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

A THESIS SUBMITTED IN TOTAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY

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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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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-1β	Interleukin-I beta
IL-6	Interleukin-6
IL-11	Interleukin-11
ISNT	*in situ* 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-κB	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
PPARγ	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
μCT	Micro-computed tomography