction. This theory is supported by one recent study which showed that IL-1 has the potential to induce osteoclast differentiation alone if sufficient levels of IL-1RI (IL-1 receptor 1) were expressed in osteoclast precursors (Kim, Jin et al. 2009). Whether MTX-induced osteoclastogenesis is induced by IL-1 alone, or enhanced by other osteoclastogenic cytokines that were not examined in this study still remain to be investigated.

Skeletal damage resulting from chronic chemotherapy is a long-term complication in which chemotherapy can directly or indirectly affect bone metabolism (Michaud and Goodin 2006). Due to the high success rates in cancer treatment, MTX remains to be an important component of chemotherapy for treating paediatric malignancies particularly acute lymphoblastic leukemia. Since MTX chemotherapy has been clinically associated with osteopenia during and after cessation of chemotherapy in children and adult survivors (Dickerman 2007), despite a decrease in severity with time after chemotherapy, early prevention and treatment are essential to protect bone growth in children during chemotherapy. Hence, this study aimed to examine the potential protective properties of FA supplementary treatment in preventing the cellular/molecular damages of growth plate chondrocytes and bone cells from chronic MTX treatment. Overall, chronic FA supplementation in this study appears to reverse the growth inhibition caused by both low- and high-doses MTX treatment, preserve bone volume by suppressing MTX-induced osteoclast formation and prevent osteoblast damages from MTX. By further investigating the protective action mechanisms of supplementary FA at both the growth plate and metaphysis separately, this study

revealed that at the growth plate, chronic FA supplementation along with high-dose MTX was able to preserve the overall growth plate structure and function by preserving chondrocyte number and mRNA expression of major matrix protein collagen-II. The preservation of chondrocyte number from FA supplementation was possibly due to FA's protective property against MTX-induced chondrocyte apoptosis via suppression of pro-apoptotic molecules (Fas and FasL) involved in the death receptor pathway. Furthermore, FA supplementary treatment was able to suppress MTX-induced chondroclast recruitment/formation, suggesting FA can prevent over-resorption of mineralized cartilage at the vascular invasion zone, hence preserve cartilage-bone conversion and bone lengthening.

Mirroring the protective properties of FA at the growth plate, chronic FA supplementary treatment was also found to preserve the primary spongiosa height and overall trabecular bone volume from MTX treatment as observed by *ex vivo* μ CT analysis. Further *in vivo* cellular analysis revealed that FA supplementation can preserve metaphyseal osteoblast numbers by preventing MTX-induced osteoblast apoptosis, as well as suppressing MTX-induced osteoclast density. While results from cellular analysis are in consistency with previous studies reporting FA supplementary treatment can moderately preserve osteoblast/pre-osteoblast numbers (Xian, Cool et al. 2008), molecular analysis from this study did not support our histological and cellular findings. Real Time RT-PCR from this study revealed chronic FA supplementation did not significantly affect metaphyseal mRNA expression of osteogenic molecules *Osx* and osteocalcin, nor RANKL/OPG mRNA ratio or mRNA expression of osteoclastogenic cytokines (TNF α , IL-1 β and IL-6), indicating local expression of osteogenic and osteoclastogenic signals were not affected significantly during maintenance MTX

chemotherapy phase. Interestingly, blood plasma obtained from the FA+MTX-treated rats was found to contain a lower level of IL-1 β and induce osteoclast formation *ex vivo* to a lesser extent compared to the plasma from the MTX-treated rats, suggesting FA supplementary treatment can suppress MTX-induced osteoclast formation and bone resorption systemically via suppression of circulating osteoclastogenic cytokine IL-1. Moreover, FA supplementation can significantly reduce bone marrow adipocyte differentiation and adiposity (as shown by PPAR- expression data) induced by MTX chemotherapy, indicating FA may potentially preserve bone volume by suppressing the MTX-induced adipocyte differentiation in the bone marrow.

5.2 Conclusions

Using a chronic MTX chemotherapy model at both low- and high-doses in young rats, this study has demonstrated that MTX toxicity in bone is dose- and time-dependent. While acute high-dose MTX treatment was found to significantly damage the growth plate and primary spongiosa, chronic low-dose MTX administration seemed to slightly suppressed formation of primary spongiosa, possibly due to toxicity accumulation. Using a 6-week high dose MTX chemotherapy model, cellular and gene expression analysis revealed that during maintenance chemotherapy phase, MTX-induced growth plate damages are mainly due to the induction of chondrocyte apoptosis, suppression of cartilage protein collagen-II production, and the increased recruitment of chondroclasts. At the metaphysis, MTX-induced bone volume reduction is mainly due to the reduction of osteoblasts, increase of osteoclasts and adipocytes. The osteoblastic damage is mainly caused by the induction of apoptosis, while osteoclastic resorption was mediated systemically by factors including IL-1 β within circulating plasma. Molecular analysis revealed lack of changes in osteoclastogenic signals locally

in bone, while osteogenic potential was increased during maintenance MTX chemotherapy phase, suggesting an initiation of recovery mechanism following the intense induction treatment phase. Due to the skeletal morbidities associated with MTX chemotherapy in long-term young survivors, it is important to develop strategies that will minimize the risk of these complications while maintaining the high cure rates. In this study, chronic FA supplementary treatment with MTX was shown to reverse MTX-induced skeletal damages, not only by protecting the growth plate from MTX-induced chondrocyte apoptosis and chondroclast recruitment, but also preserves metaphyseal bone volume by preventing osteoblasts from undergoing apoptosis, and suppressed osteoclastogenic cytokine IL-1 systemically. Based on these observations, folinic acid supplementation should be able to ameliorate MTX-associated bone growth suppression and skeletal toxicities in paediatric patients receiving chronic MTX chemotherapy.

5.3 Future Directions

Overall, histological analyses from this study suggested that chronic MTX-induced metaphyseal damages are probably mainly caused by the earlier intensive induction MTX treatment, in which the damages persist into the maintenance phase. Our real time RT-PCR analyses did not revealed obvious molecular damages during maintenance MTX chemotherapy, but an increase in osteogenic potential, which suggested an initiation of recovery mechanism during the less intensive maintenance chemotherapy, in order to compensate for the damaged bone environment. However, having only one time point during maintenance phase for such a hypothesis is a limitation for this study. For a clearer understanding of molecular damages and recovery mechanism during and after chronic MTX chemotherapy, a time-course (including induction, maintenance and recovery phases) analysis of changes in gene

expression of metaphysis may be required. Furthermore, more animals should be included at the start of trial in anticipation of death from MTX toxicity.

Recent research has discovered the viability or apoptosis of the “quiescent” bone cells “osteocytes” play a significant role in bone regulation, particularly in bone remodeling (Gu, Mulari et al. 2005; Heino, Kurata et al. 2009). Previous studies have shown that osteocyte apoptosis caused by micro-damage to the bone in a rat model was able to induce osteoclastic recruitment to the damaged site (Seeman 2006). *In vitro* study also revealed the ability of apoptotic osteolytic MLO-Y4 cells to initiate osteoclast formation and recruitment (Kogianni, Mann et al. 2008). Hence, it may be possible that chemotherapy-induced bone resorption may also be caused by osteocyte apoptosis. Recent research revealed glucocorticoid treatment can promote osteoblast and osteocyte apoptosis, and prolong osteoclast survival (O'Brien, Jia et al. 2004; Kerachian, Seguin et al. 2009). However, whether MTX chemotherapy-induced osteoclast formation is triggered by osteocyte apoptosis remains to be investigated.

Cellular and molecular analysis from this study revealed chronic MTX administration in young rats can significantly induce chondrocyte apoptosis, as well as recruiting more chondroclasts for cartilage resorption, resulting in bone loss. However, whether chondrocyte apoptosis is molecularly linked with chondroclast recruitment remains unknown. Growth plate hypertrophic chondrocytes have been shown to express OPG and RANKL (Silvestrini, Ballanti et al. 2005), which supports chondroclast differentiation by a mechanism similar to osteoblast-dependent osteoclastogenesis (Ota, Takaishi et al. 2009). Hence, it is possible that chemotherapy-induced growth plate apoptotic chondrocytes may potentially affect the secretion of these factors that mediate

the recruitment of chondroclasts. Analysis of gene expression together with functional confirmation analyses of RANKL, OPG, major matrix metalloproteinase (MMP)-9 (a gelatinase mainly expressed in osteoclasts/chondroclasts) and other molecules involved in chondroclast/osteoclast migration and differentiation in MTX-treated rats compared to normal rats would increase our understanding of the molecular changes and potential association between chondrocyte apoptosis and chondroclast recruitment.

Finally, histological analysis and gene expression of PPAR γ of metaphysis revealed that FA supplementary treatment can significantly reduce adipocyte differentiation and bone marrow adiposity from chronic MTX chemotherapy. However, the mechanisms of how FA prevents adipocyte differentiation in the bone marrow remain unclear. It is known that bone marrow MSCs have the potential to differentiate into different lineages, including osteoblasts and adipocytes. As osteoblasts and adipocytes share a common precursor, regulation of differentiation down either pathway can influence bone volume and marrow fat volume (Takada and Kato 2008). Future studies will need to investigate whether FA supplementary treatment can prevent MTX-induced bone loss by preventing the switch in the mesenchymal progenitor cell commitment more towards adipogenic differentiation, and away from osteogenic differentiation. Using bone marrow stromal cells collected from treated rats, the differentiation ability of MSCs can be examined *ex vivo* via mineralization assay for assessing osteogenic potential, or adipogenic assay for assessing adipogenic potential. Furthermore, it is known that Wnt signaling pathway plays a critical role in regulating bone or fat formation (Gimble, Zvonic et al. 2006). Previous studies have identified the importance of Wnt signaling in promoting osteoblastogenesis, while inhibiting adipogenesis (Ross, Hemati et al. 2000; Bennett, Longo et al. 2005). However, it

remains to be investigated whether altered Wnt signaling is involved in MTX-induced bone-to-fat switch, and if this switch can be inhibited following FA supplementary treatment for prevention of MTX-induced bone loss and marrow fat accumulation. As a step to examine the potential involvement of Wnt signaling in chronic MTX chemotherapy and with FA supplementation, RT-PCR gene expression analysis will need to be carried out in the future to quantify expression of Wnts, receptors, inhibitors and target genes and to examine whether altered Wnt/ β -catenin signaling is present in MTX-treated bone and whether FA supplementation can prevent this alteration.

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