

Programming of Bone and Reproductive Health by Early Life Exposure to Soy Isoflavones and Folic Acid in CD-1 Mice

by

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Nutritional Sciences
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ABSTRACT

Previous studies showed that exposure to soy isoflavones (ISO) during the first 5 days of life improves bone mineral density (BMD), structure and strength of the lumbar spine in CD-1 female mice at adulthood. In continuing this research, a first step was to optimize the CD-1 mouse model. As such, the first two studies confirmed that once-daily subcutaneous exposure to ISO results in total serum ISO levels that mimic those of infants fed soy protein formula, and that exposure for the first 10 rather than 5 days of life is needed to improve bone outcomes in vertebra as well as femurs. I hypothesized that early life exposure to ISO was improving adult bone outcomes by altering expression of genes that modulate bone development, possibly via DNA methylation, and that providing a methyl donor such as folic acid (FA) to the maternal diet during pregnancy and lactation could enhance the positive effects of ISO. Female and male CD-1 mice exposed to an adequate level of FA and ISO had higher BMD, improved trabecular connectivity and greater resistance to fracture compared to mice not exposed to ISO. In contrast, exposure to supplemental levels of FA in combination with ISO did not result in improved bone outcomes. Exposure to adequate levels of FA and ISO lowered neuropeptide Y (NPY) gene

expression by 5.7-fold, while exposure to supplemental levels of FA and ISO up-regulated the expression of beta-catenin (Ctnnb1) and parathyroid hormone receptor (PTHr1) gene by 1.6- and 1.7-fold, respectively, than mice exposed to adequate FA without ISO. NPY is a neurotransmitter which when suppressed in osteoblasts promotes bone formation while Ctnnb1 and PTHr1 are modulators of bone resorption. Thus, by inducing long-term changes in gene expression, FA and ISO may improve bone development. In contrast, reproductive health was adversely affected and identified the first 5 and 10 days of life as a sensitive window of development during which exposure to ISO can alter structural development of ovaries and uterus. In conclusion, early life exposure to ISO and FA can favourably modulate bone development but other hormone-sensitive tissues are adversely affected in this model.

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TABLE OF CONTENTS

Abstract.....	ii
Acknowledgements.....	iv
Table of Contents.....	vii
List of Figures.....	x
List of Tables	xii
Contributions	xiv
List of Abbreviations	xv
Summary of Outcomes Used in Human or Animal Studies to Understand How Bone Responds to a Dietary Intervention	xiv
1.0 INTRODUCTION	2
2.0 LITERATURE REVIEW	7
2.1 Soy protein formula	7
2.2 Soy isoflavones.....	8
2.2.1 Metabolism	11
2.3 Growth and development of infants fed soy protein formula.....	15
2.4 Animal models being used to study biological effects of ISO	23
2.5 Bone development of animals exposed to ISO.....	27
2.5.1 Prenatal exposure to ISO	28
2.5.2 Neonatal exposure	34
2.5.3 Adolescent exposure to ISO	38
2.5.4 Summary.....	39
2.6 Potential mechanisms	40
2.6.1 Epigenetic regulation.....	42
2.6.2 Altered endocrine environment	47
2.7 Fertility and reproductive health of animals exposed to ISO	52
2.8 Research Approach.....	57
2.8.1 Isoflavone metabolism.....	57
2.8.2 Bone development	60
2.8.3 Reproductive health.....	61
3.0 OBJECTIVES AND HYPOTHESES	63
3.1 Objectives	63
3.2 Hypotheses.....	65
4.0 STUDY 1 - Circulating isoflavone levels in CD-1 mice: effect of oral versus subcutaneous delivery and frequency of administration	69
4.1 Abstract.....	69
4.2 Introduction.....	70
4.3 Methods	71

4.3.1	Animals and treatment.....	71
4.3.2	Serum measures of ISO metabolism	73
4.3.3	Statistical analyses	73
4.4	Results.....	74
4.4.1	Serum measures of soy isoflavone metabolism.....	74
4.5	Discussion.....	77
5.0	STUDY 2 – Isoflavone exposure throughout suckling results in improved adult bone health in mice.....	83
5.1	Abstract.....	83
5.2	Introduction.....	83
5.3	Methods	85
5.3.1	Animals and treatment.....	85
5.3.2	BMD of femurs and LV1-LV3.....	87
5.3.3	Biomechanical strength properties of femurs and LV2.....	87
5.3.4	Microarchitecture of femur and LV4.....	87
5.3.5	Statistical analyses	88
5.4	Results.....	88
5.4.1	Body weight.....	88
5.4.2	BMD of intact LV1-3, microarchitecture of LV4 and peak load of LV2	88
5.4.3	BMD of whole femur, microarchitecture of femur neck, and peak load of femur neck and midpoint.....	92
5.5	Discussion.....	92
6.0	STUDY 3: Bone-specific gene expression patterns and whole bone tissue are programmed by early life exposure to soy isoflavones and folic acid in the developing female CD-1 mouse model	98
6.1	Abstract.....	98
6.2	Introduction.....	99
6.3	Methods	102
6.3.1	Animals and treatment.....	102
6.3.2	BMD of femurs and LV1-LV3.....	107
6.3.3	Biomechanical strength properties of femurs and LV2.....	108
6.3.4	Microarchitecture of femur and LV4.....	108
6.3.5	Serum measures of bone turnover and metabolism.....	109
6.3.6	Illumina chip-based gene expression.....	109
6.3.7	Bioanalyses of Illumina chip-based gene expression	110
6.3.8	Statistical analyses	111
6.4	Results.....	111
6.4.1	Body weight.....	111
6.4.2	Organ weight	112
6.4.3	Anthropometry of the femur.....	112
6.4.4	BMD of intact LV1-3, and peak load of LV2	117
6.4.5	BMD of whole femur, and peak load of femur neck and midpoint.....	117
6.4.6	Microarchitecture of femur and LV4.....	117
6.4.7	Serum measures of bone turnover and metabolism.....	120
6.4.8	Microarray analyses of the femur	120

6.5	Discussion.....	125
7.0	STUDY 4: Adequate but not supplemental folate combined with soy isoflavones during early life improves bone health at adulthood in male mice	138
7.1	Abstract.....	138
7.2	Introduction.....	139
7.3	Methods	143
7.3.1	Animals and treatment.....	143
7.3.2	BMD of femurs and LV1-LV3.....	145
7.3.3	Biomechanical strength properties of femur and LV2	145
7.3.4	Biochemical Analyses	146
7.3.5	Statistical analyses.....	146
7.4	Results.....	146
7.4.1	Body weight.....	146
7.4.2	Organ weight	148
7.4.3	BMD of intact LV1-LV3 and peak load of LV2	148
7.4.4	BMD of whole femur, and peak load of femur neck and femur midpoint	148
7.4.5	Serum measures of bone turnover	151
7.5	Discussion.....	151
8.0	STUDY 5 - Early life exposure to genistein and daidzein disrupts structural development of reproductive organs in female mice.....	159
8.1	Abstract.....	159
8.2	Introduction.....	160
8.3	Methods	162
8.3.1	Animals and treatment.....	162
8.3.2	Histology	164
8.3.3	Statistical analyses.....	164
8.4	Results.....	165
8.4.1	Litter size, body weight and relative organ weight	165
8.4.2	Ovary and oviduct	165
8.4.3	Uterus and cervix.....	170
8.5	Discussion.....	170
9.0	OVERALL DISCUSSION	178
9.1	Introduction.....	178
9.2	Major research findings	178
9.3	ISO-induced effects in CD-1 mice mirror those of humans with precocious puberty: Could there be a link?.....	189
9.4	Potential for transgenerational inheritance	193
9.5	Strengths and weaknesses.....	194
10.0	CONCLUSIONS	199
10.1	Future research.....	203
11.0	REFERENCES	205

LIST OF FIGURES

Figure 2-1. Chemical structures of 17 β -estradiol, ISO (genistein, daidzein, glycitein) and their secondary metabolites (equol, O-DMA).	10
Figure 2-2. Summary of ISO metabolism.....	12
Figure 2-3. Representative microcomputed tomography images of LV4, femur neck and femur midpoint from 4-month old females who in the first 5 days of life were treated with control (CON), daidzein (DAI), genistein (GEN), daidzein + genistein (DAI+GEN) or diethylstilbestrol (DES).	37
Figure 2-4. A schematic showing that early life exposure to ISO can alter phenotypic expression by changing epigenetic regulation and/or endocrine environment. ..	41
Figure 2-5. Schematic diagram of ER signaling.	49
Figure 2-6. Summary of the research approach used to characterize ISO metabolism and study the effects on bone and reproductive development in CD-1 mice.	58
Figure 4-1. Serum concentrations of genistein (A), daidzein (B), equol (C) and O-DMA (D) from female mouse pups one-hour after corn oil (CON) or equal doses of ISO were administered subcutaneously once a day, orally once a day or orally every 4 hours.....	75
Figure 4-2. Serum concentrations of genistein (A), daidzein (B), equol (C) and O-DMA (D) from male mouse pups one-hour after corn oil (CON) or equal doses of ISO were administered subcutaneously once a day, orally once a day or orally every 4 hours.....	76
Figure 5-1. Representative microcomputed tomography images of the lumbar vertebrae (LV4) and femur neck.	90
Figure 5-2. Relationship of bone volume/total volume and (A) stiffness or (B) fracture load of female mice exposed to corn oil or ISO.....	91
Figure 6-1. Study design.	104
Figure 6-2. Body weight from weaning through 16 weeks of age of female mice whose mothers' were fed low (A), adequate (B) or supplemental (C) FA during pregnancy and lactation, and who from postnatal day 1 to 10 were treated with subcutaneous injections of corn oil or ISO.	114
Figure 6-3. BMD and peak load of lumbar vertebrae (A) and femur (B) from 4-month old female CD-1 mice whose mothers' were fed a low, adequate or supplemental FA during pregnancy and lactation, and who from postnatal day 1 to 10 were treated with subcutaneous injections of corn oil or ISO.....	118

Figure 6-4. The OPG/RANKL ratio measured from serum of 4 month old female CD-1 mice whose mothers' were fed a low, adequate or supplemental FA during pregnancy and lactation, and who from postnatal day 1 to 10 were treated with subcutaneous injections of corn oil or ISO.	122
Figure 6-5. Expression profile and clustering of 949 signature genes that were identified to be statistically different between the groups.	123
Figure 6-6. Venn Diagram showing the number of genes that are up- or down-regulated in femurs of 6-week old females whose mothers were fed a diet containing either adequate or supplemental levels of FA during pregnancy and lactation and were treated with either corn oil or ISO treatment from postnatal day 1 to 10.	124
Figure 6-7. Mechanism used to describe how early life exposure to adequate FA+ISO, supplemental FA and supplemental FA+ISO programs bone development of female CD-1 mice at adulthood.	130
Figure 6-8. Effects of adequate FA+ISO on peripheral regulation of bone and fat mass by NPY.	135
Figure 7-1. The ratio of OPG to RANKL measured in serum of 4 month old male CD-1 mice whose mothers' were fed a low, adequate or supplemental level of FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO.	153
Figure 8-1. Body weight from weaning to 4 months of age of mice treated for the first 5 or 10-days of life with corn oil or ISO.	166
Figure 8-2. Representative microphotographs of ovary and oviduct from 4 month old mice treated with corn oil or ISO.	169
Figure 8-3. Representative microphotographs of uterus and cervix from 4 month old mice treated with corn oil or ISO.	171
Figure 9-1. Summary of Study 2 through 5 that have characterized the effects of 5, 10 and 21-day exposure to ISO on bone and reproductive outcomes in female and male CD-1 mice at adulthood.	181
Figure 9-2. Summary of effects and mechanisms of supplemental FA, ISO and supplemental FA+ISO on bone development in female CD-1 mice.	182
Figure 9-3. Summary of effects and mechanisms of supplemental FA, ISO and supplemental FA+ISO on bone development in male CD-1 mice.	183

LIST OF TABLES

Table 2-1. Comparison of serum or plasma ISO levels of infants and adults	9
Table 2-2. Summary of infant feeding studies: Effects of soy protein formula on bone and reproductive development	16
Table 2-3. Summary of animal studies: Effects of early life exposure to soy or ISO on bone development	29
Table 2-4. Summary of animal studies: Effects of early life exposure to soy or ISO on reproductive health.....	53
Table 5-1. Summary of ingredients in AIN-93G purified rodent diet.....	86
Table 5-2. BMD, peak load and trabecular bone parameters at the spine and femur with 21-day ISO exposure.....	89
Table 6-1. Summary of ingredients in L-amino acid rodent diet	105
Table 6-2. Body weight and total weight gain of 4 month old female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO.....	113
Table 6-3. Organ weights expressed as percent of body weight from 4 month old female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO	115
Table 6-4. Anthropometry of femur from 4 month old female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO.....	116
Table 6-5. Trabecular bone parameters of LV4 and femur neck from female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO	119
Table 6-6. Serum markers of bone turnover and metabolism measured in 4 month old female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil.....	121
Table 6-7. List of differentially expressed gene categorized according to gene ontology	126

Table 6-8. List of differentially expressed genes in mouse clones that help to explain the effects induced by adequate FA+ISO, supplemental FA and supplemental FA+ISO on bone programming	127
Table 7-1. Body weight and total weight gain of 4-month old CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO	147
Table 7-2. Organ weights expressed as percent of body weight from 4-month old CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO	149
Table 7-3. BMC, BMD and peak load of lumbar vertebrae and femur excised from 4-month old male CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO	150
Table 7-4. Serum markers of metabolism measured in 4-month old male CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO	152
Table 8-1. Organ weights from 4-month old female mice expressed both as percent of body weight and as absolute	167
Table 8-2. Histological abnormalities of ovaries, oviduct, uteri and cervix of CD-1 mice at 4 months of age	168
Table 9-1. Signs of precocious puberty known in infants and observed in CD-1 mice	191

CONTRIBUTIONS

Jovana Kaludjerović provided intellectual contribution to the study designs, was responsible for conducting the *in vivo* portion of all the experiments, performing many of the analyses and overseeing all the daily tasks. She also interpreted the results, performed all the statistical analyses and wrote the first draft of each manuscript and incorporated feedback from other authors. The following list of people, in addition to Professor Ward, assisted Jovana and contributed to the work included in this thesis.

Sarah Jones – Veterinary Technologist, Division of Comparative Medicine, University of Toronto

- Monitored mice daily and provided routine husbandry care (Study 1 through 5)

Adrian Franke – Professor, University of Hawaii Cancer Center

- Measured serum daidzein, genistein, equol and O-DMA concentrations via high-pressure liquid chromatography electrospray tandem mass spectrometry (Study 1)

Elsa Dinsdale – MSc Student, Department of Nutritional Sciences, Faculty of Medicine, University of Toronto

- Assisted in preparing and administering the 21-day ISO treatment to CD-1 mice, feeding the mice, and collected the bi-weekly body weight data (Study 2)

Julissa Tsao – Scientist, University Health Network Microarray Centre

- Performed the microarray experiments (Study 3)

Carl Virtanen - Bioinformatics Manager, University Health Network Microarray Centre

- Performed the microarray data analyses (Study 3)

Jianmin Chen - Research Associate, Department of Nutritional Sciences, Faculty of Medicine, University of Toronto

- Performed morphological and immunohistochemical analyses of ovary and uteri (Study 5).

LIST OF ABBREVIATIONS

ALP – alkaline phosphatase	Tb.N. – trabecular number
BMC – bone mineral content	Tb.Sp. – trabecular separation
BMD – bone mineral density	Tb.Th. – trabecular thickness
BMP – bone morphogenetic protein	
BV/TV – trabecular bone volume	
BS/BV – trabecular surface area	
CON – control	
Ctnnb1 – beta-catenin	
CTX – collagen crosslinks	
DES – diethylstilbestrol	
FA – folic acid	
IGF-I – insulin-like growth factor-I	
ISO – soy isoflavones	
NPY – neuropeptide Y	
LV – lumbar vertebrae	
O-DMA – 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-propan-1-one	
OPG – osteoprotegrin	
PND – postnatal day	
PTH – parathyroid hormone	
PTHr1 – PTH receptor 1	
RANK – receptor activator for nuclear factor β	
RANKL – RANK ligand	

SUMMARY OF OUTCOMES USED IN HUMAN OR ANIMAL STUDIES TO UNDERSTAND HOW BONE RESPONDS TO A DIETARY INTERVENTION

Outcome of Bone Health	Model	What information the outcome measure provides
Bone mineral density (BMD) by dual energy x-ray absorptiometry (DXA)	Human/ Animal	▪ Measures the bone mineral content (BMC) of the region of interest. ▪ BMD is a derived measurement: $BMD = BMC (g)/area (cm^2)$. ▪ There is much discussion that BMD may not be an ideal measure of fracture risk but it is the most widely used measure for assessing fracture risk in humans. ▪ Low exposure to radiation.
BMD by ultrasound	Human	▪ Non-invasive technique. ▪ How measurements of BMD by ultrasound relate to BMD measurements obtained by DXA and ultimately to risk of fragility fracture is an ongoing area of study.
Histomorphometry	Mostly Animal	▪ Measures the levels of bone cell activity (osteoblast, osteoclasts), which in turn can provide a direct measure of bone formation and resorption rate. ▪ In humans, histomorphometrical analysis can be performed on samples collected through a bone biopsy, an invasive procedure.
Micro-computed tomography (animals) or quantitative computed tomography (humans)	Human/ Animal	▪ Provides quantitative assessment of trabecular network and cortical bone as well as a three-dimensional image of bone structure at key skeletal sites (i.e. lumbar vertebrae, femur). ▪ In animals, excised bones are most often analyzed but the development of a new in vivo system allows for analyses of live animals. ▪ In humans, this measure is limited to extremities (i.e. forearm, ankle) to ensure a minimal exposure to radiation. ▪ Terms used to describe 3D outcomes of trabecular bone microarchitecture: ○ <u>bone volume fraction (BV/TV)</u> – ratio of the segmented bone volume to the total volume of the region of interest ○ <u>trabecular thickness (Tb.Th.)</u> – mean thickness of trabeculae, assessed using direct 3D methods ○ <u>trabecular number (Tb.N.)</u> – measure of the average number of trabeculae per unit length ○ <u>trabecular separation (Tb.Sp.)</u> – mean distance between trabeculae, assessed using direct 3D methods

Biochemical markers of bone turnover	Human/ Animal	- Measured in serum, plasma or urine, depending on the specific marker studied. There are two types of markers: <u>Bone formation markers include</u>: alkaline phosphatase (ALP), osteocalcin, type 1 procollagen <u>Bone resorption markers include</u>: Deoxypyridinoline, N-telopeptide of type 1 collagen, pyridinoline, tartrate-resistant alkaline phosphatase
Incidence of fragility fracture	Human	- Mostly used in studies evaluating drug interventions that are at least 2 years in duration. - Not an ideal outcome measure as the goal is to prevent fragility fractures from occurring.
Biomechanical strength testing	Animal	- Direct measure of bone fracture - Common tests and sites: <u>compression test of an individual lumbar vertebra</u> – mimics a compression fracture in humans <u>femur neck</u> – mimics a hip fracture in humans <u>femur midpoint</u> – not a common site of fragility fracture in humans but does provide information about cortical bone which predominates at this site - Each of these tests is destructive.

This table has been modified from Ward et al. 2010 [1].

Chapter One

INTRODUCTION

1.0 INTRODUCTION

Dietary estrogens called soy isoflavones (ISO) share a common phenolic ring and a 4'-hydroxyl group with 17 β -estradiol that allows them to bind to estrogen receptors and selectively stimulate or inhibit estrogen responses in many tissue including bone, adipose tissue, ovaries and uteri [2]. As a result, ISO may interfere with epigenetic programming, hormonal signaling and/or production of enzymes and transcription factors [3, 4]. These changes may induce irreversible effects on many physiological processes (i.e. growth, metabolism, stress response, sex development, behavior, ability to reproduce) if exposure occurs during sensitive stages of development. This is because endogenous concentrations of sex steroids are low in early postnatal life [5, 6] so ISO can more easily bind to estrogen receptors and induce estrogen-like effects.

In early life, infants fed commercially available soy protein formula have markedly higher serum ISO (10 fold higher) and FA (2.3 fold higher) levels than infants fed breast milk or cow milk formula [7-9]. In addition, due to mandatory fortification of the food supply and widespread use of FA supplements prior to and/or during pregnancy a portion of these infants may have markedly higher concentrations of FA in utero as 40% of North Americans have higher than recommended red blood cell folate concentrations (>1360 nmol/L) [10]. No clinical studies have evaluated the potential benefits or adverse effects of early life exposure to soy protein formula at adulthood. The need to characterize how early life exposure to a supplemental level of ISO and FA affects growth, development and future health has led to five studies presented in this thesis.

The first two studies, focused on optimizing the CD-1 mouse model to closely mimic that of human infants fed soy protein formula. The first study showed that female and male neonatal mice exposed to ISO via subcutaneous and oral dosing, once daily and every four

hours at an equivalent total daily dose have similar total ISO concentrations as infants fed soy protein formula (Study 1, Chapter 4). The second study analyzed the effects of ISO duration (5 versus 21 days of life) on BMD, bone structure and bone strength (Study 2, Chapter 5) and showed that the first 10-days of life provides a sensitive window of development during which ISO programs bone. Thereafter, with the optimized CD-1 mouse model, we investigated whether early life exposure to a supplemental level of FA and ISO provides greater benefits to bone development than exposure to adequate FA and ISO or supplemental FA alone in female (Study 3, Chapter 6) and male (Study 4, Chapter 7) CD-1 mice. The use of a comprehensive set of outcome measures including serum markers of bone metabolism (i.e. OPG, RANKL, osteocalcin, IGF-I and IGFBPs), BMD, bone structure, and bone strength at multiple skeletal sites that differ in the proportion of cortical and trabecular bone offered a robust analysis of treatment-induced effects on skeletal development. To better understand the underlying mechanism(s) driving the observed bone phenotypes, a microarray was performed to identify genes that are differentially expressed by each treatment. The use of gene ontology allowed us to identify gene candidates that may help to explain how early life exposure to adequate FA+ISO, supplemental FA and supplemental FA+ISO programs bone development. Lastly, to explore whether early life exposure to ISO has adverse effects on reproductive health we examined how early life exposure to ISO for the first 5 and 10-days of life versus corn oil affects the weight and structural development of ovaries and uteri of female CD-1 mice at adulthood (Study 5, Chapter 8).

Our published studies have shown that CD-1 mice exposed to ISO, at levels similar to that of infants consuming soy protein formula, during the first 5 days of life have higher

BMD, improved trabecular connectivity and greater resistance to fracture at 4 months of age, which is the time when peak bone mass is established in this mouse strain [11, 12]. In addition, the higher BMD, improved trabecular connectivity and greater resistance to fracture observed at adulthood were shown to attenuate deterioration of bone tissue after cessation of endogenous sex steroid production [13]. Therefore, it is possible that early life exposure to ISO provides a prevention strategy for osteoporosis. The overall objective of my PhD thesis was to delineate the mechanism(s) by which early life exposure to ISO improves bone outcomes at adulthood, while considering the potential adverse effects on reproductive health. Studies have shown that exposure to endogenous and environmental estrogens in early life alters epigenetic regulation [14]. Based on these studies, we hypothesized that through an estrogen receptor-mediated mechanism, ISO stimulate DNA methylation of cytosine-phosphodiester-guanine (CpG) sites in the promoter of bone specific genes. It is proposed that this stimulation results in increased bone formation.

To study the effects of nutrients and food components on DNA methylation the viable yellow agouti mouse is a commonly used animal model. In one study, offspring of agouti dams fed a diet rich in genistein – the most abundant ISO in soy protein - during pregnancy and lactation had reduced expression of the imprinted agouti gene due to increased methylation of CpG sites in a retrotransposon upstream of the transcription start site. The reduction in agouti gene expression was associated with a shift in coat color from yellow to dark brown and lower body weight [15]. Interestingly, these changes in coat color and body weight in agouti mice can also be imprinted by early life exposure to FA [16, 17]. From a molecular perspective, neither ISO nor FA acts directly on genes. Instead, they induce effects on developmental mechanisms that integrate genetic and epigenetic

interactions and drive phenotypic changes. According to in vitro data, through an estrogen receptor- α and - β mediated mechanism, ISO enhance core histone acetylation [18], which helps to loosen up the tightly packaged DNA around the gene promoters. These biochemical changes recruit various transcription factors and allow methyl donors such as FA to more easily donate a one-carbon group to the cytosine residue – a hallmark of DNA methylation. Thus, it is possible that administering ISO and FA in combination may have additive or synergistic effects on DNA methylation and programming of health including bone growth and development. To date, only one rodent study has combined ISO and FA intervention during early life. Male and female rats of dams fed a diet fortified with ISO and FA during pregnancy had reduced DNA damage and neuron apoptosis in the brain, and lower frequency of post-neural tube defects after exposure to cyclophosphamide (chemical that prevents neural tube closure in rodents) than either treatment alone [19]. These effects may in part be mediated through an epigenetic mechanism. No studies have investigated how combining supplemental levels of FA and ISO affects bone development. However, such findings may help to delineate whether early life exposure to ISO improves bone development through an epigenetic or an endocrine mechanism.

The overall approach undertaken in this thesis allowed us to generate a better understanding of how early life exposure to ISO improves bone and affects structural development of ovaries and uteri of CD-1 mice. No randomized controlled trials have evaluated potential benefits or adverse effects of soy protein formula at adulthood and thus, the available human data are based on extrapolations from short-term observation studies. Research presented in this thesis provides a basis for designing and conducting future prospective studies.

Chapter Two

LITERATURE REVIEW

Modified from:

1. **Kaludjerovic J**, Ward WE. Soy: Animal studies spanning the lifespan *In: Bioactive foods and chronic disease states*. Ronald, R. Watson (eds). Elsevier Ltd. Oxford, UK. 2013. In press.
2. Ward WE, Lau B, **Kaludjerovic J** & Sacco SM. Functional foods and bone health: Where are we at? *In: Functional Food Product Development*, E. Charter, J. Smith (eds), Wiley-Blackwell Publishers. Malden, MA. 2010. pg. 459-503.
3. **Kaludjerovic J**. Neonatal exposure to environmental or dietary estrogens programs bone metabolism in CD-1 mice. 2008. UMI ProQuest Dissertations & Thesis. [<http://gradworks.umi.com/MR/45/MR45167.html>]

2.0 LITERATURE REVIEW

2.1 Soy protein formula

Maternal breast milk is the ideal source of nutrition for the developing infant because it contains an optimal amount of essential proteins, fats, minerals, vitamins, hormones, enzymes and immune modulators. Since some of these components cannot be recreated artificially, the World Health Organization and UNICEF recommend that all infants be exclusively breastfed for the first 6 months of life [20, 21] with complementary breastfeeding up to 2 years of age or beyond [21]. Despite these recommendations, approximately 65% of infants worldwide are fed either cow milk formula or soy protein formula within the first 6 months of life [21].

Soy protein formula was first available in the late 1920s as a dietary substitute for infants with an allergy or an intolerance for cow milk protein [22]. To this day, the American Academy of Pediatrics recommends soy protein formula as a safe and effective alternative for providing adequate nutrition to term infants whose nutritional needs are not being met by maternal breast milk because of an intolerance or a proven allergy to cow-milk protein [23]. Despite these recommendations, many women are selecting soy protein formula for their healthy infants because they perceive soy as being a source of “high-quality protein” that is a healthier option [24].

The use of soy protein formula varies on a global scale. The percentage of infants consuming soy protein formula within the first year of life is approximately 30% in Israel [25], 25% in North America [23], 13% in New Zealand, 7% in United Kingdom and 5% in Italy [26]. The ISO content of soy protein formula range from 32 to 47 mg (67.1% genistein and 28.7% daidzein) per liter of formula [8]. Thus, infants fed soy protein formula in the first 6-months of life are exposed to approximately 50 mg of ISO daily. Per kg body weight, this

ISO intake exceeds that of adults consuming a moderate to large amount of soy in their diet by 10 fold [7, 8]. The daily intakes of infants fed cow milk formula (3.2 mg of genistein and 2.1 mg of daidzein per liter of formula) or breast milk (2.8 mg of genistein and 1.4 mg of daidzein per liter of breast milk) are negligible [8]. Therefore, infants fed soy protein formula as their primary source of nutrition are exposed to a much higher level of ISO on a body weight basis than any other population group (Table 2-1).

2.2 Soy isoflavones

ISO are plant-derived phytochemicals produced from an essential amino acid (L-phenylalanine) to help the plant inhibit pathogen attacks and mediate plant-microbe symbiotic interactions [27-29]. As such, ISO are synthesized in response to environmental stresses such as pest infection, drought or lack of nutrients [27, 30]. The most abundant and biologically active food source of ISO are legumes, such as soybean, which contain the glycoside (genistin, daidzin and glycitin) and aglycone [genistein, daidzein and glycitein] form of ISO [31]. The total ISO composition varies profoundly between different soybean products. This is in part because ISO migrate with the protein fraction during soybean processing and thus, foods rich in soy protein contain higher amounts of ISO than foods rich in soy oil or soy lecithin [7]. The ISO composition of soy protein includes genistein (58-67%), daidzein (29-34%) and glycitein (5-8%) [32]. These compounds share a common phenolic ring and a 4'-hydroxyl group with 17 β -estradiol, allowing them to bind to estrogen receptors and selectively stimulate or inhibit estrogen-like responses in many tissues (Figure 2-1) [2]. The metabolite of daidzein, called equol, lacks the ketone group on the phenolic ring and more closely resembles the chemical structure of 17 β -estradiol than any other ISO (Figure 2-1) [33]. Thus, equol has the highest estrogenicity followed by genistein, daidzein and glycitein.

Table 2-1. Comparison of serum or plasma ISO levels of infants and adults

Population	Reference	Sample	Average total ISO concentration	
			Genistein (ng/ml)	Daidzein (ng/ml)
Infants fed:				
Breast milk	Setchell et al [8]	Plasma (n=7)	2.8 ± 0.7	1.4 ± 0.3
Cow milk formula	Setchell et al [8]	Plasma (n=7)	3.2 ± 0.7	2.1 ± 0.1
Soy protein formula	Setchell et al [8]	Plasma (n=7)	684.0 ± 443.0	295.0 ± 60.0
Adults:				
Vegans/vegetarians	Peeters et al [34]	Plasma (n=70)	148.0 ± 40.0	78.7 ± 20.0
Vegetarians	Adlercreutz et al [35]	Plasma (n=14)	17.1 ± 4.6	18.5 ± 4.7
Asian women at delivery	Todaka et al [36]	Serum (n=51)	26.6 ± 7.2	7.1 ± 1.8
Asian cord serum at delivery	Todaka et al [36]	Serum (n=51)	71.8 ± 19.4	16.9 ± 4.3
Asian men	Adlercreutz et al [35]	Plasma (n=6)	90.4 ± 24	58.3 ± 15.0

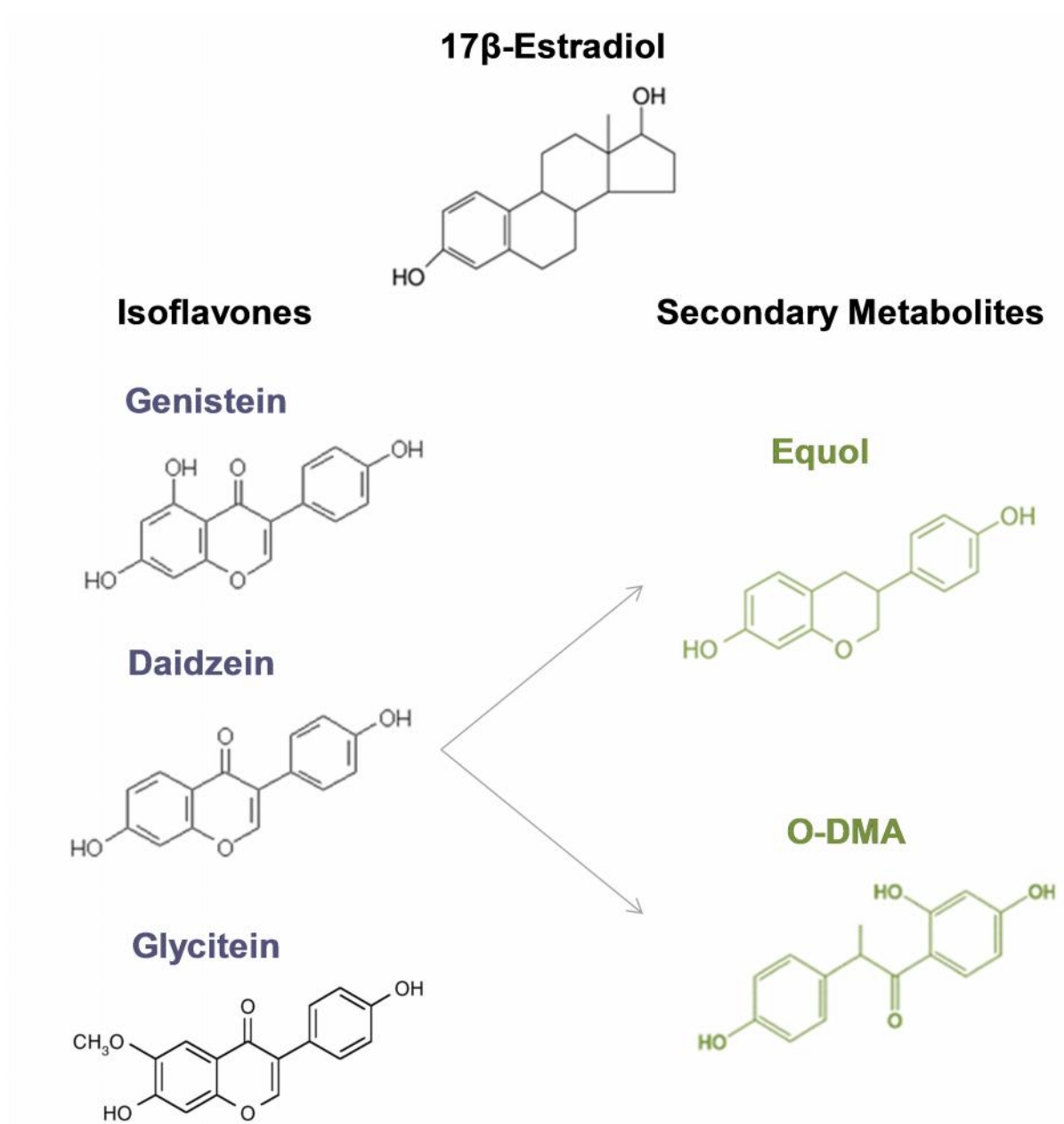


Figure 2-1. Chemical structures of 17β-estradiol, ISO (genistein, daidzein, glycitein) and their secondary metabolites (equol, O-DMA).

Genistein, daidzein, glycitein and equol share a common phenolic ring and a 4'-hydroxyl group with 17β-estradiol that allows them to bind and activate estrogen receptor-α/β. Equol, the metabolite of daidzein, lacks the ketone group on the phenolic ring so it can more tightly bind to estrogen receptor-α/β and have the greatest estrogen-like activity of all ISO. Adapted from Andrade et al., 2010 [37].

2.2.1 Metabolism

The metabolism of ISO takes place in the oral cavity as well as the intestine (Figure 2-2) [38]. In the oral cavity, buccal bacteria and epithelial cell enzymes initiate the hydrolysis of ISO [38]. The partially hydrolyzed ISO are then passed to the intestine (jejunum) where an enzyme called intestinal glycosidase cleaves the β -glucose moieties of glycosidic conjugates to release the bioactive aglycones, including daidzein, genistein and glycitein [39-41]. In order to be absorbed through the intestine, all ISO glycosides must first be hydrolyzed to their aglycone form, which have greater hydrophobicity and lower molecular weight [32]. Both infants and adults have adequate amounts of bacterial β -glucosidase in the intestine to make the ingested ISO bioavailable [7]. However, the bioavailability of ISO in human infants fed soy protein formula has not been measured [32]. Rather, the data are limited to measures of plasma and urine concentrations analyzed an hour or two after soy protein formula feeding. From these analyses, it is estimated that 30% of the bioactive aglycones are excreted in the urine [42], while the remaining 70% are absorbed into the intestinal cells by passive diffusion [7, 43, 44].

The absorption of aglycones into intestinal cells is followed by a reconjugation step, where the aglycones (i.e. genistein, daidzein, and glycitein) are conjugated to glucuronic acid or sulfate by phase II enzymes UDP-glucuronosyltransferase (i.e. UGT1A1, 1A8, 1A9 and 1A10) and sulfotransferase (i.e. SULT1A1 and SULT1A2), respectively [45-48]. Those aglycones that escape this initial phase of metabolism pass into the circulation and are quickly transported to the liver [7] where they are conjugated to glucuronic or sulfuric acid (water-soluble) by phase II enzymes (Figure 2-2). Thus, both intestinal and liver cells are sites of aglycone conjugation. The conjugated ISO are then taken up from the blood by the liver and are combined with cholesterol to form bile micelles [7]. The bile micelles are

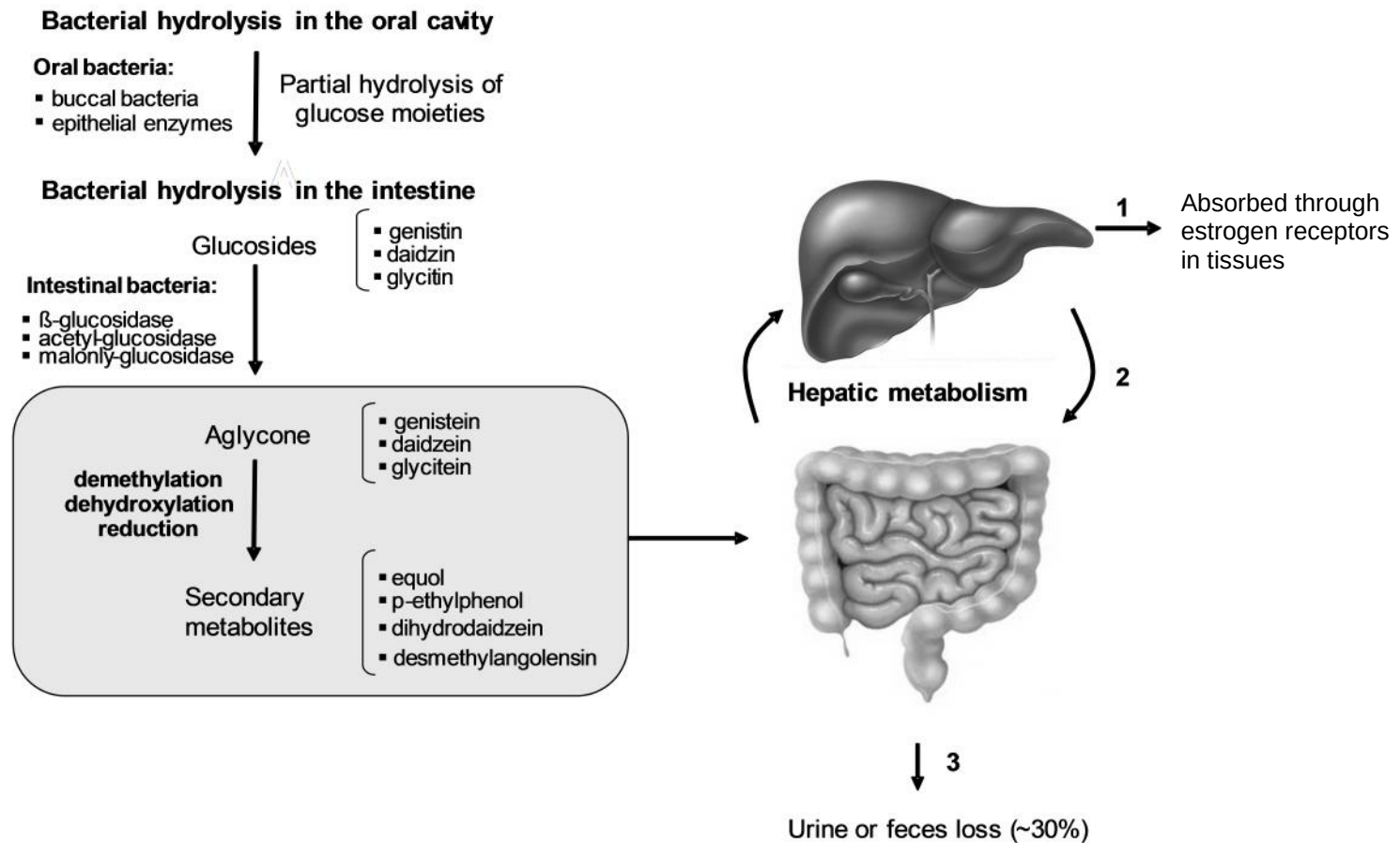


Figure 2-2. Summary of ISO metabolism

The breakdown of ISO begins in the oral cavity by buccal bacteria and epithelial enzymes initiating the hydrolysis of glucose moieties. Partially hydrolyzed ISO are then passed to the intestine where glucosidases cleave the sugar moiety from glucosides to produce the bioactive components of ISO, aglycones. Through a number of biochemical processes a portion of aglycones is metabolized to secondary metabolites including equol, p-ethylphenol, dihydrodaidzein and desmethylanholensin. Aglycones and secondary metabolites (highlighted in the grey box) pass to the liver, enter the portal blood and are 1) absorbed by surrounding tissues, 2) enter enterohepatic cycling or 3) get excreted out of the body. This figure has been modified from Kaludjerovic J, 2008 [49].

released into circulation, where they are absorbed by surrounding tissues or are reabsorbed by the intestine through a process known as enterohepatic cycling [50]. Upon the return to the intestine, bile micelles are deconjugated by β -glucuronidases and sulfatases to aglycones, which are then reabsorbed by intestinal cells or passed to the large bowel for further metabolism. In the large bowel, the bacteria metabolize the aglycone through reduction (i.e. daidzein to equol), ring opening (i.e. daidzein to O-desmethylangolensin, O-DMA) or ring cleavage (i.e. daidzein to p-ethylphenol) [51]. Interestingly, some of these metabolites (i.e. equol) have higher estrogenic and antioxidant activities than their parent compounds [52]. However, both infants younger than four months of age [8] and neonatal rodents [53, 54] lack intestinal bacteria (i.e. *Slackia Isoflavoniconvertens*) needed for secondary metabolism of aglycones and thus, have negligible amounts of circulating equol, p-ethylphenol, dihydrodaidzein and desmethylangolensin. Any metabolites not absorbed by tissues or intestine are excreted as feces [7].

The extent of ISO metabolism is subject to inter-individual differences and environmental disturbances. The rate and quality of ISO hydrolysis within the oral cavity has a 20-fold variability among subjects due to the quality and quantity of oral microbiota. Similarly, the amount of intestinal microbiota is directly correlated with the rate of ISO metabolism. Individuals with sufficient quantities of β -glucosidase can metabolize ISO, while individuals with very low quantities cannot [50]. Environmental factors such as antibacterial mouthwash and antibiotics can reduce the relative concentration of oral and intestinal bacteria and in turn lower the rate of ISO absorption [55-57]. In contrast, diets high in carbohydrates may stimulate ISO absorption and increase intestinal fermentation and conversion of daidzein to equol [57, 58]. Thus, it is possible to observe a large range of

health effects that are not correlated with the amount of ISO consumed but with the rate of ISO metabolism. Moreover, studies that treat animals with aglycones, that bypass early stages of metabolism, can provide valuable information about how ISO affect biological responses in estrogen-sensitive tissues. For this reason, a mixture of genistein and daidzein, that represents more than 90% of total aglycones in soy protein, was administered to mice in Study 1 through 5 of this thesis.

There are no pharmacokinetic studies on ISO in infants or children [32] and only two rodent studies have characterized the pharmacokinetic parameters ($c_{\max}$, $t_{\max}$, $t_{1/2}$, area under the plasma concentration-time curve [AUC]) of pups that were injected with genistein in the first 5 days of life [32, 59]. These studies demonstrated that the rate of ISO clearance is slower in early life than in adolescent or adult life [32]. If this also holds for humans it indicates that infants fed soy protein formula have higher levels of ISO than adults (Table 2-1), longer duration of ISO exposure and a higher concentration of total ISO in tissues [59]. Because ISO bind to estrogen receptor- α and - β [38, 60] in many tissues (i.e. bone, adipose, ovaries, uteri, mammary, testes, hypothalamus, lung, brain, liver, heart) there is growing concern that infants fed soy protein formula may exhibit structural or function changes in one or more of these tissues. In particular, endocrine organs including bone, ovaries and uteri, are highly responsive to environmental changes [61]. These tissues have a fundamental role in maintaining the overall homeostasis in the human body. Thus, exposure to dietary estrogens (i.e. ISO) during sensitive stages of development has the potential to induce profound effects on structural and functional properties of one or more of these tissues. There are no human data on long-term effects of early life exposure to individual ISO found in soy protein formula, so all findings pertaining to ISO effects on development have been inferred from

studies that have followed infants fed soy protein formula for less than two years of life, or have treated animals with ISO or soy protein isolate. Animal studies have primarily used the CD-1 mouse to investigate how early life exposure to ISO affects bone and reproductive development. This is partly because CD-1 mice were used to characterize biological effects of early life exposure to environmental estrogens [62, 63]. However, the effects of route, frequency and duration of exposure on serum ISO concentrations in the CD-1 mouse model have not been studied. Moreover, the mechanism(s) by which early life exposure to ISO impacts bone development and reproductive health is unknown.

2.3 Growth and development of infants fed soy protein formula

Studies investigating bone and reproductive development of healthy infants fed soy protein formula are summarized in Table 2-2. Most of these studies suggest that consumption of soy protein formula does not have a significant effect on infant length, head circumference, body weight, BMC or biochemical markers of bone formation [64-69] in the first year of life. Only one study showed that infants fed soy protein formula had lower BMC than infants fed cow milk formula [70] when treatment groups were matched for calcium and vitamin D intake. However, as this study did not include breastfed infants it cannot be concluded that soy protein formula has adverse effects on bone even in the short term. Two additional studies followed infants to 6 months of age and reported that those fed soy protein formula have lower BMC and BMD around 3 months of age than breast fed infants [68, 71]. However, these differences disappeared by 6 months of age [68] suggesting that early life exposure to ISO may temporally lower BMC and BMD. In another study, Venkataraman et al [72] followed infants who were fed breast milk, cow milk or soy protein formula in the first 6 months of life through to 2 years of age. Findings revealed that BMC at 8 weeks of

Table 2-2. Summary of infant feeding studies: Effects of soy protein formula on bone and reproductive development

Reference	Gender	Timing of exposure	Diet	Findings
<i>Bone development</i>				
Giampietro et al. 2004 [69]	Male and Female	Infants 7 to 96 months of age were followed for > 6 months	Breast milk (n=18) Soy protein formula* (n=48)	▪ Bone age was within the normal range ▪ Serum ALP, osteocalcin and parathyroid hormone were within the normal range and were not different among groups <u>Conclusion:</u> Exposure to soy protein formula did not have an effect on growth or skeletal development of neonates
Mimouni et al. 1993 [64]	Male and Female	First 12 months of life	Breast milk (n=10) Cow milk formula (n=10) Soy protein formula (n=42) Isomil** / Prosobee****	▪ There was no difference in head circumference or BMC at 8, 16, 26 and 52 weeks of age between groups <u>Conclusion:</u> Exposure soy protein formula did not have an effect on growth or skeletal development of neonates
Venkataraman et al. 1992 [72]	Male and Female	First 6 months of life	Breast milk (n=17) Cow milk formula (n=19) Soy protein formula* (n=20) All infants received 400 IU of vitamin D/day	▪ Serum calcium, phosphorus, magnesium, ALP and PTH did not differ between groups at 8, 16, 24 or 26 weeks of life ▪ Infants fed soy protein formula had higher BMC at 16, 24 and 26 weeks of life than breast fed infants ▪ At 16 weeks of life bone width was higher among soy protein formula fed infants than other groups <u>Conclusion:</u> Exposure to soy protein formula improved BMC and bone width when compared to breast milk
Hillman et al. 1988 [65]	Male and Female	First 12 months of life	Breast milk (n=9) Cow milk formula (n=11) Soy protein formula* (n=11) All infants also received 400 IU of vitamin D/day	▪ In the first year of life, BMC of the middle region of the humerus increased in all treatment groups, which may in part be attributed to vitamin D intake <u>Conclusion:</u> Exposure to soy protein formula did not have an effect on growth or skeletal development of neonates

Reference	Gender	Timing of exposure	Diet	Findings
Hillman et al. 1988 [66]	Male and Female	First 12 months of life	Breast milk (n=9) Cow milk formula (n=11) Soy protein formula* (n=11) All infants also received 400 IU of vitamin D/day	▪ Serum calcium, phosphorus, PTH, BMC and bone growth were not statistically different across groups at 2, 4, 6, 9 and 12 months of life Conclusion: Exposure to soy protein formula did not have an effect on growth or skeletal development of neonates
Bainbridge et al. 1988 [67]	Male and Female	First 12 months of life	Breast milk (n=27) Cow milk formula (n=27) Soy protein formula* (n=57)	▪ BMC and BMD in the first year of life were not statistically different between groups Conclusion: Exposure to soy protein formula did not have an effect on growth or skeletal development of neonates
Chan et al. 1987 [71]	Male and Female	First 4 months of life	Breast milk (n=10) Soy protein formula* (n=40)	▪ Treatment did not have an effect on weight, length, head circumference, 25-hydroxyvitamin D, calcium, phosphorus, magnesium, copper or ALP at 2 and 4 months of age ▪ Infants fed breast milk had higher zinc, BMC and BMD at 2 and 4 months of age than soy protein formula infants Conclusion: Exposure to soy protein formula resulted in lower BMC and BMD than breast milk feeding
Steichen et al. 1987 [70]	Male and Female	First 12 months of life	Cow milk formula (n=17) Soy protein formula** (n=18)	▪ Infants fed soy protein formula had lower BMC at 3, 6, 9 and 12 months of age compared to infants fed cow milk formula ▪ Type of formula feeding had no effect on weight, length and head circumference of infants in the first 12 months of life Conclusion: Exposure to soy protein formula resulted in lower BMC in the first year of life compared to cow milk formula

Reference	Gender	Timing of exposure	Diet	Findings
Kohler et al. 1984 [68]	Male and Female	First 12 months of life	Breast milk Cow milk formula Soy protein formula*	- Treatment did not have an effect on body length or skin fold - Soy protein formula fed infants displayed a slower mineralization and maturation of bone at 3 months of age, but not at 6 months of age Conclusion: Exposure to soy protein formula did not have an effect on growth or skeletal development of neonates
Sellars et al. 1971 [73]	Male and Female	First 12 months of life	Breast milk (n=401) Cow milk formula (n=839) Soy protein formula (n=239)	No difference in length or weight were detected between groups Conclusion: Exposure to soy protein formula did not have an effect on growth or skeletal development of neonates
Reproductive development				
Gilchrist et al. 2010 [74]	Female	First 4 months of life	Breast milk Cow milk formula Soy protein formula*	Conclusion: There was no difference in reproductive organ size, as measured by ultrasound, between infants fed soy protein formula, breast milk or cow milk formula
Bernbaum et al. 2008 [75]	Male and female	First 6 months of life	Breast milk Cow milk formula Soy protein formula*	- Breast tissue and vaginal wall cells of infants fed soy protein formula showed maximal estrogen effect at birth and then reverted. - Genital development did not differ between groups - At 6 months of age, females fed soy protein formula showed re-estrogenization of vaginal wall cells and had higher maturation index¹ Conclusion: Exposure to soy protein formula had some estrogenic effects on female breast and reproductive development

¹ Maturation index - an index indicating the degree of maturation attained by the vaginal epithelium

Reference	Gender	Timing of exposure	Diet	Findings
Zung et al. 2008 [76]	Female	First 3 months of life	Milk group (n=602) (breast milk + cow milk formula) Soy protein formula* (n=92)	▪ Breast tissue was more prevalent in the second year of life in girls fed soy protein formula versus those fed breast milk or cow milk formula (22.0% vs 10.3%; $P = 0.02$) with an odds ratio of 2.45 (95% CI, 1.11-5.39). ▪ No differences in breast bud prevalence were observed among groups during the first year of life Conclusion: Exposure to soy protein formula promoted breast development of girls in the second but not first year of life
Strom et al. 2001 [77]	Male and female	Exposed during infancy and recalled for a telephone interview 20 to 34 years later	Cow milk formula (n=563) Soy protein formula* (n=248)	▪ Thirty measured outcomes did not differ between groups including: height, weight, body mass index, age of sexual maturation, length of menstruation, missed periods, spotting, cramps, breast tenderness, pregnancy, birth defects, number of miscarriage, sexual orientation, libido dysfunction, cancer incidence, reproductive tumors, hormonal dysfunction ▪ Longer menstrual bleeding (difference of 8 hrs) and greater discomfort was reported among women fed soy protein formula than cow milk formula Conclusion: Exposure to soy protein formula does not lead to different general health or reproductive outcomes than exposure to cow milk formula

Reference	Gender	Timing of exposure	Diet	Findings
Freni-Titulaer et al. 1986 [78]	Female	During infancy	Age matched control subjects through retrospective questioning of parents about infant feeding (n=130)	▪ Unable to detect a significant association overall between premature thelarche² and soy protein formula intake ▪ Restriction of multivariate analysis to subjects with thelarche before 2 years of age showed significant association with soy protein formula intake (OP 2.7; 95% CI 1.1-6.8, p=0.029) Conclusion: Exposure to soy protein formula maybe associated with onset of secondary breast development

Abbreviations: ALP – alkaline phosphatase; BMC – bone mineral content; BMD – bone mineral density; PTH – parathyroid hormone.

* Type of SPF was not specified; however all commercial SPFs contain between 40 and 50 mg per liter of ISO.

** Isomil SPF contains 44 mg ISO per liter of formula.

*** Prosobee SPF contains 45 mg ISO per liter of formula.

² Thelarche is the onset of secondary breast development

age, and serum markers of bone metabolism (i.e. calcium, magnesium, phosphorus, ALP, and PTH) at 8, 16, and 24-26 weeks of age were not significantly different among the groups. However, infants fed soy protein formula had greater bone width at 16 weeks of age and higher BMC at 16 and 24-26 weeks of age compared to infants fed breast milk. Based on these findings, authors concluded that exposure to soy protein formula – abundant in ISO – during early life may provide benefits to bone that are imperceptible at the time of exposure, but become apparent with time.

No randomized clinical trials have characterized bone health of adolescents or adults who were fed soy protein formula during infancy. However, a longitudinal prospective study aiming to characterize the growth, development and future health of infants fed breast milk, cow milk formula or soy protein formula is underway at the Arkansas Children's Nutrition Center. Preliminary findings show that infants fed soy protein formula have lower percent body fat at 6 months of age and accelerated bone growth from 6 to 12 months of age than infants fed breast milk or cow milk formula [79-81]. While these findings suggest that feeding soy protein formula to infants may provide some health benefits, this data is preliminary and more definitive results await completion of the study, which is expected to be in 2017.

To date, only one retrospective study compared the growth and development of young men and women who were fed soy protein formula (n=248) or cow milk formula (n=563) in the first 4 months of life [77]. Findings from this study showed no differences in adult height, body weight, body mass index, pubertal maturation or fertility as measured by pregnancy, miscarriage, or ectopic pregnancy rates due to type of feeding. There were no differences in cancer, reproductive or hormonal disorders, sexual orientation, libido

dysfunction or birth defects between infants fed soy protein formula or cow milk formula. However, women fed soy protein formula reported slightly longer (8 hour) duration of menstrual bleeding and greater discomfort with menstruation, with no difference in severity of menstrual flow. The authors concluded that exposure to soy protein formula does not lead to different general and reproductive health compared to cow milk formula. However, taking that the number of subjects fed soy protein formula was fairly small and that participants were only followed to their mid-30s and were questioned via a telephone interview it is possible that authors reported some false-negative conclusions (type II error).

Four clinical studies have examined the reproductive development of infants who were fed soy protein formula within the first year of life (Table 2-2) [75, 76, 82]. One study reported no difference in reproductive organ size, as measured by ultrasound, between infants fed soy protein formula, breast milk or cow milk formula at 4 months of age [82]. In contrast, prospective studies of healthy infant fed breast milk, cow milk or soy protein formula showed that females fed soy protein formula had enhanced vaginal wall cell maturation at 6 months of age [75] and more developed breast tissue at 2 years of age than infants who were fed breast milk or cow milk formula [76]. Moreover, a case-control study published in 1986 reported that soy protein formula feeding is significantly associated with the onset of secondary breast development (termed thelarche) before 2 years of age (OR 2.7, 95% CI 1.1–6.8) [78]. Therefore, despite its long history of use, there is some concern that exposure to soy protein formula may have adverse effects on developing infants.

In 2010, the United States National Toxicology Program Center for the Evaluation of Risks to Human Health Reproduction concluded that there is minimal concern (level 2) that soy protein formula, containing ISO, cause adverse reproductive and/or developmental

effects in exposed humans, but acknowledged the paucity of well-designed clinical studies to fully address concerns [32]. In contrast, the European Society for Pediatric Gastroenterology Hepatology and Nutrition has taken a more cautious approach and is advising the public to avoid the use of soy protein formula, especially for infants less than 6 months of age, because of uncertainties regarding safety in infants and young children [26]. Therefore, there is an ongoing need to better characterize growth, development and future health of adults who were fed soy protein formula during development. Although well designed case-control studies that follow infants fed soy protein formula from birth through adulthood provide a direct evaluation of ISO effects on human health, these studies require a large time commitment from both the subject and the investigator, are expensive, have many environmental confounders, and are limited in the type of analyses that can be performed. Thus, use of animal models can help characterize the potential long-term biological effects of early life exposure to ISO. Findings from such studies can provide a rationale for long-term clinical trials.

2.4 Animal models being used to study biological effects of ISO

The choice of animal model depends on the scientific question and the stage of the life cycle being investigated. Practical considerations such as body size, length of time required to reach adulthood and the intervention form (soy protein isolate, ISO, soy protein formula) often dictate the choice of animal model used. Studies that investigate effects of prenatal or neonatal exposure to ISO at adulthood most often use a mouse model because mice can metabolize ISO, have a short gestation period and a rapid rate of development where sexual maturity is reached by 2 months of age and peak bone mass is established by 4 months of age [11-13, 54, 83-90]. From all the available mouse strains the CD-1 mouse has been largely

used to investigate how early life exposure to ISO affects bone and reproductive development for several reasons. Firstly, the CD-1 mouse has been extensively used to characterize the effects of environmental estrogens, including diethylstilbestrol (DES) and bisphenol A, which allows for direct comparison between ISO and environmental estrogens [91]. Secondly, the effects induced by prenatal exposure to environmental estrogens (i.e. DES and bisphenol A) in the CD-1 mouse model mimic those observed in humans, which provides support that CD-1 mice and humans respond similarly to estrogen-like compounds. Some of the reported effects include reduced fertility, higher incidence of benign tumors in reproductive organs and vaginal carcinomas, reduced testicular size and sperm count, higher body weight and greater incidence of autoimmune complications [62, 63]. Lastly, CD-1 mice have a low rate of cannibalism when cross-fostered and handled by humans in the first week or two of life, which makes them a useful model for predicting potential ISO effects in human infants with similar exposures, and studying the underlying molecular mechanisms.

A drawback of using a mouse model is its small body size. It is not possible to feed a sufficient quantity of soy protein formula to rodents, particularly mice, and achieve serum ISO levels that resemble those of human infants fed soy protein formula. For this reason ISO, in the aglycone form, are most often administered via subcutaneous injection to mouse pups. A recently published summary from an *NIH Workshop on Designing, Implementing and Reporting Clinical Studies of Soy Interventions* identified that it is extremely important that blood levels of animal models be evaluated and be comparable to the blood levels observed in human populations consuming ISO containing products [92]. Indeed, our published findings showed that ISO levels of CD-1 pups, injected subcutaneously with 5 mg of genistein and 2 mg of daidzein/kg of body weight/day, resembled the circulating ISO levels

of human infants fed soy protein formula [8, 54]. Thus, despite the fact that pure ISO were given rather than soy protein formula, serum levels of genistein, daidzein and equol of CD-1 pups resembled those of human infants fed soy protein formula. Data from human and rodent studies have shown that at adulthood most of the ISO compounds detected in circulation are present in the conjugated form with less than 5% of total plasma ISO being present in the aglycone form [93, 94]. However, during development enzymatic activity is low in both human infants and neonatal rodents so developing organisms likely have a limited capacity to catalyze glucuronidation of ISO or generate secondary metabolites such as equol [8, 53, 54]. Although, no studies have reported the aglycone fraction in serum of infants fed soy protein formula, reports from 5-day old CD-1 mice demonstrate an elevated fraction of aglycones (~30%) in serum [59]. Thus, it seems likely that the bioavailability of the aglycone form of ISO is higher during development and may thereby have marked estrogenic effects in tissues. Doerge et al. [95] showed that in Sprague-Dawley rats steady state concentrations of genistein in tissues are significantly associated with estrogen receptor mediated responses but no studies have been done in humans.

Study 1 was designed to determine how route and frequency of ISO exposure affect plasma ISO concentrations in the commonly used CD-1 mouse model. Human infants are fed soy protein formula every few hours, while the CD-1 pups are administered ISO once daily via subcutaneous injection. To be able to more accurately translate findings from the CD-1 mouse model to human infants, it was important to evaluate whether there is a difference in circulating ISO levels after oral versus subcutaneous delivery, and once daily versus 4 hours treatments of an equivalent total daily dose in the CD-1 mouse model. *Thus the first study in this thesis compared the serum levels of genistein, daidzein, and its metabolites, equol and*

O-DMA [1-(2,4-dihydroxyphenyl)-2-2(4-hydroxyphenol)-propan-1-one] one hour after subcutaneous injection and oral dosing with once daily and 4-hour treatments of ISO in the CD-1 mouse model.

Investigators have also used piglets and rats to characterize the effects of early life exposure to ISO [96-98]. The primary advantage of using a piglet model is that soy protein formula can be fed directly. However, piglets do not reach peak bone mass until 3 years of age and are large, making it extremely difficult to investigate how ISO modulate long-term programming of health from a time and resource perspective [99]. The rat model is typically not used for investigating the effects of ISO on bone development because its growth plates do not fuse during sexual maturation and thus peak bone mass cannot be defined [99]. Interventions at early adulthood, when endogenous concentrations of sex steroid are adequate, have primarily used the sexually intact rodent model. In contrast, studies designed to investigate if soy or its ISO attenuate deterioration of bone tissue when endogenous concentrations of sex steroid are minimal have most often used the ovariectomized rat model. The ovariectomized rat is a preclinical model approved by the Food and Drug Administration for investigating postmenopausal osteoporosis [100]. Acute ovarian estrogen deficiency leads to a high rate of bone turnover in which the rate of bone resorption exceeds the rate of bone formation. This high rate of bone turnover post-ovariectomy is followed by a slower phase of bone loss and is consistent with postmenopausal loss of BMD and structure. Moreover, both ovariectomized rodents and postmenopausal women exhibit a greater loss of trabecular than cortical bone, have reduced intestinal calcium absorption and exhibit similar skeletal responses to commonly used drug therapies including estrogen, tamoxifen, bisphosphonate, PTH and calcitonin [101]. Inevitably, by investigating each one of the outlined animal

models researchers can gain a more comprehensive understanding of how ISO affect health outcomes including both skeletal and reproductive health. For the purpose of investigating how early life exposure to ISO affects bone and structural development of ovaries and uteri at adulthood the CD-1 mouse model is the most appropriate animal model.

2.5 Bone development of animals exposed to ISO

The shape, size and structure of bone, as well as its biological activity, are constantly changing throughout the life cycle. Bones lengthen by interstitial growth of the epiphyseal plates and expand by appositional growth [102]. Beginning in early gestation, condensation of mesenchymal cells differentiate into chondrocytes, which through continued proliferation form the cartilage and an extracellular matrix that facilitate angiogenesis and invasion of osteoblasts (bone forming cells) [102]. When osteoblasts invade the cartilage, they lay down matrix that becomes mineralized and forms bone. The bone is then continuously remodeled by osteoclasts (bone resorbing cells) that digest mature packets of bone in the skeleton, and osteoblasts (bone forming cells) that deposit newly formed bone. At any one time, approximately 20% of bone is being remodeled. During development and early adulthood, the amount of bone resorbed by osteoclasts is in balance with the amount of bone formed by osteoblasts; however, during aging bone resorption dominates. Thus, the quantity of bone tissue present at any time during adult life is the difference between the amount of bone mass achieved at maturity (i.e. peak bone mass) and that lost with aging. Individuals who acquire a high peak bone mass during adolescence have more bone to lose and are at a lower risk of fracture in later life [103-105]. Thus, optimizing peak bone mass is crucial for prevention of osteoporosis.

Sex steroids, in particular, estrogens play an important role in attainment and maintenance of peak bone mass [106-109]. Findings from patients with aromatase and/or estrogen deficiency have revealed that estrogen withdrawal in the first two decades of life prevents the epiphyseal fusion that stops bones from lengthening, up-regulates bone resorption and decreases BMD [110]. In contrast, estrogen therapy restores BMD and induces epiphyseal closure after several months but, only if treatment is introduced in early adolescence [110, 111]. If estrogen therapy is initiated after puberty the skeleton receives minimal benefits, thus timing of estrogen exposure is a crucial predictor of skeletal development. Similarly, dietary estrogens such as ISO may have a significant influence on attainment of peak bone mass [12, 54, 85].

2.5.1 Prenatal exposure to ISO

Two animal studies (Table 2-3) have investigated the effects of in utero exposure to ISO on skeletal development but only one of these studies examined BMD and bone strength at adulthood [96, 112]. In the first study, mouse pups born to mothers treated with vehicle, genistein, daidzein or a combination of genistein and daidzein had comparable BMD and strength at the femur and lumbar spine at young adulthood (4 months of age) demonstrating that in utero exposure to ISO does not result in nutritional programming of bone metabolism [112]. In the second study, newborn pigs whose mothers were exposed to daidzein from gestation day 85 to 114 had reduced estrogen receptor- β gene expression in the hypothalamus and elevated insulin-like growth factor-I (IGF-I) receptor gene transcription in skeletal muscle [96]. However, the long-term implications of altering IGF-I signaling in bone and hypothalamus are unknown and require further investigation.

Table 2-3. Summary of animal studies: Effects of early life exposure to soy or ISO on bone development

Reference	Model	Time of Exposure	Studied Until	Treatment	Route	Findings
Prenatal exposure						
Ward WE & Piekarz A. 2007 [112]	Mouse (CD-1) n=12/ group male & female	Gestation day 9 to 21	17 weeks of age	Daidzein (3.75 mg/kg body weight/day) Genistein (3.75 mg/kg body weight/day) Daidzein + Genistein (7.5 mg/kg body weight/day)	Subcutaneous injection to dams	- Offspring exposed to control or genistein treatment had higher femur BMD than those exposed to daidzein or daidzein + genistein - ISO exposure did not have an effect on femur peak load - Females exposed to daidzein had lower LV1-4 BMD than all other groups, but LV4 peak load did not differ between groups Conclusion: <i>In utero</i> exposure to ISO did not result in functional benefits to bone at young adulthood.
Ren et al. 2001[96]	Piglet n=6-8 /group male	Gestation day 85 to 114	6 hrs post birth	Daidzein (8 mg/kg feed)	Oral administration to dams	- Male offspring exposed to daidzein had: - higher birth weight - lower levels of estrogen receptor-β mRNA expression in the hypothalamus - higher IGF-I receptor expression in skeletal muscle - Exposure to daidzein had no effect on IGF-I receptor expression in the pituitary, hypothalamus, thymus and liver Conclusion: Daidzein may promote fetal growth by increasing IGF-I receptor expression in skeletal muscle and decreasing estrogen receptor-β expression in the hypothalamus

Reference	Model	Time of Exposure	Studied Until	Treatment	Route	Findings
Neonatal exposure						
Kaludjerovic J & Ward WE, 2010 [13]	Mouse (CD-1) n=8-18 /group male & female	Postnatal day 1 to 5	32 weeks (sex organs were excised at 16 weeks)	Daidzein (2 mg/kg body weight/day) Genistein (5 mg/kg body weight/day) Daidzein + Genistein (7 mg/kg body weight/day)	Subcutaneous	- Females treated with daidzein + genistein had higher BMD and improved trabecular connectivity at the femur neck and lumbar spine 4 months post-OVX compared to corn oil treated mice. Importantly, these improvements translated to stronger bones that were more resistant to compression fractures - Exposure to daidzein + genistein had no benefit, nor adverse effects on bones of orchidectomized males Conclusion: Neonatal exposure to daidzein + genistein attenuated deterioration of bone tissue in ovariectomized females but not orchidectomized males
Kaludjerovic J & Ward WE, 2009 [54]	Mouse (CD-1) n=8-16 /group male & females	Postnatal day 1 to 5	16 weeks	Daidzein (2 mg/kg body weight/day) Genistein (5 mg/kg body weight/day) Daidzein + Genistein (7 mg/kg body weight/day)	Subcutaneous	- Females treated with daidzein, genistein or daidzein + genistein had improved BMD, structure and strength at the lumbar spine compared to corn oil treated mice - In males, ISO exposure did not have a benefit nor an adverse effect Conclusion: Neonatal exposure to daidzein, genistein or daidzein + genistein improved peak bone mass of female mice but, compared to individual treatments daidzein + genistein did not provide great benefits to bone

Reference	Model	Time of Exposure	Studied Until	Treatment	Route	Findings
Julius et al. 2009 [97]	Piglet n=6 /group male & female	Postnatal day 9 to 41	6 weeks	Cow milk formula* Soy protein formula*	Oral	▪ There was no difference in body weight or growth between pigs fed soy protein and cow milk formula ▪ Pigs fed soy protein formula had lower plasma cholesterol than those fed cow milk formula ▪ Bone calcium, measured as percent of dry, fat-free femur or whole carcass ash, was lower in pigs fed soy protein formula than cow milk formula Conclusion: Similar growth and development were observed in pigs fed soy protein and cow milk formula, but exposure to soy protein formula resulted in lower bone calcium
Chen et al. 2009 [98]	Piglet n=3-5 /group male & female	Postnatal day 2 to 21/35	3 or 5 weeks	Cow milk formula (Similac Advance powder)* Soy protein formula (Enfamil Prosobee Lipil powder)*	Oral	Compared to sow-fed piglets male and female piglets fed soy protein formula for 21 days had: ▪ higher osteoblastogenesis in ex vivo bone marrow cells ▪ higher serum osteocalcin and bone ALP ▪ lower serum CTX ▪ higher tibial BMP-2 and ALP mRNA expression ▪ higher tibial expression of extracellular kinases ▪ lower tibial RANKL expression Compared to sow-fed piglets, male piglets fed soy protein formula for 35 days had: ▪ higher osteoblast number ▪ lower osteoclast number ▪ higher trabecular bone formation rate and higher mineral apposition rate in the tibia Conclusion: Soy protein formula fed piglets had higher bone quality in part due to enhanced BMP-2

Reference	Model	Time of Exposure	Studied Until	Treatment	Route	Findings
Piekarz AV & Ward WE. 2007 [12]	Mouse (CD-1) n=4-14 /group male & female	Postnatal day 1 to 5	16 weeks	Genistein (4 mg/kg body weight/day) DES positive control (2 mg/kg body weight/day)	Subcutaneous	- DES- and genistein-treated females had higher BMD and peak load of the femur and LV compared to the control group - Genistein-treated males had higher LV BMD and peak load than control or DES group Conclusion: Neonatal exposure to genistein had positive effects on femur and lumbar spine of females and lumbar spine of males at adulthood
Adolescent exposure						
Fujioka et al. 2007 [113]	Mouse (ddy) n=8/ group male & female	5 to 9 weeks	9 weeks	Daidzein (0.08% of diet) (~3 mg/kg body weight/day) Genistein (0.08% of diet) (~3 mg/kg body weight/day)	Oral (Pair-fed)	- Daidzein-treated males had higher bone formation rate, whole body BMD, lumbar spine and femur BMD than control group - Genistein-treated males had higher bone formation rate and femur BMD than controls - Daidzein-treated females had lower bone formation rate, whole body BMD and femur BMD than control group - Genistein had no effect on female bone health Conclusion: Daidzein has a sexually dimorphic effect on bone formation and BMD in mice with positive effects in males and adverse effects in females
De Wilde et al. 2007 [114]	Piglet n=8/ group female	6 to 12 weeks	12 to 18 weeks	Control (6.6 mg ISO/kg body weight/day) SoyLife (35.6 mg ISO/kg = 2.8 mg/kg body weight/day)	Oral (Pair-fed)	- SoyLife did not alter body weight, bone turnover, BMD or bone strength of piglets - Stromal cells from SoyLife fed piglets had higher ALP, OPG, RANKL and osteocalcin, as well as more mineralized nodules compared to control fed piglets Conclusion: SoyLife has no effect on BMD of growing piglets but stimulated bone marrow osteoprogenitor cells via estrogen receptors.

Reference	Model	Time of Exposure	Studied Until	Treatment	Route	Findings
Peterson et al. 2008 [115]	Rats Sprague Dawley n=8-9 /group female	3 to 11 weeks	11 weeks	Casein (200 g/kg diet) Soy protein isolate: Low (0.11mg/g protein) Medium (2.16mg/g protein) or High (3.95mg/g protein) amount of ISO	Oral	▪ High ISO consumption suppressed serum estradiol levels but had no effect on total serum estrogenicity ▪ Rats fed a low ISO diet had lower body weight from 4 to 11 weeks of age, and lower calcium content in the tibia than all other groups ▪ Rats fed a medium or high ISO diet had higher uterine weight relative to body weight compared to the control group ▪ Soy intake had no effect on whole body or tibial BMD Conclusion: Bone growth and BMD were unaffected by ISO intake in soy protein isolate.

This table has been modified from Kaludjerovic J & Ward WE, 2013 [116].

* There is no published data on the ISO content or composition in the diet

Abbreviations: ALP - alkaline phosphatase; BMD - bone mineral density; BMP-2 - bone morphometric protein 2; BV/TV – trabecular bone volume; CTX - collagen crosslinks; DES - diethylstilbestrol; IGF-I - insulin growth factor-I; ISO – isoflavone; LV – lumbar vertebrae; OPG – osteoprotegerin; RANKL - receptor activator for nuclear factor β ligand.

2.5.2 Neonatal exposure

A growing body of literature indicates that exposure to dietary estrogens (ISO) has a measurable effect on bone when endogenous estrogen production is low (Table 2-3) [12, 13, 54, 98]. This may be because ISO bind to estrogen receptor- α and - β with a markedly lower affinity than endogenous estrogen [117, 118]. There are two critical stages of the life cycle when endogenous estrogen production is low: early postnatal life, and late adulthood [38].

At delivery, cutting of the umbilical cord subjects the neonate to an abrupt withdrawal of maternal and placental sex hormones, which results in a drastic drop of its endogenous sex steroid concentrations [38, 119]. The low estrogen and testosterone levels stimulate the hypothalamic-pituitary-gonadal axis to produce luteinizing hormone and follicle stimulating hormone, which in turn stimulate production of sex steroids [119]. By 4 to 6 months of age, most infants can produce low quantities of luteinizing and follicle stimulating hormones that weakly stimulate production of estradiol and testosterone from ovaries and testes [6, 119, 120]. This weak activation of sex steroid production persists from infancy to puberty and is critical for sensitizing the tissues so they can respond to higher doses of sex steroids in adulthood. It also may provide a strategy for optimizing peak bone mass.

The postnatal withdrawal of sex steroids that is common to many animals including the mouse, rat, monkey and human may provide a critical time of exposure for programming bone cell differentiation and inducing long-term programming effects [5, 119]. In rodents, the postnatal withdrawal of sex steroids occurs within the first two weeks of life, while in humans it occurs within the first 6 months of life [5, 6].

Findings from our group [12, 13, 54] and others [98] indicate that targeting the early postnatal life provides a window of opportunity for ISO to improve bone health. For example, piglets fed soy protein formula for the first 21 or 35 days of life had greater BMC,

BMD and trabecular number (Tb.N.) in the tibia than sow-fed piglets [98]. These piglets also exhibited a greater number of osteoblasts, higher expression of bone forming genes (ALP and BMP-2), higher concentration of serum bone formation markers (osteocalcin, ALP) and lower concentration of serum bone resorption markers (CTX; RANKL). Whether these benefits improve bone health at adulthood (i.e. BMD, bone turnover, bone structure and bone strength) has not been investigated in a piglet model.

In CD-1 mice, exposure to ISO (genistein or daidzein alone or in combination) for the first 5 days of life improves bone mineral accrual and bone structure at young adulthood [12, 54], and attenuates deterioration of bone tissue during aging [13]. Male and female mice treated with genistein had higher BMD at the lumbar spine (LV1-LV4) and stronger vertebra as demonstrated by the significantly higher peak load of LV3 [12]. Effects of genistein on bone in females were similar to those induced by DES (an environmental estrogen) while in males genistein and DES had divergent effects [12]. These findings suggest that genistein enhances bone development through an estrogen-dependent mechanism in females but not males. A follow-up study used a similar experimental design but included daidzein and a combination of genistein and daidzein at a dose that mimicked the serum ISO concentrations of infants fed soy protein formula [54]. The key finding from this study (my M.Sc. thesis research) was that the combination of genistein and daidzein does not induce greater benefits to bone than either treatment alone, suggesting that genistein and daidzein may be competing for the same estrogen receptors. Female mice treated with daidzein, genistein or the combination had greater BMC and BMD at the lumbar spine compared to the control group but not to each other.

As in the previous study, the ISO-induced effects on bone in female mice were similar to those seen with early life exposure to DES, demonstrating that ISO have a potential estrogen-like effect on bone development. To our surprise the most profound effects on bone mineral were observed with daidzein exposure, as it was the only treatment to improve BMC and BMD at the femur. Microstructural analyses (Figure 2-3) revealed that although exposure to all ISO groups resulted in higher trabecular thickness (Tb.Th) and lower trabecular separation (Tb.Sp) at the lumbar spine daidzein had the most profound effect. Accordingly, the improvements in bone mineral and bone structure among females treated with daidzein were translated into stronger vertebrae that were more resistant to compression fracture, while exposure to genistein or daidzein + genistein had intermediate effects. This study demonstrated that 5-day exposure to daidzein has the most profound biological effect on bone development. However, because soy-based foods contain a mixture of both daidzein (29-34%) and genistein (58-68%) it remained more important to investigate how these two compounds rather than daidzein alone program bone development. Thus, rather than studying individual ISO we continued to investigate how the combination of daidzein and genistein programs bone development. Doses of daidzein (2 mg/kg body weight/day) and genistein (5 mg/kg body weight/day) at a level and ratio (29% daidzein and 71% genistein) found in commercially available soy protein formulas were used to minimize complications due to nonlinear pharmacokinetics and mimic the bioavailability and biological activity of infants fed soy protein formula.

In our third study, which build on our two previous studies, we showed that improvements in BMD, trabecular connectivity and bone strength at adulthood provide protection against the deterioration of bone tissue in ovariectomized females but not

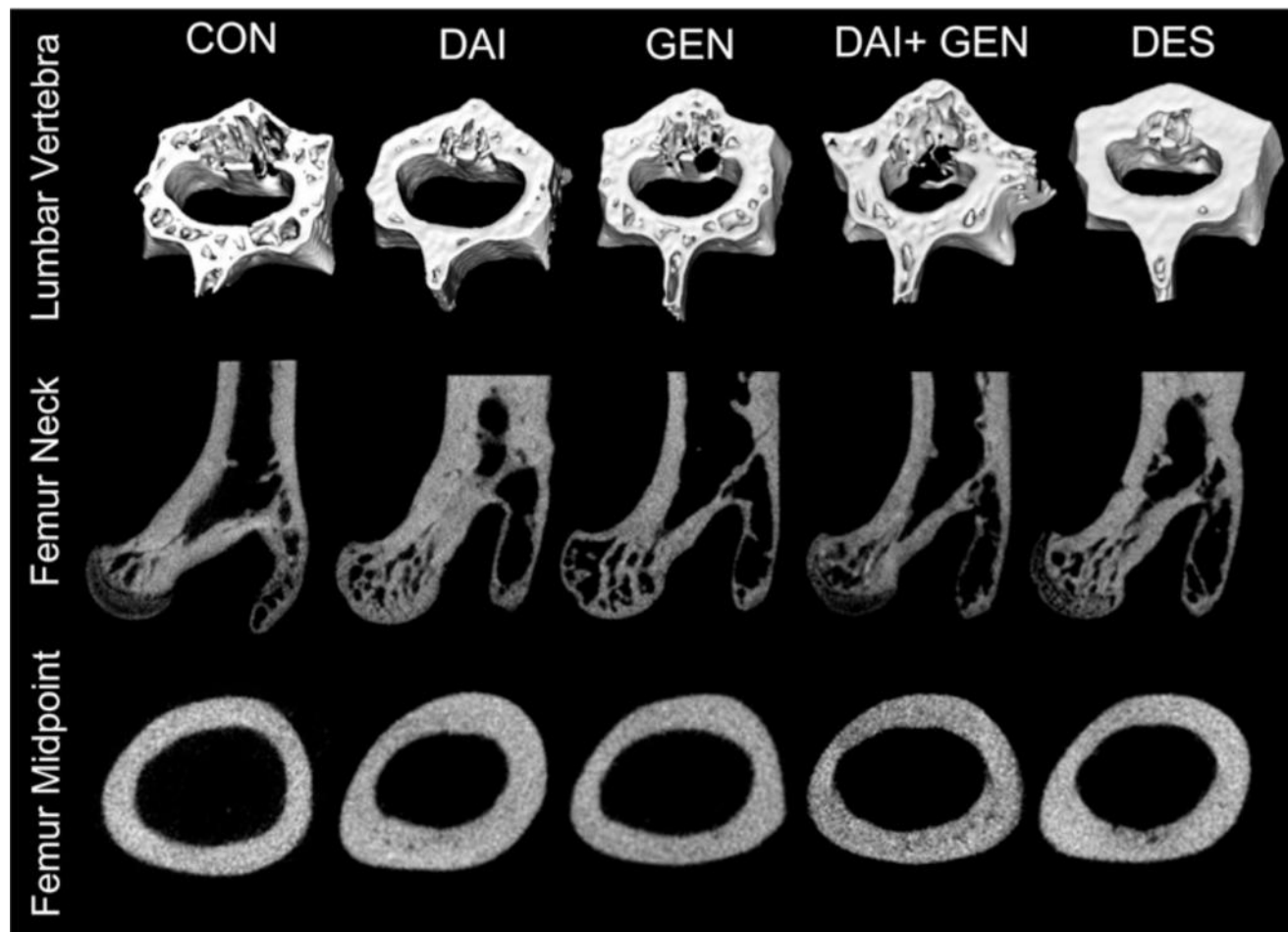


Figure 2-3. Representative microcomputed tomography images of LV4, femur neck and femur midpoint from 4-month old females who in the first 5 days of life were treated with control (CON), daidzein (DAI), genistein (GEN), daidzein + genistein (DAI+GEN) or diethylstilbestrol (DES).

This figure has been modified from Kaludjerovic J and Ward WE, 2009 [54].

orchidectomized males [13]. Ovariectomized females treated with genistein plus daidzein had a 1.6 fold increase in lumbar spine peak load 4 months post-ovariectomy relative to control [13]. Therefore, early life exposure to ISO may provide a prevention strategy against deterioration of bone disease during aging.

2.5.3 Adolescent exposure to ISO

Few animal studies have investigated the effects of adolescent exposure to ISO on bone development [113-115] (Table 2-3). In one study, oral consumption of daidzein or genistein from 5 to 9 weeks of age had gender-specific effects on bone in a mouse model with a higher bone formation rate and femur BMD in males, and a lower bone formation rate and whole body BMD in females [113]. These findings are challenging to explain because estrogen has positive effects on female bone development. The reason why weak dietary estrogens would induce adverse effects in females, but not males who are more sensitive to estrogen fluctuations, is unclear. In another study, female rats were fed with soy protein isolate that has low, medium or high levels of ISO from 3 to 11 weeks of age, and serum estradiol, BMD and histomorphometry of tibia were measured [115]. Growth and BMD of tibia was unaffected by soy intake. However, serum estradiol concentrations were significantly lower in the high ISO group compared to the low ISO group suggesting that higher doses of ISO suppress endogenous estrogen production by contributing to the total estrogenic activity. The third study fed a commercial product “SoyLife” containing a mixture of daidzein and genistein to pigs from 6 to 12 weeks of life [114]. There was no significant effect on growth rate, biochemical markers of bone formation (i.e. ALP and osteocalcin), tibial or metacarpal BMD or metatarsal strength. However, analyses of bone cells isolated from the treated piglets revealed that ISO stimulate production of bone formation markers (i.e. ALP and osteocalcin), expression of estrogen-receptor- α and $-\beta$ and synthesis of

molecular factors that enhance osteoblastic activity such as OPG. Therefore, it is possible that a longer intervention (> 6 weeks) may have measurable benefits to bone.

2.5.4 Summary

In summary, while not all studies investigating early life exposure to ISO suggest positive effects on bone, most animal studies do demonstrate that bone tissue responds positively to ISO when exposure occurs during neonatal life. This is likely because the neonate is exposed to higher amounts of ISO than the developing fetus. According to a study by Doerge et al [121] fetal serum concentrations of total and aglycone genistein were 20 and 5-fold lower, respectively, than maternal serum concentrations in a Sprague–Dawley rat model. Thus, while ISO can cross the placenta it is possible that the amount of ISO transferred may not be high enough to induce biological effects on fetal bone tissue. It is also possible that ISO have more profound biological effects on bone when endogenous concentrations of sex steroids are negligible, so that they can more freely bind to estrogen receptors [38]. In utero, the level of endogenous estrogen is high due to maternal and placental estrogen production but, at delivery cutting of the umbilical cord subjects the organism to an abrupt withdrawal of estrogen that persists to 2 weeks of age in mice and 4 months of age in humans [38, 119]. Thus, exposure to ISO during early postnatal life in rodents or humans may provide a strategy for optimizing bone development. The 5-day window of exposure (estimated to represent the first 4 months of human life) that we [12, 13, 54, 122] and others [86-90, 123, 124] used in the CD-1 mouse model was selected to mimic published studies investigating the effects of potent environmental estrogens (i.e. DES and bisphenol A) on bone development and reproductive health [125-130]. However, a question arose whether longer duration of ISO exposure could induce greater benefits to bone health. Mice suckle for the first 21 days of life, which is the stage of development when human

infants are fed SPF. *Thus, the objective of our second study was to determine if exposure to ISO from birth throughout suckling enhances the previously observed positive effects of 5-day exposure to ISO on bone development in female CD-1 mice at young adulthood.*

2.6 Potential mechanisms

Animal and human studies have shown that exposure to nutrients or bioactive food components during development can affect the rate of extra-uterine growth and programming of long-term metabolic outcomes by altering gene expression or endocrine regulation [131]. Thus, the term “nutritional programming” has emerged from the fetal-origin hypothesis to help characterize permanent changes in the structure or function of an organism caused by a nutrient or a bioactive food component during development [131]. The exact mechanism by which early life exposure to ISO improves skeletal development has not been elucidated. Recent efforts in defining estrogen signaling pathways and modes of epigenetic programming have provided some insight into potential mechanisms of action. By binding and activating nuclear receptors such as estrogen receptor- α and - β , ISO may interfere with hormonal signaling and/or the production of enzymes and transcription factors [3, 4]. These changes may induce irreversible effects on many physiological processes (i.e. growth, metabolism, stress response, sex development, behavior, ability to reproduce) if exposure occurs during sensitive stages of development. This is because during development endocrine factors (i.e. hormones/enzymes) can alter epigenetic regulation by increasing histone acetylation, the availability of DNA methyltransferases, nucleosome positioning or the production of non-coding RNAs, as well as program long-lasting changes in hormone secretion and tissue hormone sensitivity (Figure 2-4) [14]. In line with this, studies have shown that through an estrogen receptor- α and - β mediated mechanism, ISO enhance

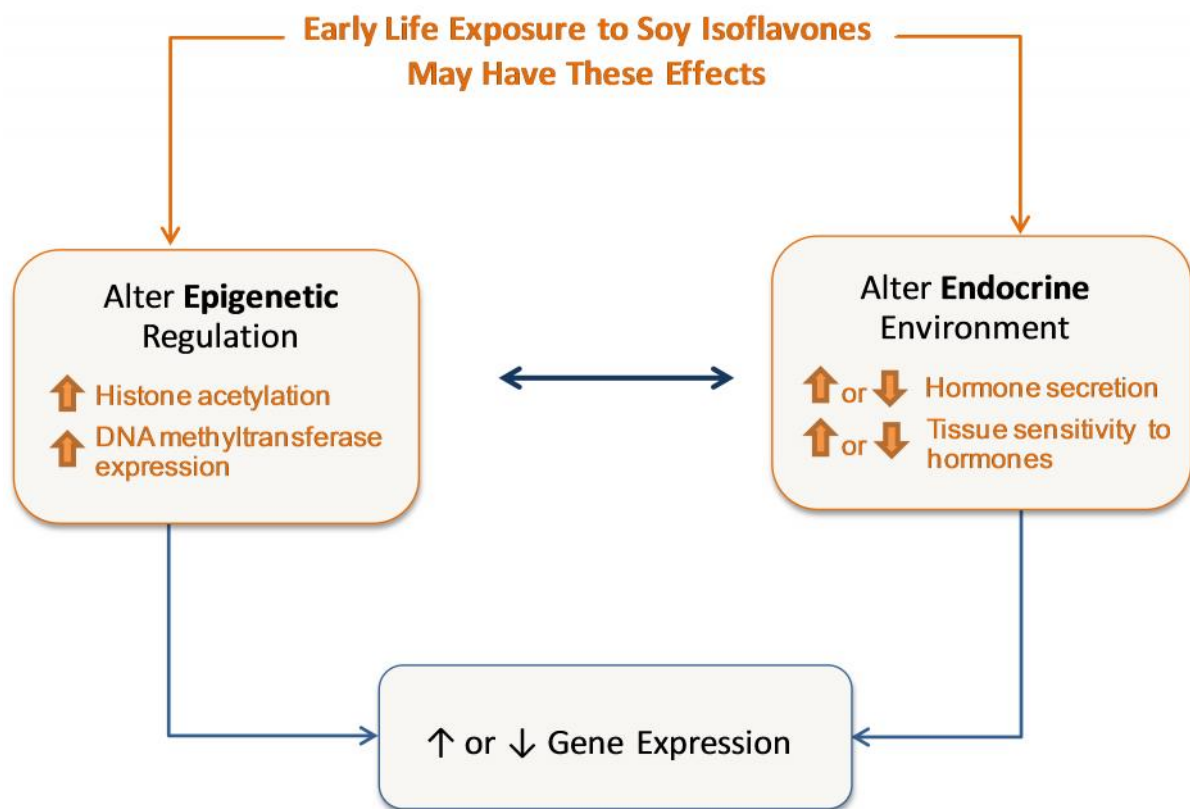


Figure 2-4. A schematic showing that early life exposure to ISO can alter phenotypic expression by changing epigenetic regulation and/or endocrine environment.

acetylation of core histones [18], increase the overall expression of DNA methyltransferases in uterine tissue [132] and alter the circulating concentration of growth and stress hormones. Taking that epigenetic and endocrine processes are not mutually exclusive, changing one may inherently affect the other (Figure 2-4), and so it is important to consider the role of ISO in epigenetic and endocrine regulation.

2.6.1 Epigenetic regulation

By definition, epigenetics is a term used to describe heritable changes in gene expression that occur without altering the genomic sequence [61]. These heritable changes are established during cell differentiation by methylation of cytosine bases in DNA, post-translational modification of histone proteins or positioning of nucleosomes along the DNA, and are maintained throughout multiple cycles of cell division [133]. In addition, each of the epigenetic mechanisms includes regulation by non-coding RNAs, such as microRNAs, small RNAs and long or large RNAs [134]. Therefore, by influencing the accessibility of several factors (i.e. transcription factors, enzymes, co-activators, co-repressors, etc.) to the genetic material of the cell, epigenetics provides a mode for cells to develop distinct identities while containing the same genetic information. These distinct identities can create a range of adaptive phenotypes some of which are well suited to survive in their given environment and some of which are not. A rapidly increasing body of literature is showing that early life exposure to nutrients and bioactive food components can modify epigenetic processes and reduce the risk of disease. As such, we searched the literature for epigenetic studies and found that early life exposure to ISO can increase DNA methylation of CpG sites upstream or near the promoters of protein-coding genes.

Offspring of viable agouti (A^{vy}) mouse dams fed a diet rich in genistein, at a level of exposure that is comparable to that of human infants fed soy protein formula, had increased

DNA methylation of CpG islands upstream of the agouti promoter that persisted into adulthood [15]. These marked changes in DNA methylation were associated with a lower body weight and a shift from the yellow to a dark brown coat color. In line with this evidence, a recently published study showed that 12 week old C57BL mice who were exposed to genistein during gestation had increased DNA methylation of certain repetitive elements, which were associated with a marked down-regulation of estrogen-responsive genes (*Grin2d*, *ApoE*, *Cdkn1a*, *MacroD*, *Vegfa*, *Hdac6*, *Tacc1*, *Abcc5*, *Ctsd*, *Ccnd1*, *Pcna*, and *Igf2*) and genes involved in hematopoiesis of bone marrow cells [135]. Thus, lifelong changes induced by early life exposure to ISO may in part be mediated by DNA methylation of CpG islands³.

From a molecular perspective, DNA methylation is driven by a group of enzymes called DNA methyltransferases. To date, three DNA methyltransferases have been identified that regulate DNA methylation [61]. DNA methyltransferase-1 (Dnmt-1) exists in two different isoforms (Dnmt-1o and Dnmt-1s) and is responsible for maintaining patterns of CpG dinucleotides throughout replication cycles. The Dnmt-1o isoform is expressed in oocytes and early embryos where it maintains the expression of imprinting genes but is absent from adult tissue, while the Dnmt-1s isoform is expressed in oocytes, embryos and all adult tissues. DNA methylation de novo is catalyzed by Dnmt-3a and -3b, which are strongly expressed in differentiating cells such as early embryos and developing germ cells. In general, DNA methyltransferases recognize the CpG dinucleotides of palindromic sequences and catalyze the transfer of methyl groups from methyl donors to the cytosine residues. As such, changes in DNA methyltransferase expression or the availability of methyl

³ DNA regions of 200 base pairs in length that have more than 50% of base pairs represented by CpG dinucleotides

donors/acceptors can alter DNA methylation patterns. While DNA methylation can occur in many parts of the DNA it has most profound effect on gene expression when it occurs in or near a gene promoter [134]. In the human genome, at least 60% of the promoters of protein coding genes contain CpG islands [136], and the density of methylation at these sites determines the rate of gene transcription.

A recently published study showed that exposure to an environmental estrogen (i.e. DES) for the first 5 or 14 days of life reduced the expression of DNA methyltransferases (Dnmt-1 and Dnmt-3a) in the uteri of C57BL mice [132]. These changes in DNA methyltransferase expression resulted in altered DNA methylation with 5 genomic loci being demethylated and 5 other loci being hypermethylated. No studies to date have investigated whether early life exposure to ISO alters DNA methyltransferase expression but, such information may help to uncover the mechanism(s) by which early life exposure to ISO improves bone outcomes at adulthood.

Estrogen-like compounds may also modulate DNA methylation and gene transcription by affecting histone acetylation [134]. An in vitro study showed that through an estrogen receptor- α and - β mediate mechanism, both 17 β -estradiol and ISO (i.e. genistein, equol) increase acetylation of core histone NH₂-terminal tails [18]. In general, histone acetylation is characterized by an attachment of an acetyl group to a lysine residue and results in the neutralization of its positive charge. Given the role of histone tails in the organization of high order structure, the neutralization of the positive charge on the histone tail decreases the interaction of the N termini of histones with negatively charged phosphate groups of DNA. As a result, the tightly packed chromatin structure is transformed into a more relaxed structure that is associated with recruitment of various transcription factors and enzymes.

When this happens, DNA methyltransferases in the presence of methyl donors and cofactors work together to increase DNA methylation. In agouti mice, supplementation with dietary methyl donors (i.e. FA, vitamin B12, choline and betaine) during pregnancy and lactation increased the rate of methylation at seven A^{vy} pseudoexon 1A sites, which in turn caused a shift in coat color from yellow to dark brown and reduced total body weight [16, 137]. These effects were similar to those observed with genistein exposure [15].

A follow-up study compared how FA and genistein affect DNA methylation in bisphenol A-treated agouti mice that exhibit hypomethylation at nine CpG sites upstream of the A^{vy} pseudoexon promoter. Maternal supplementation with FA or genistein negated the DNA hypomethylating effect of bisphenol A and in turn, caused a similar shift in coat color from yellow to dark brown [91]. No studies have examined how the combination of FA and ISO modulates epigenetic regulation. However based on the published studies, it is possible that during sensitive windows of development ISO and FA (a methyl donor) have a common mechanism through which they regulate DNA methylation and gene transcription. We hypothesized that exposure to ISO will activate core histone acetylation, which will loosen up the DNA and enable enzymes and transcription factors to more easily catalyze the formation and breakage of disulfide bonds. These biochemical reactions in the presence of supplemental levels of FA – a methyl donor – will increase DNA methylation of CpG site in the promoter of protein-coding genes and in turn, alter gene expression. In bone, the genes activated (i.e. OPG, BMP) or suppressed (i.e. RANKL) by early life exposure to supplemental FA+ISO will promote bone morphogenesis and bone formation, which in turn will lead to higher BMD, bone structure, bone turnover and bone strength at adulthood in both male and female CD-1 mice. If our hypothesis holds true, this work will provide a basis

for future prospective studies as many infants in North America are being exposed to high levels of FA and/or ISO during development and the risks of suboptimal bone development are rapidly increasing with the ever-growing aging population.

Currently, women of child-bearing age are encouraged to eat folate-rich foods and take a supplement containing 400 µg of FA [138] in order to prevent pregnancy induced anemia and correct abnormal folate metabolism or subtle folate inadequacy that may result in neural tube defects. In addition, many countries, including United States and Canada, have introduced mandatory FA fortification of grain products to ensure that all women of childbearing age have adequate levels of folate before getting pregnant and reduce the rate of neural tube defects. Due to these initiatives many women and men have higher (>1360 nmol/L) than recommended folate concentrations [10]. If some of these individuals also feed their neonates with soy protein formula that naturally contain high amounts of ISO (32-47 mg/L) and are fortified with FA (level of fortification is 0.2-0.4 mg of FA per 100 mL of formula) [8, 9], their infants may have markedly higher concentration of circulating ISO (10 fold higher) and FA (2 fold higher) than infants fed breast milk or cow milk formula [7-9]. For this reason, there is a need to better understand how early life exposure to FA and ISO affects growth, development and future health of vertebrates. To date, one study showed that male and female rats exposure to FA and ISO in combination during development had greater protective effects on post-neural tube closure in male and female offspring than either treatment alone [19]. This effect was in part due to reduced DNA damage and neuron apoptosis. No studies have investigated how combining FA and ISO affects bone development. *Thus, we examined whether early life exposure to ISO in combination with a supplemental level of FA provides greater benefits to bone outcomes (i.e. BMD, bone*

structure, serum markers of bone metabolism and bone strength) of female (Study 3) and male (Study 4) CD-1 mice at adulthood than either treatment alone.

2.6.2 Altered endocrine environment

In vitro and animal studies have shown that ISO, like other selective estrogen receptor modulators, bind to estrogen receptors and exert estrogen agonistic and antagonistic effects. Two estrogen receptors have been identified to date: estrogen receptor- α (expressed in the uterus, ovaries, testes, hypothalamus, kidney and bone) and estrogen receptor- β (expressed in the ovaries, prostate, heart, vessels, brain, bladder and bone). Both receptors have an N- and a C-terminal region. The N-terminal region, which regulates gene transcription at a promoter, is highly conserved between the two estrogen receptors [139] while, the C-terminal region that is involved in ligand binding is poorly conserved, sharing only 56% homology between the two estrogen receptors [140]. ISO can bind to both estrogen receptor subtypes but, have a slightly higher binding affinity for estrogen receptor- β [141] which in turn can lead to different conformations of the activated receptor, different recruitment of co-regulators and different transactivation capacities [142]. Therefore, the tissue specific responses to ISO are somewhat determined by the level of expression of estrogen receptor- α and - β .

It is well documented that estrogen receptor- α and - β have expression patterns that are tissue-specific. Estrogen receptor- α is highly expressed in the uterus, kidney and the pituitary gland, while estrogen receptor- β is the predominant receptor in the prostate, ovaries, vascular endothelium and the central nervous system [143]. Because estrogen receptor- α and - β are both present in bone [144, 145] and breast tissue [146], it makes these organs more difficult to study on a molecular level. As discussed in section 2.5, early life exposure to ISO improves skeletal development of CD-1 mice at sites rich in trabecular bone (i.e. femur neck

and lumbar spine) [12, 54]. Interestingly, trabecular bone expresses both estrogen receptor subtypes with higher abundance of estrogen receptor- β , while cortical bone expresses high levels of estrogen receptor- α with little or no estrogen receptor- β [147, 148]. Estrogen receptor- α has at least eight promoters that contain CpG sites and is tightly regulated by DNA methylation [149]. An in vitro study using Caco-2 cell line showed that exposure to genistein increase promoter methylation of estrogen receptor- α as well as gene transcription of both estrogen receptor- α and - β . Thus, it is possible that by increasing DNA methylation of the CpG sites in the promoter of estrogen receptor- α , early life exposure to ISO can alter estrogen receptor signaling mechanisms. The major estrogen receptor-mediated mechanisms, through which ISO may be able to trigger biological responses that are normally evoked by physiological estrogens, include: classical ligand-dependent activation of gene transcription, gene activation independent of DNA binding, ligand independent activation of gene transcription, and activation of non-genomic signaling cascades (Figure 2-5).

In the classical pathway, ISO diffuse through the plasma membrane and bind to the estrogen receptors (Figure 2-5-1) [151]. The binding induces a conformational change in the receptor that causes the dissociation of chaperone proteins, such as heat-shock protein 90. The hormone-receptor complex then dimerizes and is translocated into the nucleus, where it binds to a specific estrogen response element in the promoter region of the response genes. This triggers the recruitment of co-regulatory proteins including chromatin-remodeling complexes, co-activators and co-repressors, which help to activate or suppress gene transcription. X-ray crystallography studies examining the dimerization of estrogen receptor complex indicate that estrogen receptor- α and - β protein helices are crucial in determining whether ISO induce an agonistic or an anti-agonistic effect [150]. When both estrogen

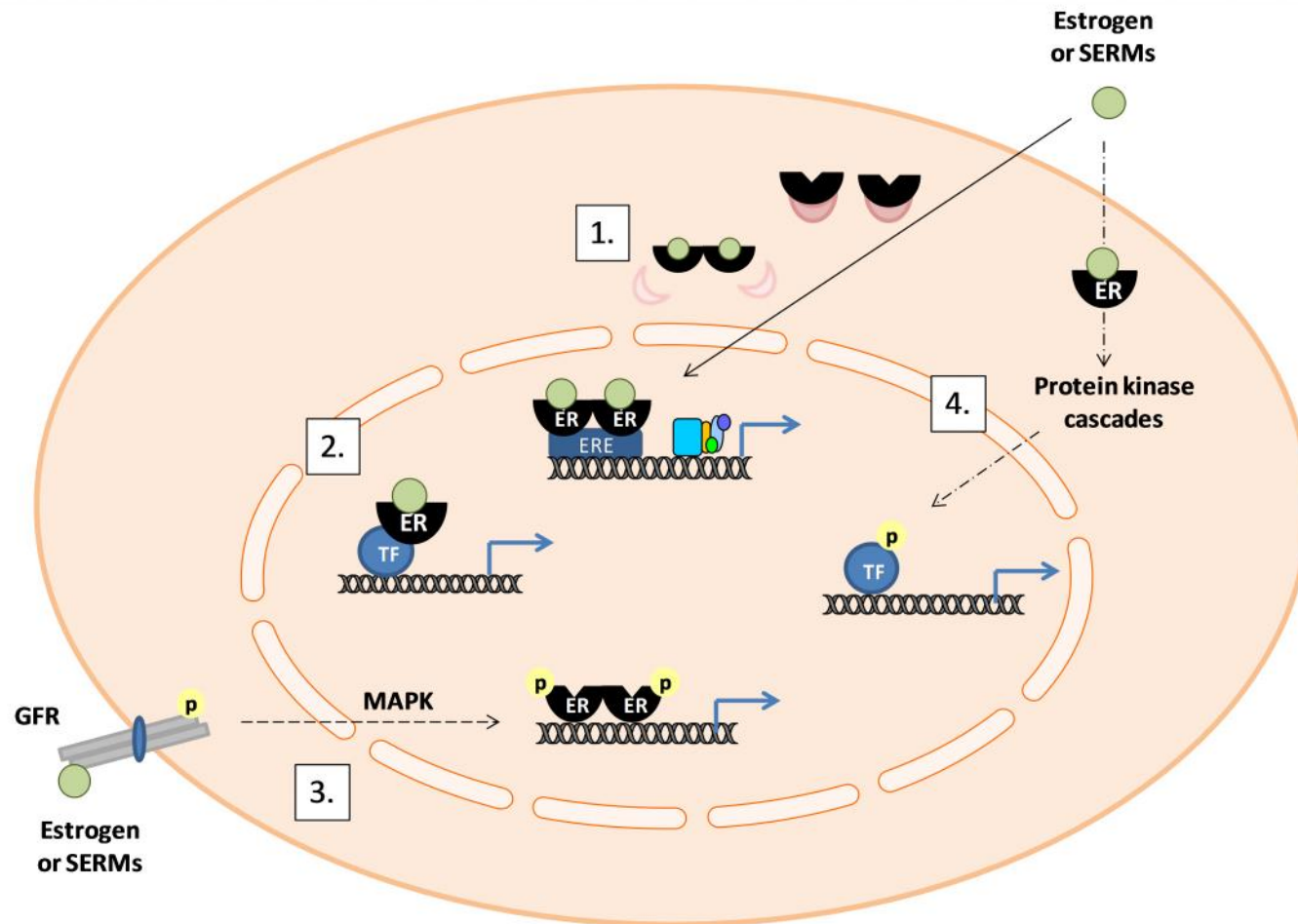


Figure 2-5. Schematic diagram of ER signaling.

1. Classical ligand-dependent activation of gene transcription. 2. Gene activation independent of DNA binding. 3. Ligand-independent transcriptional activation. 4. Activation of non-genomic signaling cascades. Modified from Björnström & Sjöberg, 2005 [151].

receptor subtypes are present in a single cell, the ability to form homodimers or heterodimers provides a potential mechanism for tissue specificity that may be ligand specific. For this reason, it is important to investigate how individual ISO as well as ISO mixtures modulate biological responses in estrogen sensitive tissues including bone, ovaries, uteri and adipose tissue. We have previously shown that a mixture of genistein and daidzein does not induce greater benefits or adverse effects on bone than either treatment alone [54]. Thus, in the presented research the combination of genistein and daidzein was investigated.

Gene activation independent of DNA binding can occur without the ISO-estrogen receptor complex binding to the estrogen response element (Figure 2-5-2) [151]. Instead, the hormone-receptor complex interacts with DNA bound proteins that function as transcription factors or co-regulators and activate gene transcription. Some examples of transcription factors that have been shown to interact with estrogen receptors include activator protein-1, specificity protein-1 and cyclic AMP response element binding protein [152-154]. To date, no studies have explored how ISO regulate the expression of these transcription factors. However, exposure to DES for the first two weeks of life was shown to decrease specificity protein-1 expression in the uteri of C57BL mice [132] indicating that estrogen-like compounds may evoke estrogen receptor induced gene transcription without physically interacting with estrogen response element. Based on this research, we examined how exposure to ISO for the first 10 days of life affects the expression of genes in the femur of CD-1 mice exposed to ISO and FA.

In the non-classical ligand independent pathways, estrogen-like molecules indirectly regulate gene transcription by stimulating cross talk between estrogen receptors and growth factor signaling pathways (Figure 2-5-3) [151]. For example, 17β -estradiol can activate

growth factor receptors, such as epidermal growth factor receptor and IGF-I receptor, which trigger the recruitment of specific promoter complexes that interact with DNA-bound transcription factors and stimulate phosphorylation and activation of estrogen receptor-mediated gene transcription.

It has also been hypothesized that ISO can interact with steroid or non-steroid hormone receptors on the cell membrane and cytoplasm to trigger non-genomic signaling cascades that recruit secondary messengers (i.e. nitric oxide, receptor tyrosine kinases, G-protein-coupled receptors and protein kinases) and lead to downstream cytoplasmic or transcriptional events (Figure 2-5-4) [155, 156]. For example, activation of estrogen receptors in the plasma membrane of osteoblasts and osteoclasts can induce rapid signaling pathways that stimulate apoptosis of osteoclasts, inhibit apoptosis of osteoblasts and in turn promote bone growth [157-160].

In summary, there are several estrogen receptor-mediated signaling mechanisms through which ISO have the potential to induce biological responses. However, the underlying details of these mechanisms are poorly understood. One strategy for uncovering some of the mechanistic details (i.e. enzymes, transcription factors and receptors) is to identify the plasticity genes directly responsive to ISO. Thus, in this thesis a genome-wide search was performed and a list of potential gene candidates was created. Further investigations into the biological significance of the uncovered genes will not only shed light on our current understanding of how ISO and FA program bone development but, will also lead to predictions of other physiological responses that may be evoked by early life exposure to ISO and FA. Moreover, a particular area of interest is reproductive health as we have seen through history that estrogen-like compounds can disrupt the structure and

function of uteri, ovaries and testes. Therefore, examining how early life exposure to ISO affects gene expression as well as structural and functional development of reproductive organs is important for determining whether or not ISO should be recommended or restricted in early life.

2.7 Fertility and reproductive health of animals exposed to ISO

Since the 1940's there has been interest in the relationship between ISO and fertility. Initially, it was observed that sheep grazing on a particular type of clover that is rich in ISO developed an infertility syndrome [161, 162]. Sheep had reduced ovulation, structural abnormalities in the reproductive track combined with high urinary concentrations of equol (metabolite of daidzein). Reduced fertility was also observed in cows grazing on red clover fields and cheetahs eating soy-based diet [163, 164] suggesting that ISO may function as endocrine disrupting chemicals. By definition, endocrine disrupting chemicals are environmental substances that “interfere with synthesis, secretion, transport, metabolism, binding action or elimination of natural hormones in the body that are responsible for homeostasis, reproduction, development and behaviour” [165]. For this reason, studies investigating biological effects of ISO in laboratory animal models should consider the wide range of physiological effects that may occur as a result of endocrine disrupting chemical exposure. In terms of reproductive health, this involves examining structural and functional development of reproductive organs, endocrine function, timing of puberty, sexual behavior and gamete production or fertility.

Early observations that showed adverse effects of ISO on reproductive health in farm animals (sheep and cow) have led to evaluating the estrogenic activity of ISO and their effect on reproductive development in laboratory animals (Table 2-4). Collectively, these studies

Table 2-4. Summary of animal studies: Effects of early life exposure to soy or ISO on reproductive health

Author	Model	Exposure Time	Followed to	ISO Dose	Route	Findings
Jefferson et al. 2007 [90]	Mouse (CD-1) male + female	Postnatal day 1 to 5	24 weeks	Genistein (0.5, 5, 25, 50 mg/kg body weight/day)	Subcutaneous	▪ Low doses of genistein (0.5-5 mg/kg body weight/day) did not disrupt ovarian function, estrous cycling, fertility, ovarian differentiation and uterine wet weight at 6 months of age ▪ Higher dose of genistein (25 mg/kg body weight/day) increased uterine wet weight and reduced fertility by 50% ▪ At 4 months of age, mice treated with genistein (50 mg/kg body weight/day) had altered ovarian differentiation that increased multioocyte follicles and at 6 months ovarian function, estrous cycling and mammary gland development were altered Conclusion: Neonatal exposure to a low dose of genistein did not have an adverse effect on reproductive health but a high dose of genistein altered structural and functional development of reproductive organs
Padilla-Banks et al. 2006 [167]	Mouse (CD-1) female	Postnatal day 1 to 5	4, 5 or 6 weeks	GEN (0.5, 5 and 50 mg/kg body weight/day)	Subcutaneous	▪ Exposure to 50 mg of genistein altered mammary gland growth and development (lower number of terminal buds and branching); while 5 mg of genistein induced minor changes in dilation of mammary ducts ▪ All doses of genistein reduced estrogen receptor-α expression, and increased estrogen receptor-β and progesterone receptor expression ▪ Mice exposed to 50 mg of genistein did not deliver live pups Conclusion: Neonatal exposure to a high dose of genistein altered the structure of reproductive organs and prevented delivery

Author	Model	Exposure Time	Followed to	ISO Dose	Route	Findings
Takashima-Sasaki et al. 2006 [168]	Mouse male + female	Beginning of gestation to postnatal day 56	3, 5 or 8 weeks	Genistein (30 mg/kg body weight/day) daidzein (18.5 or 1.5 mg/kg body weight/day)	Oral via lactation and diet	- Exposure to genistein or daidzein had no adverse effects on male reproductive function - Female exposed to ISO had early puberty and production of multioocyte follicles Conclusion: Early life exposure to genistein had an adverse effect on female but not male reproductive health
Jefferson et al. 2005 [123]	Mouse (CD-1) female	Postnatal day 1 to 5	24 weeks	Genistein (0.5, 5 or 50 mg/kg body weight/day)	Subcutaneous	- No change was seen in the day of vaginal opening - In a dose-dependent manner genistein exposure resulted in abnormal estrous cycles, altered ovarian function, and early reproductive senescence - Fertility was reduced by 40% and 60% with genistein treatment (0.5 and 5 mg of genistein, respectively) 50 mg of genistein resulted in infertility Conclusion: Neonatal exposure to genistein altered estrous cycling and ovarian function which reduced fertility by up to 60%
Fielden et al. 2003 [169]	Mouse male	Gestation day 12 to postnatal day 20	15 and 45 weeks	Genistein (0.1, 0.5, 2.5, 10 mg/kg body weight/day)	Oral gavage	- Genistein exposure had no adverse effects on body weight, anogenital distance, seminal vesicle weight, testis weight, sperm count, or sperm motility <i>In vitro</i> fertilization of epididymal sperm was 17% higher in the high-dose group Conclusion: Perinatal exposure to genistein did not have an effect on structural development of male sex organs but resulted in higher sperm fertilization

Author	Model	Exposure Time	Followed to	ISO Dose	Route	Findings
Jefferson et al. 2002 [87]	Mouse (CD-1) female	Postnatal day 1 to 5	PND 5, 12 or 19	Genistein (0.5, 5 or 50 mg/kg body weight/day)	Subcutaneous	Dose-dependent increase in incidence of multi-oocyte follicles Conclusion: Neonatal exposure to genistein had an adverse effect on structural development of reproductive organs
Klein et al. 2002 [170]	Rat male	Beginning of gestation to postnatal day 21	10 weeks	Genistein (Dams were exposed to 0.42 or 25 mg/kg body weight/day)	Oral via lactation	Males exposed to genistein (25 mg/kg body weight/day) had higher thymus mass and T-cell count but lower testosterone concentrations compared to control treated animals Conclusion: Perinatal exposure to genistein altered endocrine secretion
Newbold et al. 2001 [126]	Mouse (CD-1) female	Postnatal day 1 to 5	72 weeks	Genistein (50 mg/kg body weight/day)	Subcutaneous	At 18 months of age, 35% of genistein-treated mice had uterine adenocarcinomas Conclusion: Neonatal exposure to genistein increased the incidence of uterine adenocarcinoma
Delclos et al. 2001 [171]	Rat male + female	Gestation day 7 to postnatal day 50	7 weeks	Genistein (0.5, 2.5, 10, 25, 62.5, 125 mg/kg body weight/day)	Maternal + oral via mother's milk or direct administration	- Genistein (125 mg/kg body weight/day) reduced body weight but increased pituitary glands and renal tube mineralization - In males, genistein (125 mg/kg body weight/day) lowered ventral prostate weight and sperm count - In females, genistein (25-125 mg/kg body weight/day) caused mammary gland hyperplasia, abnormal cellular maturation of the vagina and abnormal development of ovarian antral follicles Conclusion: Exposure to genistein altered structural development of reproductive organs in males and females

This table has been modified from Dinsdale E, 2010 [172].

have provided a wealth of evidence that exposure to ISO during early life negatively impacts reproductive development in adulthood. A review by Dinsdale and Ward [166] provides a thorough overview of these investigations and addresses the strengths and limitations of studying ISO in animal models.

In general, animal studies have identified that exposure to ISO, in particular genistein, can disrupt the estrous cycle, reduce the number of oocyte follicles, impair fertility, reduce the number of live pups, and increase the risk of tumour formation (i.e. uterine fibroids, ovarian and mammary gland cancer) several weeks or months post-treatment (Table 2-4) [84, 87, 89, 90, 123, 124, 126, 127, 167, 173-180]. However, the relevance of these findings to human health has been questioned. First, the doses of ISO used in animal studies has often been higher than can be attained by diet alone [87, 90, 123, 126, 167, 175]. Secondly, genistein – the most abundant ISO in soy – has often been investigated in isolation [87, 90, 123, 126, 167, 169, 175] while soy protein-based foods contain a mixture of genistein, daidzein and glycitein. Finally, the ISO intervention has almost always been introduced in the first 5 days of life [86-90, 123, 124, 181], which represent a shorter time of exposure than infants are fed soy protein formula. Because timing of ISO exposure may determine the physiological effects of ISO, it is important to investigate reproductive health (i.e. structural development of reproductive organs, timing of puberty, sexual behavior and fertility of sexually mature animals) after exposure to ISO at different developmental time points. Exposure to ISO for the first 5 days of life is the traditional exposure time that has been used in many studies while, exposure for the first 10 days of life more closely mimics the period when human infant are fed soy protein formula but avoids the time when mice may have elevated sex steroid concentrations. As already mentioned, mice suckle for the first 21 days of life, which is the stage of development in which human infants are

fed soy protein formula. However, unlike human infants, mice start to reach sexual maturation 3 weeks post weaning - a much shorter duration between neonatal life and sexual maturity – and thus, may have higher concentrations of circulating sex steroids in the last week of suckling than infants fed soy protein formulas have in the first year of life. Avoiding the stage of life when mice approach sexual maturity or have elevated sex steroid concentrations is important when investigating reproductive health as ISO and endogenous sex steroids compete for the same estrogen receptors. For the purpose of studying reproductive health, the first 10-days of life in a mouse model more closely mimics the developmental time frame when infants are fed soy protein formula than 21-day exposure. *Thus, the objective of our fifth study was to determine how 5 versus 10-day exposure to a combination of daidzein and genistein – the most abundant ISO present in soy protein formula - modulates weight and structural development of ovaries and uterus of female CD-1 mice at adulthood.*

2.8 Research Approach

To conduct this research and generate findings that can be used to design prospective clinical studies we first optimized the CD-1 mouse model to closely mimic that of human infants fed soy protein formula; and then tested the effects and mechanism of ISO and supplemental FA, alone and in combination, on BMD, bone structure, bone strength, bone turnover, bone-specific gene expression and reproductive development (Figure 2-6).

2.8.1 Isoflavone metabolism

The first study was designed to determine how route and frequency of ISO exposure affect circulating concentrations of primary and secondary metabolites an hour after ISO administration, which is the time when serum ISO concentrations peak in the CD-1 mouse model [122]. High-pressure liquid chromatography electrospray tandem mass spectrometry was used to

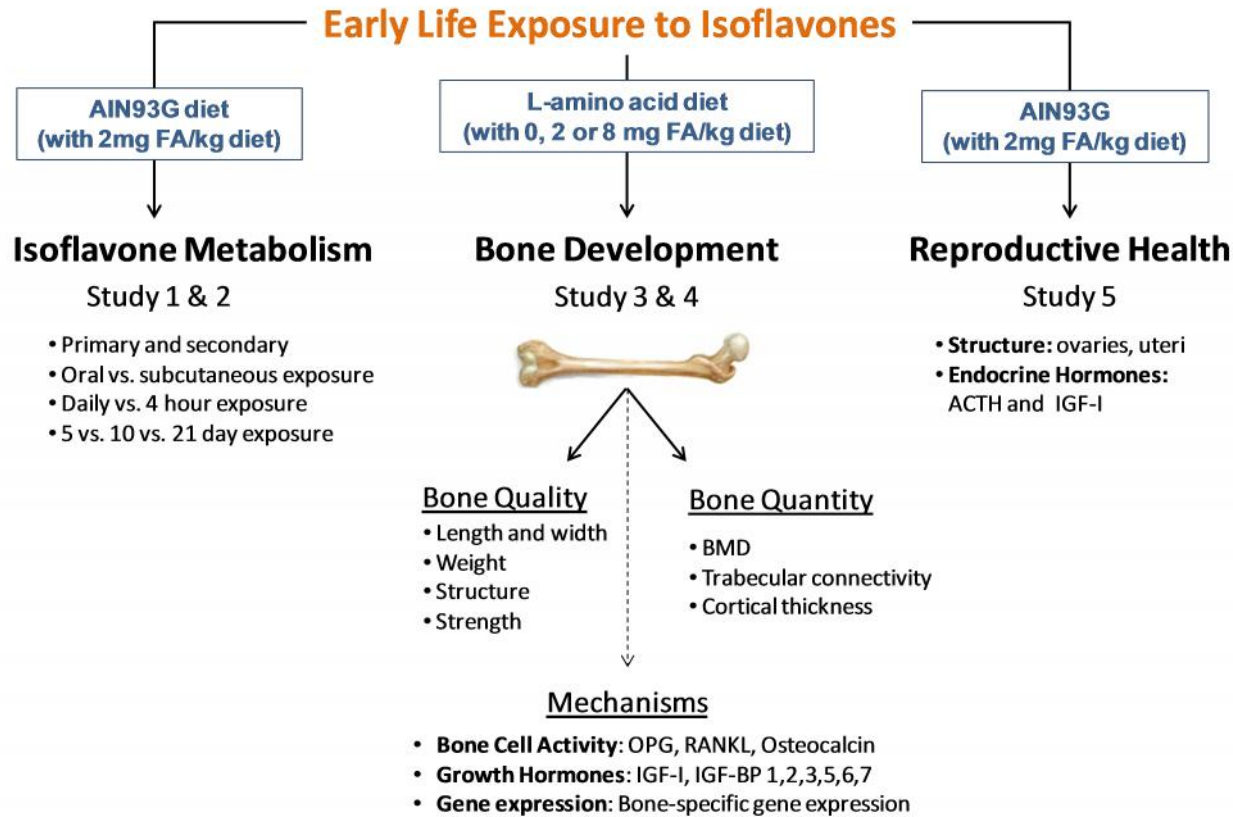


Figure 2-6. Summary of the research approach used to characterize ISO metabolism and study the effects on bone and reproductive development in CD-1 mice.

Study 1 was designed to determine the effects of route and frequency of ISO exposure on circulating concentrations of primary and secondary metabolites an hour after ISO administration using high-pressure liquid chromatography electrospray tandem mass spectrometry. **Study 2** was designed to determine the optimal duration of ISO exposure needed to improve bone quantity, bone quality and bone strength of female mice at adulthood. **Studies 3 and 4** were designed to investigate how early life exposure to low, adequate and supplemental levels of FA with or without ISO modulate bone quality, bone quantity and bone turnover of female and male CD-1 mice at adulthood. Study 3 was also designed to identify whether improvements induced by early life exposure to supplemental FA, ISO or the combination are in part mediated by changes in bone-specific gene expression. **Study 5** was design to how duration of ISO exposure during neonatal life affects structural development of ovaries and uteri in female CD-1 mice. Note: L-amino acid diet has a similar composition of minerals and vitamins as the AIN93G diet.

measure serum daidzein, genistein, equol and O-DMA concentrations because this is the only available technique that can reliably measure ISO metabolites from very small volumes (<100 μ l). This study showed that female and male neonatal mice exposed to ISO via subcutaneous and oral dosing, once daily and every four hours at an equivalent total daily dose have similar total serum genistein, daidzein, equol and O-DMA concentrations as infants fed soy protein formula [122]. As a result, once daily subcutaneous exposure was used to administer ISO to CD-1 mice in Study 2 through 5.

Study 2 followed from Study 1 and was designed to investigate the effects of ISO duration (5 versus 21 days of life) on BMD, bone structure and bone strength. Measurements of BMD at sites susceptible to fracture (i.e. femur and lumbar spine) were performed using dual energy x-ray absorptiometry (DXA). These measures of BMD are very useful in translating animal finding to humans as BMD is routinely used in diagnosing osteoporosis and assessing bone mass over the life span in humans [182]. Bone structure of the femur neck, femur midpoint and lumbar spine was analyzed using microcomputed tomography (μ CT). As part of trabecular analyses, trabecular bone volume (BV/TV), trabecular thickness (Tb.Th.), trabecular number (Tb.N.) and trabecular separation (Tb.Sp.) were measured to provide information about trabecular integrity and bone morphometry. Peak load – the ultimate force a bone can withstand before fracture - was measured by using a materials testing system to determine the overall bone strength and provide information about mechanical integrity [183, 184]. The advantage of using an animal model is that biomechanical testing of bone can be used in combination with other measures of bone integrity to determine the ultimate load that the bone can withstand before it fractures. Since fracture is the major complication of bone diseases the aim of any prevention or treatment strategy is to decrease the incidence of fragility fracture. Thus, the evaluation of peak bone mass

as a major end point in animal models is relevant to clinical scenarios [185]. Performed only on excised bones and more appropriate for small specimens, a three-point bending test and a compression test were used in studies 2, 3 and 4 to assess the mechanical properties of bone.

2.8.2 Bone development

In discovering that longer than 5-day exposure to ISO is needed to provide a benefit to femur BMD and peak load, in our subsequent studies we treated CD-1 mice with ISO for the first 10-days of life. The 10-day window of exposure allowed for epigenetic programming but, avoided the window of development when ISO-induced effects may be less pronounced due to endogenous concentrations of sex steroid becoming elevated. Subsequently, to determine whether ISO improve bone by altering DNA methylation, we examined how exposure to low, adequate and supplemental levels of FA (a methyl donor) during pregnancy and lactation with or without 10-day exposure to ISO affects bone outcomes (i.e. anthropometry, BMD, bone structure, bone strength, bone turnover and bone-specific gene expression) of female and male CD-1 mice at adulthood. Because many bone outcomes were analyzed in this study, to make findings easier to interpret we reported female (Study 3) and male (Study 4) data separately. For both genders, BMD and peak load were measured at the femur and lumbar spine via dual energy x-ray absorptiometry (DXA) and biomechanical stress testing, respectively. The inclusion of both femur and lumbar spine measures provided insight about treatment-induced effects on skeletal sites that differ in the proportion of trabecular and cortical bone. Serum osteocalcin and OPG were measured as markers of osteoblast activity. Serum RANKL was measured as a marker of osteoclast activity. The ratio of OPG to RANKL was determined because it is a common measure of bone formation. In addition, serum concentrations of IGF-I, IGFBP-1,2,3,5,6,7 and ACTH were measured to investigate whether early life exposure to supplemental FA or ISO can alter the growth hormone or stress hormone pathway.

Since females were identified to have greater biological responses to supplemental FA and ISO than males, bone structure and bone-specific gene expression were assessed only in female mice. Both types of analyses provided mechanistic insight. Assessment of BV/TV, Tb. Th., Tb.N. and Tb.Sp. at the femur neck and lumbar spine by μ CT allowed for identification of treatment induced effects on trabecular connectivity, integrity and morphology; while mouse genetics analyzed by microarray was performed on RNA samples extracted from fresh femurs in order to identify a list of genes that may help to explain how supplemental FA, ISO and supplemental FA+ISO modulate bone development.

2.8.3 Reproductive health

In discovering that 10-day exposure to ISO improves bone development of CD-1 mice we wanted to explore its effect on other estrogen-sensitive tissues. Taking that ovaries and uteri of female CD-1 mice had previously been shown to be adversely affected by 5-day exposure to genistein, study 5 was designed to determine the effects of 5 versus 10-day exposure to ISO on markers of ovarian and uterine health using crude weight measurements, qualitative morphologic analysis such as hematoxylin and eosin staining of ovarian and uterine sections, and immunohistochemical analysis of cell proliferation in the endometrial epithelium. Male reproductive organs have been collected but analyses have not been performed to date.

Chapter Three

OBJECTIVES AND HYPOTHESES

3.0 OBJECTIVES AND HYPOTHESES

3.1 Objectives

Overall:

- To delineate the mechanism(s) by which early life exposure to ISO improves bone outcomes at adulthood, while considering adverse effects on reproductive health.

Study 1:

In the CD-1 mouse model:

- Compare the serum levels of genistein, daidzein and its metabolites, equol and O-DMA after subcutaneous injection and oral administration of 5 mg of genistein and 2 mg of daidzein per kg of body weight.
- To determine if frequency of oral administration (daily versus every 4 hours) results in different circulating levels of genistein, daidzein, equol and O-DMA in CD-1 mice after daily exposure to 5 mg of genistein and 2 mg of daidzein per kg of body weight.

Study 2:

- To determine if exposure to ISO (5 mg of genistein and 2 mg of daidzein per kg of body weight) from birth throughout suckling enhances the previously observed positive effects of 5-day exposure to ISO on lumbar spine and, unlike the 5-day exposure, has favorable effects on femur outcomes at young adulthood.

Study 3:

- Determine how early life exposure to low, adequate and supplemental levels of FA (0, 2 and 8 mg of FA per kg of diet, respectively) with or without ISO (5 mg of genistein and 2 mg of daidzein per kg of body weight) affects BMD, bone structure, bone strength and serum markers of bone turnover of female CD-1 mice at adulthood.
- Determine whether early life exposure to supplemental FA and ISO provides greater benefits to bone in female CD-1 mice at adulthood than either treatment alone
- Identify whether early life exposure to supplemental FA or ISO, alone or in combination, can permanently alter the expression of bone-specific genes in **female** CD-1 mice.

Study 4:

- Determine whether early life exposure to ISO (5 mg of genistein and 2 mg of daidzein per kg of body weight) in combination with supplemental FA (8 mg per kg of diet), a methyl donor, results in higher BMD and greater bone strength at femur and lumbar spine than either treatment alone in **male** CD-1 mice at adulthood.

Study 5:

- Determine how 5 versus 10-day exposure to ISO (5 mg of genistein and 2 mg of daidzein per kg of body weight) modulate organ weight and structural development of ovaries and uteri of female CD-1 mice at adulthood.

3.2 Hypotheses

Study 1:

- Because subcutaneous delivery of ISO bypasses the gastrointestinal tract and liver metabolism, mice exposed to 5 mg of genistein and 2 mg of daidzein per kg of body weight via subcutaneous treatment will have significantly higher serum daidzein and genistein concentrations than mice given the same level of ISO orally. Route of ISO delivery will not affect serum equol and O-DMA concentrations of 5-day old CD-1 mice because their intestinal microbiota is not yet developed. Thus, the concentration of equol and O-DMA will be minimal in early life.
- The serum half-life of ISO in neonatal mice is 12-19 hours and thus, the frequency of ISO exposure will not have an effect on serum daidzein and genistein concentrations of 5-day old CD-1 mice administered a daily dose of 5 mg of genistein and 2 mg of daidzein per kg of body weight.

Study 2:

- Female mice exposed to 5 mg of genistein and 2 mg of daidzein per kg of body weight for the first 21 days of life will have higher BMD, trabecular connectivity, and greater bone strength of the femur and lumbar spine at young adulthood compared to 5-day treated animals. This is because the 21-day window of exposure will allow ISO to bind to estrogen receptors for a longer period of time than 5-day exposure and in turn have more profound estrogen-like effects in tissues.

Study 3:

- Because both ISO and FA can increase methylation of CpG sites in the promoter of protein coding genes, early life exposure to one or both of these compounds will improve bone outcomes of female mice at adulthood by permanently changing the expression of genes that regulate the production of enzymes and transcription factors involved in bone morphogenesis and bone formation. As a result, **female** offspring whose mother's are exposed to adequate or supplemental levels of FA (2 or 8 mg of FA per kg of diet, respectively) and are treated with ISO (5 mg of genistein and 2 mg of daidzein per kg of body weight) for the first 10 days of life will have stronger bones with greater BMD and improved bone microarchitecture at young adulthood than females who are treated with corn oil or whose mother's are fed with a diet containing low levels of FA during pregnancy and lactation.

Study 4:

- Because both ISO and FA can enhance DNA methylation of CpG sites in the promoter of protein coding genes, and in turn alter gene transcription, **male** mice exposed to supplemental FA and ISO (8 mg of FA per kg of diet, 5 mg of genistein and 2 mg of daidzein per kg of body weight) during early development will have higher BMD and strength at the femur and lumbar spine than males exposed to either treatment alone.

Study 5:

- Because reproductive tissues are highly sensitive to estrogen-like compounds female mice exposed to 5 mg of genistein and 2 mg of daidzein per kg of body weight for the first 10-days of life will have a higher incidence of hyperplasia in the oviduct, lower number of ovarian corpora lutea, higher number of multiple oocyte follicles, and higher incidence of ovarian and uterine cysts at adulthood than control or 5-day treated females.

Chapter Four

STUDY 1

CIRCULATING ISOFLAVONOID LEVELS IN CD-1 MICE: EFFECT OF ORAL VERSUS SUBCUTANEOUS DELIVERY AND FREQUENCY OF ADMINISTRATION

Modified from:

Kaludjerovic J, Franke A, Ward WE. Circulating isoflavone levels in CD-1 mice: effect of oral versus subcutaneous delivery and frequency of administration. *J Nutr Biochem*. 2012 May; 23(5):437-42.

4.0 STUDY 1 - CIRCULATING ISOFLAVONE LEVELS IN CD-1 MICE: EFFECT OF ORAL VERSUS SUBCUTANEOUS DELIVERY AND FREQUENCY OF ADMINISTRATION

4.1 Abstract

The CD-1 mouse is a commonly used animal model to understand the biological effects of early life exposure to ISO in infants. Most studies using CD-1 mice have administered ISO by daily subcutaneous injection while infants receive oral feeds every few hours. The study objectives were to compare the serum levels of genistein, daidzein and the daidzein metabolites equol and O-DMA, after subcutaneous injection and oral dosing; and to determine if frequency of oral administration results in different circulating levels of ISO using the CD-1 mouse model. From postnatal day 1 to 5, pups randomly received corn oil or ISO (total daily dose=0.010 mg daidzein + 0.025 mg genistein) by subcutaneous injection once a day, orally once a day or orally every 4 hours. On postnatal day 5, one hour post-treatment, mice were sacrificed and serum was collected. Mice treated with ISO had higher ($p<0.05$) serum genistein (female: 2857-3391 ng/mL and male: 483-578 ng/mL) and daidzein (female: 1186-1580 ng/mL and male: 248-322 ng/mL) concentrations versus control (5-20 ng/mL) mice, regardless of route or frequency of administration and were similar among dosing strategies. Serum concentrations of genistein and daidzein were higher ($p<0.05$) among females (genistein: 2714 ± 393 ng/mL and daidzein: 1205 ± 164 ng/mL) than males (genistein: 521 ± 439 ng/mL and daidzein: 288 ± 184 ng/mL) across treatment groups. Serum equol and O-DMA concentrations were negligible (<3 ng/mL) across groups. In conclusion, different routes of delivery and frequency of administration resulted in similar serum levels of genistein, daidzein, equol or O-DMA.

4.2 Introduction

ISO, with potential hormonal effects, are abundant in the food supply. To date, more than 300 plants have been identified that contain phytoestrogens such as ISO, lignans and coumestans [186]. ISO are most predominant phytoestrogens leading to particularly high exposure during early life when infants are fed soy protein formula that contain substantial levels of ISO [7]. Developing rodent models that mimic the exposure of human infants fed soy protein formula have proven challenging. While two studies have provided genistein once daily by oral feeding [88, 181], a rodent's small size at birth, particularly for mice, can complicate this route of administration from birth through the first few days of life. Multiple oral feedings per day to mimic infant feeding patterns can also result in marked stress and a higher risk of death due to perforating tissues or aspiration. Moreover, the volume of soy formula needed to deliver levels that are equivalent to human doses of ISO is not possible; thus purified ISO are required to attain appropriate serum levels in young rodent models. Because of some or all of these reasons, the majority of studies that have used rodent models to study the biological effects of soy during early life have administered purified or synthetic ISO by subcutaneous injection during the first days of life [12, 13, 54, 87, 89, 90, 123, 124, 187].

As noted in the recent report of the *NIH Workshop on Designing, Implementing and Reporting Clinical Studies of Soy Interventions*, existing animal model systems provide useful information that supports the results from clinical and epidemiological studies, but are not entirely comparable to humans when assessing metabolism or health effects of soy because of fundamental differences in ISO exposure [92]. The report also states that "while dietary exposure in preclinical models is preferred, it is critical that blood levels be evaluated and be comparable to the blood levels observed in human populations consuming ISO

containing products" [92]. Indeed, we have previously shown that subcutaneous administration of synthetic ISO, daidzein and genistein, to young CD-1 mice resembles circulating levels of human infants fed soy protein formula [8, 54]. Thus, despite the fact that subcutaneous delivery of ISO bypasses the gastrointestinal tract and liver metabolism, similar serum ISO levels are observed. There are also similarities in the metabolism of ISO among young CD-1 mice and human infants; both have a limited ability to metabolize daidzein to its metabolite, equol [8, 54]. These similarities in metabolism are important because some studies have shown that equol is more biologically active than daidzein [188].

Although the CD-1 mouse model is a commonly used animal model to understand the biological effects of early life exposure to ISO in human infants, no studies have directly compared the differences in circulating levels of ISO and their metabolites after oral versus subcutaneous delivery of synthetic ISO in this mouse model. Since human infants feed every few hours, it was also important to identify whether once daily oral administration versus multiple oral dosing per day results in similar levels of circulating ISO in CD-1 mice. The objectives of this study were twofold: to compare the serum levels of daidzein, genistein and their metabolites, equol and O-DMA after subcutaneous injection and oral dosing; and to determine if frequency of oral administration results in different circulating levels of the ISO and their metabolites using the CD-1 mouse model. Findings from this study will more fully characterize this model system.

4.3 Methods

4.3.1 Animals and treatment

Seven-wk old CD-1 mice (n=13) (Charles River Laboratories, St. Constant, QC) were bred harem-style in the Department of Comparative Medicine at the University of Toronto.

Once females were identified as being pregnant they were housed in individual cages under standard environmental conditions (23°C, 50% humidity, 12:12h light-dark cycle), and were fed a control diet (AIN93G, Dyets Inc., Bethlehem, PA) devoid of any estrogenic compounds [189]. Water was provided *ad libitum*. Thirteen litters with 8-12 pups were cross-fostered at birth and randomized into 1 of 6 groups. Control pups received corn oil as it is used as the vehicle for ISO [12, 13, 54]. ISO mixtures were prepared fresh prior to treatment by solubilizing 12.5 mg of synthetic genistein and 5 mg of synthetic daidzein in dimethyl sulfoxide [54]. The volume of both the oral and subcutaneous treatment was 20 µl, and the total daily dose that each pup was exposed to was 0.010 mg of daidzein and 0.025 mg of genistein. Oral treatment was administered using a 0.6 cc syringe that has a plastic tip and subcutaneous injection was administered using a 3/10 cc insulin syringe that has a 30-gauge needle and a 12.7 mm needle length. To promote ingestion of oral dose, pups were held ventral side up while suckling on the syringe and their abdomen was stroked until the treatment was swallowed. On postnatal day 5, one hour after the last treatment, pups were euthanized by decapitation and their samples were collected. Blood samples were transferred into BD Vacutainer tubes containing clot activator and gel for serum separation, and were left to sit at room temperature for 15 min. Thereafter, blood samples were centrifuged at 3000 g for 15 min and serum samples were carefully removed from the BD Vacutainer tubes and transferred into plain polypropylene tubes. The samples from 2-4 pups were pooled and stored in the -80°C until serum analysis was performed. All protocols for animal use and treatment were approved by the University of Toronto Animal Care Committee and were in compliance with the guidelines of the Canadian Council on Animal Care [190].

4.3.2 Serum measures of ISO metabolism

Serum concentrations of genistein, daidzein, equol and O-DMA were determined by high pressure liquid chromatography electrospray tandem mass spectrometry as previously updated [191, 192]. In brief, 0.065 mL serum pooled from several pups were mixed with 0.01 mL of a methanolic solution of triply labeled ^{13}C daidzein, equol, and O-desmethyldaidzein (obtained by synthesis from the University of St. Andrews, Scotland) and $^2\text{H}_4$ labeled genistein (obtained by synthesis from the University of Helsinki, Finland) as internal standards followed by incubation overnight with 0.02 mL beta-glucuronidase (from *E. coli* K12, 200 U/mL; Roche Applied Sciences Indianapolis, IN) and 0.075 mL arylsulfatase (from *Helix pomatia* 24,190 U/g; 2mg/mL in 1M pH5 acetate buffer; type H-1 #9626, Sigma, St. Louis, MO) at 37°C. 0.065 mL acetonitrile was added to this mixture, then vortexed briefly, and partitioned into 2 mL dichloromethane. The organic phase was evaporated followed by redissolving the residue in 0.1 mL methanol/0.01% aq. formic acid (1:1). 0.02 mL of this hydrolysate were separated on a Gemini C18 (150 x 2.0 mm; 5 μm) reversed phase column coupled to a Gemini C18 (4.0 x 2.0 mm; 5 μm) direct-connect guard column (Phenomenex, Torrance, CA) using a linear gradient at 0.2 mL/min of Methanol/Acetonitrile/Water=20/20/60 to 30/30/40 in 2.5 min., to 40/40/20 in 5.5 min and back to 15/15/70 in 0.1 min for equilibration before subsequent injections. The mass spectrometric conditions using a model TSQ Quantum Ultra triple quadrupole instrument (Thermo Electron, San Jose, CA) were applied as described in detail previously [191]. Intra- and inter-assay variability ranged from 5-12% depending on the analyte concentration.

4.3.3 Statistical analyses

Based on a published study that measured serum genistein concentration of 5-day old CD-1 mice [59], and consultation with our collaborator (Dr. Adrian Franke), who routinely

measures ISO via mass spectrometry, a sample size of 6 mice/gender/group was considered adequate to observe differences in serum ISO measures between groups.

Statistical analyses were performed using Sigma-Stat (version 3.5, Jandel Scientific). Data are expressed as mean $\pm$ standard error mean (SEM). To analyze serum daidzein, genistein, equol and O-DMA concentrations, 2-way ANOVA was used with treatment and route of exposure as the main effects. Student-Newman-Keul's post hoc test was used to determine differences among groups. Significance was determined as $p < 0.05$.

4.4 Results

4.4.1 Serum measures of soy isoflavone metabolism

Serum genistein (female: 2857-3391 ng/mL and male: 483-578 ng/mL) and daidzein (female: 1186-1580 ng/mL and male: 248-322 ng/mL) concentrations of mice treated with the combination of genistein + daidzein were significantly greater ($p < 0.05$) than that of control (5-20 ng/mL) mice regardless of gender (Figure 4-1, Figure 4-2). The serum concentrations of genistein and daidzein were significantly higher ($p < 0.05$) among females (genistein: 2714 ± 393 ng/mL and daidzein: 1205 ± 164 ng/mL) than males (genistein: 521 ± 439 ng/mL and daidzein: 288 ± 184 ng/mL) across treatment groups. Mean serum concentrations of genistein and daidzein in females administered the combination of genistein + daidzein subcutaneously once a day (genistein: 3391 ± 1334 ng/mL and daidzein: 1580 ± 649 ng/mL), orally once a day (genistein: 2857 ± 888 ng/mL and daidzein: 1186 ± 323 ng/mL) or orally every 4 hours (genistein: 3271 ± 908 ng/mL and daidzein: 1396 ± 568 ng/mL) did not differ (Figure 4-1). Similarly, route of administration or frequency of administration did not result in significantly different circulating levels of genistein or

FEMALES

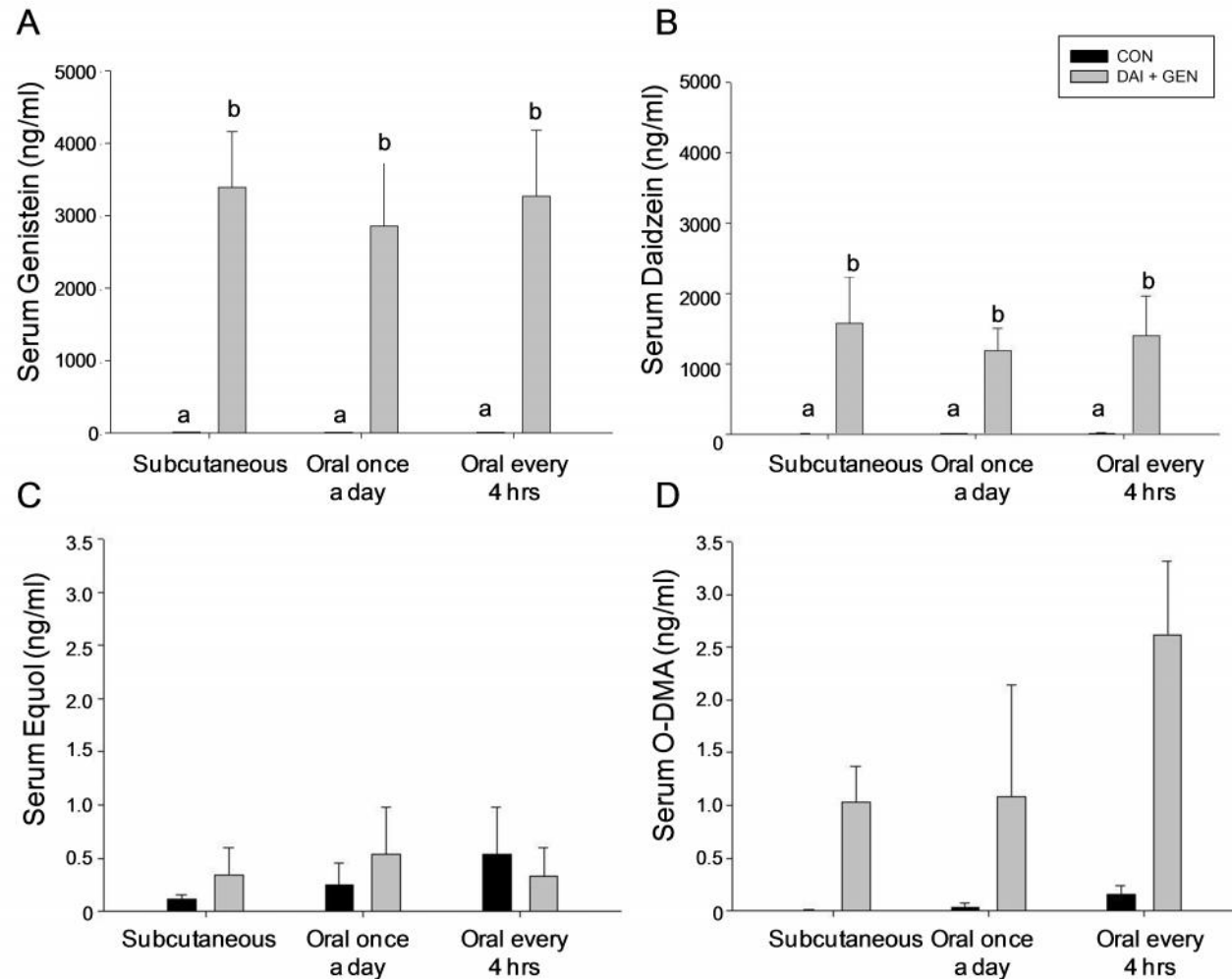


Figure 4-1. Serum concentrations of genistein (A), daidzein (B), equol (C) and O-DMA (D) from female mouse pups one-hour after corn oil (CON) or equal doses of ISO were administered subcutaneously once a day, orally once a day or orally every 4 hours.

* Values are expressed as means $\pm$ SEM. Sample size was n= 5-6 per group with each sample representing serum pooled from 2-4 mouse pups. Labeled means without a common letter differ, $P < 0.05$.

MALES

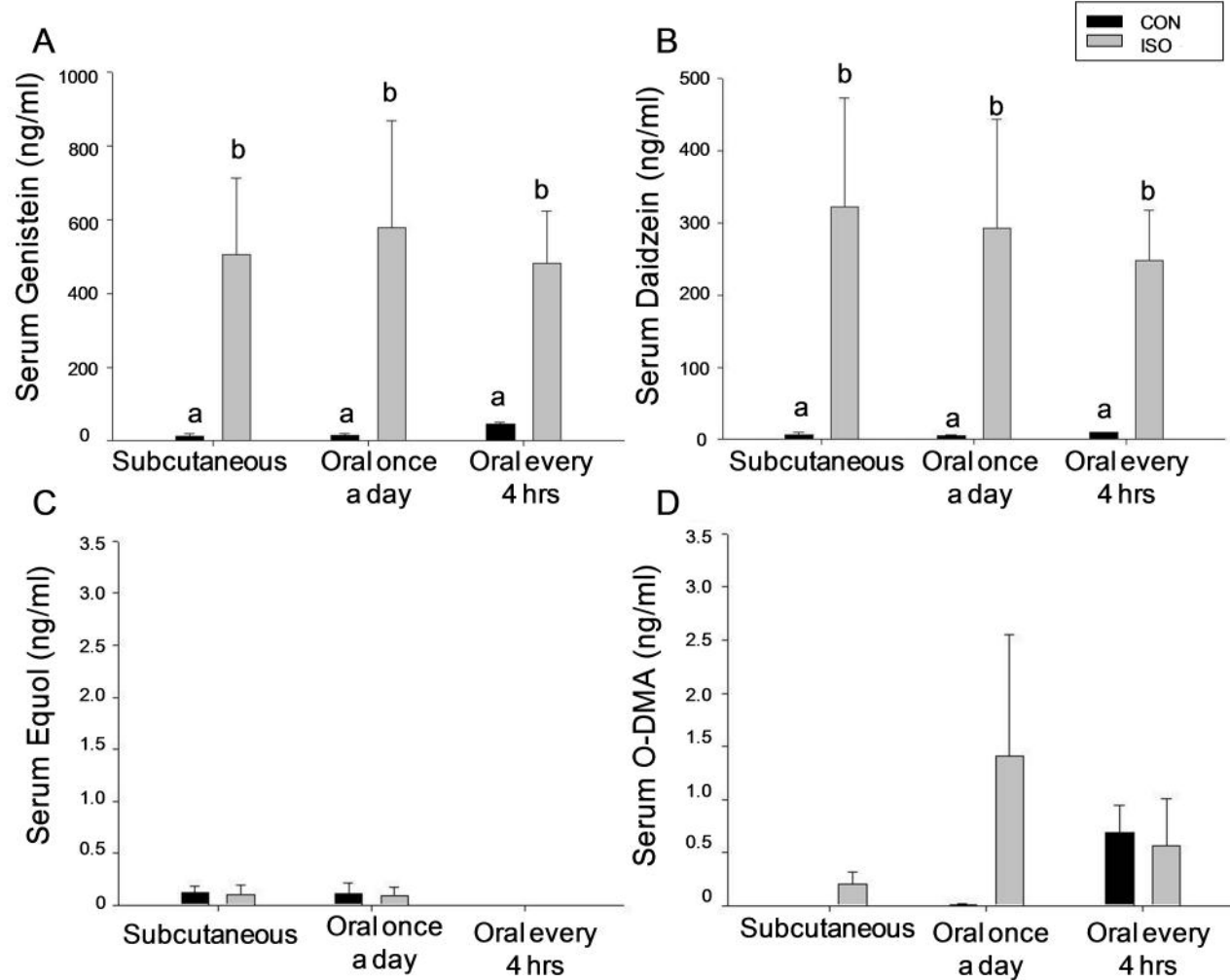


Figure 4-2. Serum concentrations of genistein (A), daidzein (B), equol (C) and O-DMA (D) from male mouse pups one-hour after corn oil (CON) or equal doses of ISO were administered subcutaneously once a day, orally once a day or orally every 4 hours.

* Values are expressed as means $\pm$ SEM. Sample size was $n=4-6$ per group with each sample representing serum pooled from 2-4 mouse pups. Labeled means without a common letter differ, $P < 0.05$.

daidzein among males (Figure 4-2). Males administered genistein + daidzein subcutaneously once a day, orally once a day or orally every 4 hours had mean genistein concentrations that were 504 ± 207 ng/mL, 578 ± 290 ng/mL and 483 ± 141 ng/mL and mean daidzein concentrations that were 322 ± 151 ng/mL, 293 ± 150 ng/mL and 248 ± 69 ng/mL, respectively. Serum equol and O-DMA concentrations were negligible (<3 ng/mL) across groups (Figure 4-1, Figure 4-2).

4.5 Discussion

Findings from this study are first to demonstrate that oral versus subcutaneous delivery, and daily versus multiple oral dosing of genistein in combination with daidzein did not result in significantly different levels of serum genistein, daidzein, equol or O-DMA one hour after treatment was administered on the postnatal day 5. This finding is likely because the aglycone form of genistein and daidzein was administered to neonatal pups both subcutaneously and orally rather than the conjugated form that is present in soy protein. Thus, the hydrolysis of the β -glucose moiety by intestinal microbiota that normally limits the bioavailability and biological activity of ISO given orally was not required prior to uptake [193]. Moreover, the intestinal microbiota that partially metabolizes the aglycones in the intestine is underdeveloped during early neonatal life [7, 8, 194]. Therefore, the aglycones could readily be absorbed in the intestine without being degraded which could explain why the oral administration was as effective as the subcutaneous treatment in elevating total serum ISO. The unconjugated forms were administered to mimic previously published studies that administered ISO as aglycone forms to mouse pups [12, 13, 54, 87, 89, 90, 123, 124, 195]. However, the unconjugated form of daidzein and genistein can be re-conjugated by UDP-glucuronosyl transferases in the intestine and re-secreted into circulation. Thus, it is

possible that if orally ingested aglycones first reach the intestine they may be more readily re-conjugated such that the overall area under the curve for total genistein and daidzein may be less following oral versus subcutaneous exposure. Subcutaneous or oral administration of genistein, but not in combination with daidzein, on serum concentrations has been reported over a 24-hour period [59, 88]. In a recently published study, Jefferson et al. [88] compiled the published data and showed that the dose-adjusted area under the curve for total genistein is lower (12.8 versus 147, respectively) after oral versus subcutaneous administration in 5-day old CD-1 mice. Although care must be taken when extrapolating data across studies, this difference in area under the curve for total genistein between oral and subcutaneous administration likely reflects the bypass of re-conjugation in the gut after injection.

The finding that serum genistein and daidzein concentrations were similar following oral and subcutaneous treatment is consistent with a previous study in which uterine weight was increased to a similar extent regardless of whether ISO was provided orally (25 mg of genistein/kg body weight/day) or subcutaneously (20 mg of genistein/kg body weight/day) from postnatal day 1 through 5 in CD-1 mice [124]. This previous study did not report serum ISO concentrations of mice [124], but the serum ISO data presented in this study suggests that subcutaneous exposure using the aglycone form of ISO may be a suitable model for oral exposure of ISO aglycones. More recently, once daily oral administration of 50 mg genistein/kg body weight/day – a dose that the authors note exceeds the level of exposure to human infants fed soy protein formula - resulted in peak serum levels (genistein: 3.8 ± 1.1 $\mu\text{mol/L}$) [59] that in males were of the same order as measured in the plasma of infants (genistein: 2.53 ± 0.62 $\mu\text{mol/L}$) fed soy protein formula [8], but in females were two fold higher (genistein: 6.8 ± 1.4 $\mu\text{mol/L}$) [59]. However, serum levels of genistein ranged from 1-

5 μ M for most of the day post-dose [59]. A previous study by our group, using a lower dose of ISO (2 mg daidzein + 5 mg genistein/kg body weight/day) in neonatal mice resulted in serum ISO levels (genistein: 2.86 ± 1.78 μ mol/L and daidzein: 1.18 ± 0.21 μ mol/L) [54] that were similar to concentrations measured in the plasma of human infants (genistein: 2.53 ± 0.62 μ mol/L and daidzein: 1.16 ± 0.09 μ mol/L) consuming soy protein formula [8]. To date, the present study is the first to administer multiple oral doses of ISO and directly compare the serum ISO levels achieved with oral and parenteral administration protocols. Whether animal models that administer the aglycone form of ISO can predict the biological effects of glycosides or soybean is uncertain because different forms of ISO vary in bioavailability. The area under the curve and mean concentration for plasma genistein were reported to be approximately 3.7- and 6-times higher in the adult rat model following oral genistein (15 mg/kg body weight/day) exposure compared to genistin (15 mg/kg body weight/day), respectively [196]. In contrast, findings by Jefferson et al. revealed that in young CD-1 mice, 80% of administered genistin (25 mg/kg body weight/day) is converted to genistein and results in higher uterine weight, comparable to the effect of administering genistein at a dose of 20 mg/kg body weight/day [88, 124]. Therefore, developmental factors and timing of ISO exposure may be responsible for variations in bioavailability reported in different studies.

One study [59] to date has shown that the half-life of genistein is longer in the neonatal mouse (12-19 h) than in the adult rat (2.9 h) or adult human (~ 9 h) [197, 198]. Because of this finding, the authors predicted that the longer half-life in the neonatal mice would result in higher serum genistein concentrations with repeated daily dosing from postnatal day 1 through 5 than with a single daily dose [59]. Our findings may suggest to reinvestigate genisteins' half-life in developing CD-1 mice. Early programming of human health has

raised concern that higher concentrations of genistein during critical stages of development may cause permanent and long-lasting changes in human health [199]. To assess whether serum ISO concentrations fluctuate with different dosing regimens, daidzein and genistein were administered orally to mice either once daily or every 4 hours while keeping the total daily ISO dose constant. Our finding that frequency of ISO exposure did not have a significant effect on serum levels of genistein and daidzein one hour post-treatment at the doses tested suggests that future studies using this developing animal model can administer ISO via a single daily administration when the dose is kept at levels applied in this study.

In the present study, serum genistein and daidzein concentrations were significantly higher in female than male neonatal mice. This finding is consistent with another study in which the maximum serum concentrations for total genistein (conjugated and aglycone) was higher among female compared to male CD-1 pups administered 50 mg genistein/kg body weight/day [59]. The difference between males and females in the present study was 85% for genistein and 80% for daidzein while others reported a 44% difference in total genistein concentration between males and females [59]. The lower levels of ISO in males compared to females suggest that males may metabolize ISO faster than females. This finding is supported by the fact that the pharmacokinetics of ISO have been reported to be faster in males than females [187]. The average total excretion of radioactivity in urine and feces of rats administered [^{14}C] genistein was 66% and 33% of the dose in males and females, respectively. In the present study all serum samples were collected approximately one hour post-treatment, so it is expected that males who generally metabolize ISO faster than females [187] have substantially lower serum genistein and daidzein concentrations. A limitation of this study is that the conjugated form of genistein and daidzein was not measured. Thus,

comparison of ISO forms, i.e. conjugated versus aglycone, after subcutaneous versus oral exposure should be determined in a future study.

Serum concentrations of O-DMA and equol, the two metabolites of daidzein, were negligible (<3.5 ng/ml) in 5-day old male and female CD-1 mice. This finding is consistent with studies investigating ISO metabolism during neonatal stages of development. Metabolism of ISO in neonatal mice and human infants is minimal [54] because intestinal bacteria and phase II metabolism needed for conversion of aglycones to secondary metabolites (i.e. equol and O-DMA) is underdeveloped during early life [8, 43]. The specific bacterium/bacteria responsible for equol and O-DMA production in humans have yet to be identified, but *in vitro* and animal studies have suggested that equol and O-DMA are more biologically active than their precursor, DAI. Observational and intervention studies in humans have suggested that the ability to produce equol and O-DMA may be associated with reduced risk of breast and prostate cancers [188].

In conclusion, oral versus subcutaneous delivery, and daily versus multiple oral dosing do not result in significantly different levels of serum genistein, daidzein, equol and O-DMA in the developing CD-1 mouse model at the investigated daily doses. These findings suggest that dose and timing of exposure, rather than route of administration and frequency of exposure, may more strongly modulate biological effects of ISO during early development.

Chapter Five

STUDY 2

ISOFLAVONE EXPOSURE THROUGHOUT SUCKLING RESULTS IN IMPROVED ADULT BONE HEALTH IN MICE

Modified from:

Dinsdale EC*, **Kaludjerovic J***, Ward WE. Longer duration of soy isoflavone exposure during early life results in improved adult bone health in the CD-1 mouse model. Journal of Developmental Origins of Health and Disease (JDOHaD). Aug 2012. 3(4):271-5. (*indicates that first author is shared)

5.0 STUDY 2 – ISOFLAVONE EXPOSURE THROUGHOUT SUCKLING RESULTS IN IMPROVED ADULT BONE HEALTH IN MICE

5.1 Abstract

Exposure to ISO, abundant in soy protein infant formula, for the first 5-days of life results in higher BMD, greater trabecular connectivity and higher peak load of LV at adulthood. The effect of lengthening the duration of exposure to ISO on bone development has not been studied. This study determined if providing ISO for the first 21-days of life, which more closely mimics the duration that infants are fed soy protein formula, results in higher BMD, improved bone structure and greater strength in femurs and LV than a 5-day protocol. Female CD-1 mice were randomized to subcutaneous injections of ISO (7 mg/kg body weight/day) or corn oil from postnatal day 1 to 21. BMD, structure and strength were measured at the femur and LV at 4 months of age, representing young adulthood. At the LV, exposure to ISO resulted in higher ($p<0.05$) BMD, trabecular connectivity and peak load compared to control. Exposure to ISO also resulted in higher ($p<0.05$) whole femur BMD, higher ($p<0.05$) BV/TV and lower ($p<0.05$) trabecular separation at the femur neck, as well as greater ($p<0.05$) peak load at femur midpoint and femur neck compared to the control group. Exposure to ISO throughout suckling has favorable effects on LV outcomes and unlike previous studies using 5-day exposure to ISO femur outcomes are also improved. Duration of exposure should be considered when using the CD-1 mouse to model the effect of early life exposure of infants to ISO.

5.2 Introduction

“Nutritional programming” is a concept used to describe a permanent change in the structure or function of an organism caused by a food or food component during early

development. Early diet may provide a safe and practical approach for optimizing bone health throughout life. While some aspects of programming by early diet can be investigated directly in humans through prospective and retrospective studies, mechanistic studies often require appropriate use of animal models to closely mimic the human scenario. The CD-1 mouse model is commonly used to assess biological effects of exposure to ISO during early life, including effects on bone development [12, 13, 54]. We previously reported in CD-1 mice that neonatal exposure to ISO, at a dose that mimics that of human infants fed soy protein formula, favorably programs the lumbar spine of females at adulthood with no significant improvements at the femur [54]. The benefits included higher vertebral BMD and improved structure (greater trabecular thickness and connectivity) that translate to stronger LV at 4 months of age, which represents young adulthood [13, 54]. While the mechanism of these programming effects has not been elucidated, ISO are selective estrogen receptor modulators that can bind to estrogen receptors to elicit estrogen-like responses in bones, ovaries, uteri, prostate, the central nervous system and the cardiovascular system but inhibit estrogen stimulation in the breast and endometrium [92]. As such, exposure to ISO during sensitive stages of development offers the possibility of permanent alterations in bone-specific gene expression [200] or rapid non-genomic action that modulates a diverse array of intracellular signal transduction cascades that affect processes associated with bone metabolism [201]. To date, published studies investigating early life exposure to ISO and bone development have used a 5-day dosing protocol, starting at postnatal day 1 and ending on postnatal day 5. To more closely represent the duration of exposure (first year of life) that human infants are fed soy protein formula, the protocol was lengthened such that mice are exposed to ISO throughout suckling (the first 21 days of life). The objective of this study

was to determine if exposure to ISO from birth throughout suckling enhances the previously observed positive effects of 5-day exposure to ISO on LV and, unlike the 5-day protocol, has favorable effects on femur outcomes at young adulthood.

5.3 Methods

5.3.1 Animals and treatment

Six-week old outbred CD-1 mice (Charles River Laboratories, St. Constant, QC) were housed in the Department of Comparative Medicine at the University of Toronto under standard environmental conditions (12 h light and 12 h dark cycle; 23°C), were provided water *ad libitum* and fed a semi-purified casein-based diet devoid of ISO (AIN93G, Dyets Inc., Bethlehem, PA) (Table 5-1) [189]. After two weeks of acclimatization to the environment and diet, mice were bred harem style. Once females were identified as being pregnant they were housed in individual cages until pups were born. Offspring of six different dams that delivered on the same day were studied. To avoid a littermate effect, cross-fostering was performed at birth and a maximum of one male and one female pup from each dam was assigned to each of the six litters. Cross-fostered litters were subsequently randomized to corn oil or ISO treatment from postnatal day 1 through 21. Control pups (n=15) received corn oil because it is used as the vehicle for ISO [13, 54]. The group receiving ISO (n=15) was administered a daily dose containing daidzein and genistein, the two major ISO in soy infant formula. The dose of ISO, 2 mg of daidzein and 5 mg of genistein/kg body weight/day, resembles the quantity and ratio of each ISO in soy protein formula [7], and mimics the total circulating ISO levels of human infants fed soy protein formula [8, 54]. Treatments were administered via a single daily subcutaneous injection with a total volume of 20 μ L/pup/day. On postnatal day 21, gender was determined and females

Table 5-1. Summary of ingredients in AIN-93G purified rodent diet

Ingredients	Units	
Casein, High Nitrogen	200	g/kg
L-Cystein	3	g/kg
Sucrose	100	g/kg
Cornstarch	397.49	g/kg
Dyetrose	132.00	g/kg
Soybean Oil	70	g/kg
t-Butylhydroquinone	0.014	g/kg
Cellulose	50	g/kg
Choline Bitartrate	2.5	g/kg
Mineral Mix #210025	35	g/kg
<i>Calcium</i>	5000	mg/kg
<i>Phosphate</i>	1561	mg/kg
<i>Potassium</i>	3600	mg/kg
<i>Sodium</i>	1019	mg/kg
<i>Chloride</i>	1571	mg/kg
<i>Sulfite</i>	300	mg/kg
<i>Magnesium</i>	507	mg/kg
<i>Iron</i>	35	mg/kg
<i>Copper</i>	6	mg/kg
<i>Manganese</i>	10	mg/kg
<i>Zinc</i>	30	mg/kg
<i>Chromium</i>	1.0	mg/kg
<i>Iodine</i>	0.2	mg/kg
<i>Selenium</i>	0.15	mg/kg
Vitamin Mix # 310025	10	g/kg
<i>Thiamin HCl</i>	6	mg/kg
<i>Riboflavin</i>	6	mg/kg
<i>Pyndoxine HCL</i>	6	mg/kg
<i>Niacin</i>	180	mg/kg
<i>Ca pantothenate</i>	16	mg/kg
<i>Folic acid</i>	2	mg/kg
<i>Biotin</i>	0.2	mg/kg
<i>Cyanocobalamin B12, 0.1%</i>	25	mg/kg
<i>Menadion Na Bisulfite</i>	0.8	mg/kg
<i>Vitamin A Palmitate</i>	4000	IU
<i>Vitamin E Acetate</i>	50	IU
<i>Vitamin D3</i>	1000	IU

were housed 3-4 per cage (male offspring were not studied beyond postnatal day 21). Body weight was measured once weekly and mice were studied to 4 months of age, which is the time when peak bone mass is established in this mouse strain [11]. Femurs and LV1-LV4 were excised and stored at -80°C until analyses were performed. All experimental procedures respected the policies set out by the Canadian Council on Animal Care and were approved by the University of Toronto Animal Ethics Committee [190].

5.3.2 BMD of femurs and LV1-LV3

BMC and BMD of the femur and intact spine (LV1-LV3) was determined by dual energy x-ray absorptiometry (DXA) (pSabre, Orthometrix, White Plains, NY) and a specialized software program (Host Software Version 3.9.4; Scanner Software Version 1.2.0) that scanned bones in air at a speed of 2 mm/min with a resolution of 0.01 mm X 0.01 mm as previously described [13, 54].

5.3.3 Biomechanical strength properties of femurs and LV2

Biomechanical strength properties of the right femur and LV2 were measured using a material testing system (Model 4442, Instron Corp., Canton, MA) and specialized software (Series IX Automated Materials Tester, Version 8.15.00) as previously described [12, 13, 54, 202].

5.3.4 Microarchitecture of femur and LV4

Microcomputed tomography (μ CT; GE Healthcare System, Model # MS0900325-0010) was used to analyze the microarchitecture of trabecular bone as previously described [13, 54]. Trabecular bone was evaluated at LV4 and femur neck. To analyze a specific bone volume, a contoured region of interest (ROI) was created using the advanced ROI module (MicroView Version ABA 2.2). For femur neck analysis, the ROI was defined from the top of the growth plate to the narrowest part of the femur shaft.

5.3.5 Statistical analyses

Statistical analyses were performed using SigmaStat (Version 3.5, Jandel Scientific, CA). Results are expressed as mean $\pm$ SEM. Student's t-test was used to compare the outcomes between the control and ISO treated groups. Pearson coefficient of determination (r^2) was used to examine the relationship between vertebral BV/TV and stiffness as well as BV/TV and peak load for the control and ISO treated groups. Statistical significance was defined as $p < 0.05$.

5.4 Results

5.4.1 Body weight

Body weight at weaning (CON 12.2 ± 0.36 g; ISO 12.1 ± 0.25 g) and week 4 (CON 22.4 ± 0.68 g; ISO 25.4 ± 0.4 g) did not differ between control and ISO treated mice. At week 6 (CON 25.0 ± 0.87 g; ISO 29.6 ± 0.70 g) and week 8 (CON 26.7 ± 1.17 g; ISO 32.2 ± 0.69 g) ISO treated mice had higher ($p < 0.05$) body weight than the control group.

5.4.2 BMD of intact LV1-3, microarchitecture of LV4 and peak load of LV2

The ISO intervention resulted in higher ($p < 0.05$) BMD of LV1-3 (Table 5-2); higher ($p < 0.05$) trabecular number (Tb.N.) and lower ($p < 0.05$) trabecular separation (Tb.Sp.) of LV4 (Table 5-2); and greater ($p < 0.05$) peak load of LV2 (Table 5-2) compared to the control group. The ISO intervention also resulted in higher ($p < 0.05$) BV/TV of LV4 (Table 5-2) compared to the control group. Qualitative assessment demonstrated that the ISO group had improved trabecular network at LV4 compared to the control group (Figure 5-1). There was no significant coefficient of determination for vertebral BV/TV and stiffness, or BV/TV and peak load among mice exposed to corn oil. In contrast, the coefficient of determination of

Table 5-2. BMD, peak load and trabecular bone parameters at the spine and femur with 21-day ISO exposure

Measured Outcomes ^b	Control	ISO	<i>p</i> value
Lumbar Spine			
LV1-3 BMC, mg	0.024 ± 0.002	0.029 ± 0.002	NS
LV1-3 BMD, mg/cm ²	0.066 ± 0.004	0.078 ± 0.003*	0.024
LV2 peak load, N	62.0 ± 4.85	84.0 ± 6.22*	0.013
LV4 BV/TV, %	29.7 ± 1.00	37.3 ± 0.70*	<0.001
LV4 BS/BV, mm ² /mm ³	39.5 ± 2.29	38.9 ± 2.83	NS
LV4 Tb.Th, mm	0.051 ± 0.003	0.053 ± 0.004	NS
LV4 Tb.N., mm ⁻¹	5.69 ± 0.309	7.23 ± 0.512	0.031
LV4 Tb.Sp., mm	0.123 ± 0.009	0.089 ± 0.006	0.008
Femur			
Whole Femur BMC, mg	32.4 ± 1.30	41.0 ± 0.988*	<0.001
Whole Femur BMD, mg/cm ²	79.7 ± 2.57	97.8 ± 1.87*	<0.001
Femur Midpoint Peak Load, N	31.0 ± 2.14	37.0 ± 1.87*	0.014
Femur Neck Peak Load, N	23.1 ± 1.36	31.8 ± 3.87*	0.049
Femur Neck BV/TV, %	52.5 ± 4.30	61.9 ± 4.10*	<0.001
Femur Neck BS/BV, mm ² /mm ³	19.8 ± 3.43	19.8 ± 3.70	NS
Femur Neck Tb.Th., mm	0.104 ± 0.020	0.104 ± 0.018	NS
Femur Neck Tb.N., mm ⁻¹	5.20 ± 0.956	6.14 ± 1.18	NS
Femur Neck Tb.Sp., mm	0.094 ± 0.020	0.065 ± 0.018*	0.011

Values are expressed as mean ± SEM. Statistical significance was defined as $p < 0.05$ using a two tailed t-test.

^b Sample size was n=10-14 for BMC, BMD and peak load; and n=6-7 for outcomes of bone structure.

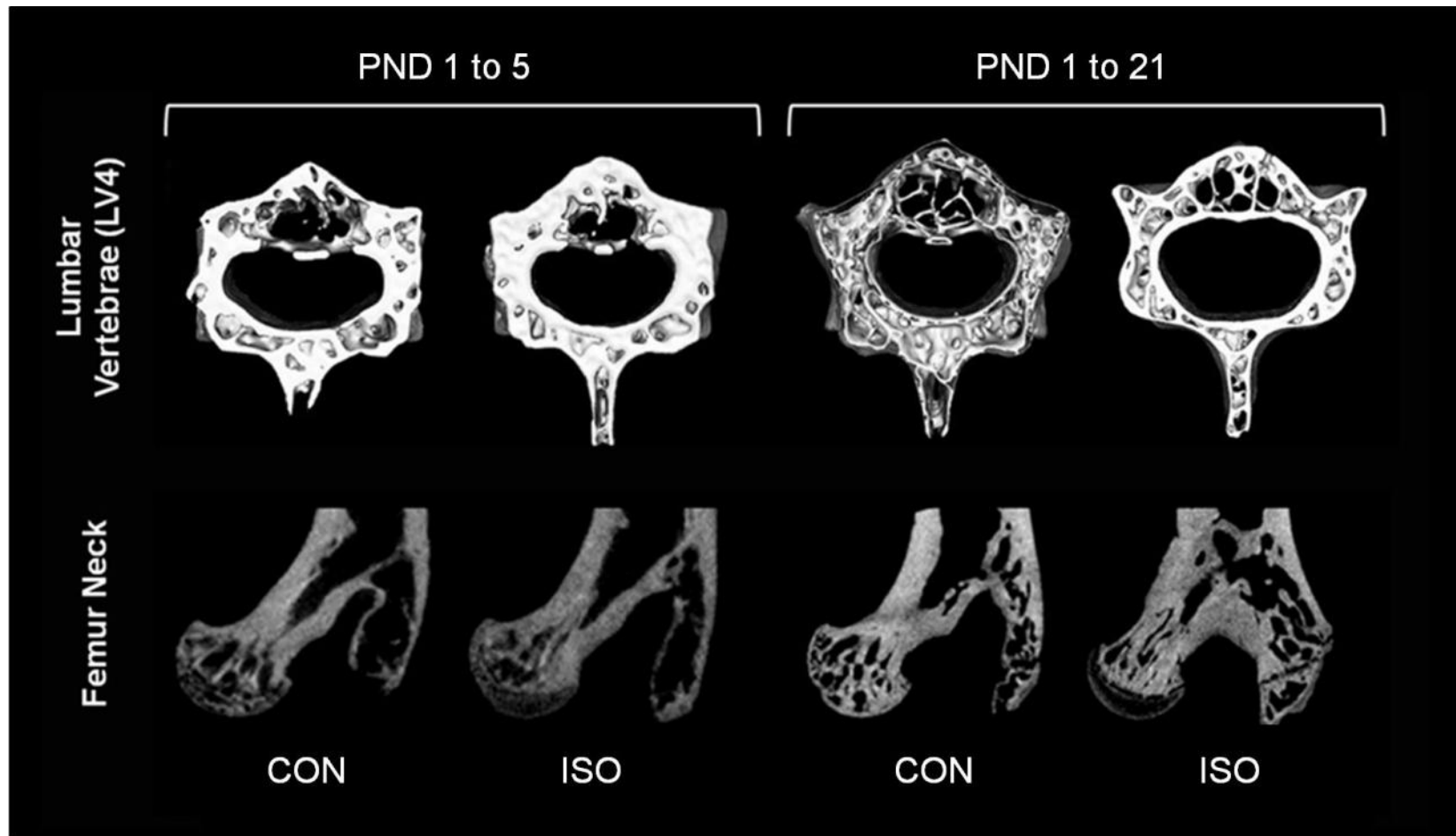


Figure 5-1. Representative microcomputed tomography images of the lumbar vertebrae (LV4) and femur neck. The trabecular network is visibly improved at the lumbar spine in ISO-treated females compared with control. The cortical thickness of ISO-treated females is visibly improved at the femur neck compared with all other treatment groups. Abbreviations: PND, postnatal day; CON, control; ISO, isoflavones.

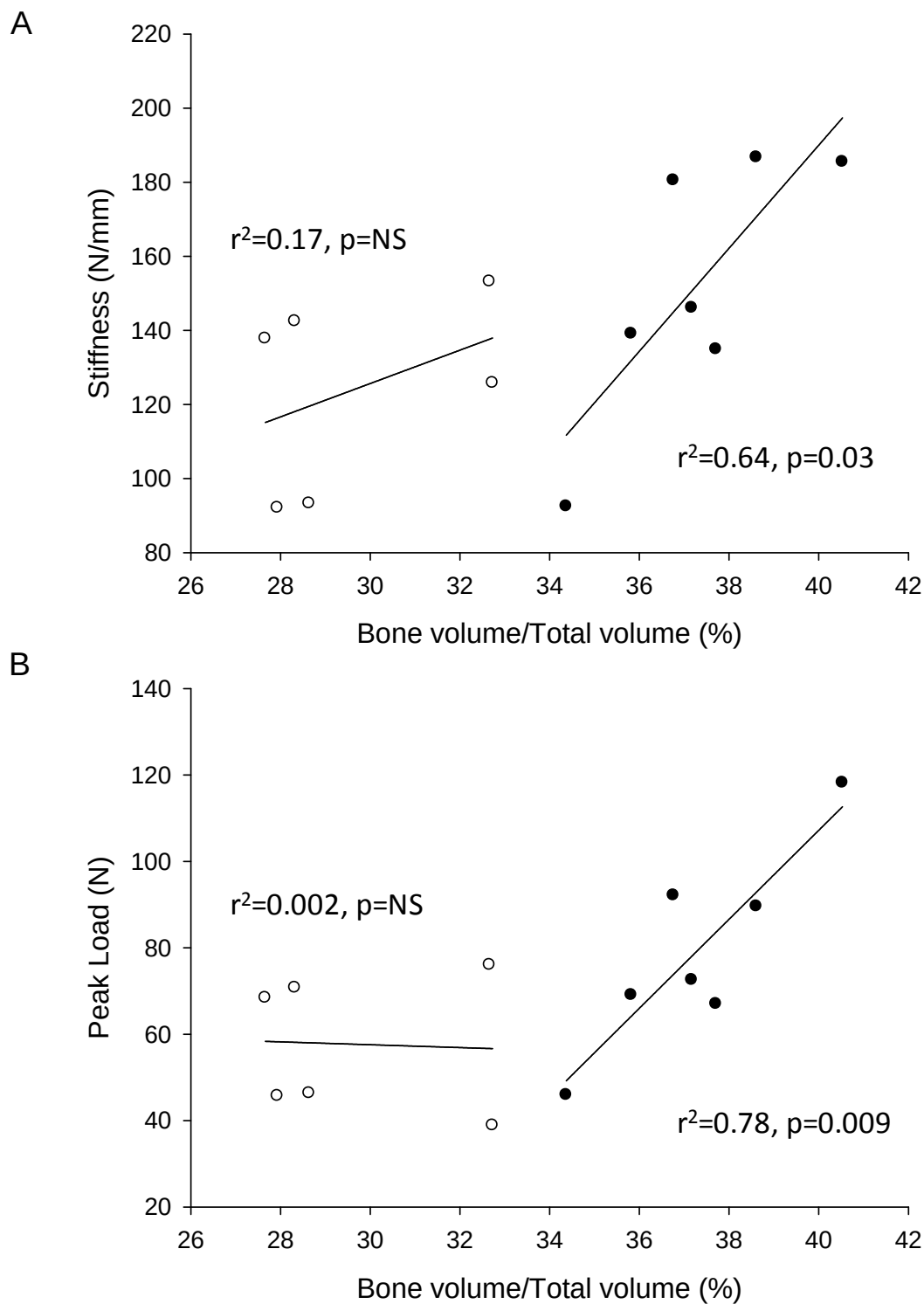


Figure 5-2. Relationship of bone volume/total volume and (A) stiffness or (B) fracture load of female mice exposed to corn oil or ISO.

Open circles represent females treated with corn oil while solid circles represent females treated with ISO.

vertebral BV/TV and stiffness ($r^2=0.64$) as well as vertebral BV/TV and peak load ($r^2=0.78$) were significant ($p<0.05$) for the ISO group (Figure 5-2). These findings indicate that the variability in vertebral BV/TV can predict the variability in vertebral stiffness and peak load by 64% and 78%, respectively.

5.4.3 BMD of whole femur, microarchitecture of femur neck, and peak load of femur neck and midpoint

Whole femur BMC and BMD, as well as femur neck and femur midpoint peak load were higher ($p<0.05$) in the ISO group compared to the control group (Table 5-2). BV/TV was higher ($p<0.05$) and Tb.Sp. was lower ($p<0.05$) in the ISO group compared to the control group (Table 1). Qualitative assessment at the femur neck for females exposed to ISO showed greater trabecular connectivity and cortical thickness than all other treatment groups (Figure 5-1).

5.5 Discussion

A previous study from our group showed that exposure to ISO in the first 5-days of life improves bone outcomes (i.e. BMD, bone structure and bone strength) of the lumbar vertebrae with no significant improvements at the femur [12, 13, 54]. Findings from this study demonstrate that exposure to ISO from birth to 21-days of age has a more significant effect on bone with improvements in both the lumbar vertebrae and the femur. Thus, duration of exposure to ISO is a factor to consider when using the CD-1 mouse model to mimic the human infant scenario.

Our studies have shown that skeletal sites rich in trabecular bone (i.e. lumbar spine and femur neck) are more easily influenced by ISO than sites rich in cortical bone (i.e. femur midpoint) [12, 13, 54]. This effect may be observed because trabecular bone has a higher

surface-to-volume ratio and is more metabolically active. While both the 5 and 21-day exposure to ISO improved trabecular connectivity of the lumbar spine in female CD-1 mice by increasing Tb.N. and decreasing Tb.Sp. [54], only the 21-day exposure improved the apparent bone density (BV/TV) at the lumbar spine as well as the femur neck. These improvements in apparent bone density (BV/TV) suggest that longer duration of ISO exposure is needed for trabeculae to be significantly resorbed with bony tissue and merged into cortical bone, which is abundant in the femur. This finding is important because in humans a small improvement in bone mass and structure during adolescence lowers the risk of fracture in later life [103]. Observational studies indicate that a 5% increase in bone mass at young adulthood can reduce fracture risk by 40% during aging [104, 105].

The apparent bone density (BV/TV) of ISO group was significantly correlated with stiffness ($r^2=0.63$) and peak load ($r^2=0.78$) at the lumbar spine. This discovery is similar to findings in humans and identifies that improvements in bone volume induced by ISO exposure can predict the probability of vertebral fracture by 78% in the mouse model. Chevalier et al. [203] showed that there is a strong correlation between increases in BV/TV and increases in vertebral stiffness and failure load among alendronate and risedronate treated postmenopausal women with osteoporosis. Thus, the relationship of BV/TV and stiffness, and BV/TV and failure load exists in both this mouse model and older humans. Vertebral fractures are the most common manifestation of osteoporosis and account for nearly half of all fractures [204]. Fracture prevention has largely focused on attenuating the rate of age-related bone loss and reducing falls during older age. Findings from this study suggest that early life nutrition is also an important consideration when developing lifestyle approaches to improve bone health throughout the life cycle.

In addition to effects on bone, prolonged ISO treatment induced weight gain at an earlier stage of life than 5-day exposure. Our published data shows that female mice exposed to ISO (7 mg/kg body weight/day) from birth to 5-days of life have higher body weight than control mice from 28 weeks of age, but not at earlier stages of development [3]. Findings from other researchers indicate that higher doses of ISO (50 mg/kg body weight/day) from postnatal day 1 to 5 result in higher body weight at 12 and 16 weeks of age [17]. In this study, the body weight of ISO treated females was higher at 6 and 8 weeks of age. Therefore, the dose as well as the duration of ISO exposure may program the time when female mice begin to gain weight.

The timing of when ISO exposure should be introduced in the developing mouse model to mimic human infants fed soy protein formula has been debated [38, 205]. Mice suckle for the first 21 days of life and thus, it could be argued that ISO exposure should take place during suckling to mimic the stage of development in which human infants are fed soy protein formula. However, unlike human infants, mice reach sexual maturation 3 weeks post-weaning, a much shorter duration between neonatal life and sexual maturity. To better understand the time of life when peak bone mass and strength is reached in the CD-1 mouse, we previously established that BMC, BMD and peak load of femur and lumbar spine were similar between 3 and 4 months of age, demonstrating that peak bone mass is attained by 4 months of age in the CD-1 mouse [11].

It is hypothesized that the programming effects on bone may be mediated through permanent estrogen receptor-induced changes in gene expression [200] or through non-genomic action that modulates a diverse array of intracellular signal transduction cascades [38, 206]. Moreover, exposure to ISO during early postnatal life has the potential to exert

biological effects that would otherwise be diluted in the presence of higher endogenous sex steroid concentrations that exist at later stages of the life cycle.

To establish an animal model that investigates ISO exposure to human infants requires careful consideration of the dose and composition of ISO, the route of ISO administration, as well as the duration and frequency of ISO exposure. Our previous research has shown that the dose and ratio of ISO (5 mg of genistein and 2 mg of daidzein/kg body weight/day) used in this study result in total serum levels of ISO, specifically daidzein and genistein, that are similar to those of human infants fed soy protein formula [54]. Moreover, during early postnatal life, both human infants and rodents have a poorly developed microbiota that limits their ability to metabolize daidzein to equol, the most estrogenic metabolite of ISO. Route of administration has also been studied [122]. Because of small size of mice from birth through the first days of life it is technically challenging to administer ISO orally and thus, administration of ISO by subcutaneous injection is more commonly used. Recent findings have shown that oral versus subcutaneous delivery, and single versus multiple oral treatments of an equivalent daily dose does not result in significantly different levels of total serum ISO in neonatal CD-1 mice [122]. This study is the first to evaluate how duration of ISO exposure during postnatal life affects peak bone mass, bone structure and bone strength of female CD-1 mice.

In conclusion, the present study further characterizes the CD-1 mouse model by identifying that longer duration of exposure to ISO has more profound benefits to bone health at multiple skeletal sites than the previously used 5-day exposure. Future studies should consider the duration of ISO exposure when using the CD-1 mouse model to evaluate the effect of early life exposure to ISO on programming of bone development. This mouse

model may also be useful for studying effects of other environmental estrogens on bone development. Examples include bisphenol A, DES, dichlorodiphenyltrichloroethane and dioxin. Duration of exposure to such environmental estrogens may be an important consideration when extrapolating findings to understand effects of exposure to human infants.

Chapter Six

STUDY 3

**BONE-SPECIFIC GENE EXPRESSION PATTERNS AND WHOLE BONE TISSUE
ARE PROGRAMMED BY EARLY LIFE EXPOSURE TO SOY ISOFLAVONES
AND FOLIC ACID IN THE DEVELOPING FEMALE CD-1 MOUSE MODEL**

6.0 STUDY 3: BONE-SPECIFIC GENE EXPRESSION PATTERNS AND WHOLE BONE TISSUE ARE PROGRAMMED BY EARLY LIFE EXPOSURE TO SOY ISOFLAVONES AND FOLIC ACID IN THE DEVELOPING FEMALE CD-1 MOUSE MODEL

6.1 Abstract

Female CD-1 mice exposed to ISO during early postnatal life, at levels similar to that of human infants consuming soy protein formula, have higher BMD, improved trabecular connectivity and greater resistance to fracture at the femur and lumbar spine at adulthood compared to mice fed a control diet devoid of ISO. We hypothesized that these benefits may in part be mediated through DNA methylation, and thus could be enhanced by providing supplemental levels of FA (a methyl donor) during early life. Thus, the objective of this study was to determine whether early life exposure to a supplemental level of FA in combination with ISO provides greater benefits to female bone development than ISO alone. CD-1 dams (n=36) were randomized to a low (0 mg/kg diet), adequate (2 mg/kg diet) or supplemental (8 mg/kg diet) level of FA during pregnancy and lactation, and offspring received corn oil or ISO (7 mg/kg body weight/day) for the first 10 days of life. At weaning, all females were placed on a control diet containing an adequate level of FA, and were studied to 4 months of age - the time when peak bone mass is established in this mouse strain. Females exposed to adequate levels of FA+ISO had improved bone outcomes - higher ($p<0.05$) BMD, improved ($p<0.05$) trabecular connectivity and greater resistance to fracture at the femur and lumbar spine – at adulthood compared to females exposed to adequate FA without ISO. Exposure to supplemental levels of FA alone also provided benefits to bone with exposed females having higher BMD at the femur and lumbar spine, improved trabecular connectivity at the lumbar spine, and greater resistance to fracture at the femur neck compared to those exposed to adequate FA without ISO. In contrast, exposure to

supplemental levels of FA+ISO was limited to higher ($p<0.05$) femur BMD compared to adequate FA alone. Illumina gene-chip microarray analyses of bone tissue identified that these effects were in part mediated through changes in gene expression. Females exposed to adequate level of FA+ISO had a 5.7 fold lower expression of neuropeptide Y (NPY), while those exposed to supplemental levels of FA+ISO had a 1.7-fold higher expression of beta-catenin (Ctnnb1) and 1.6-fold higher expression of parathyroid hormone receptor 1 (PTHr1) than females exposed to adequate FA alone. In bone, low levels of NPY promote bone formations, while high levels of Ctnnb1 and PTHr1 promote bone resorption. Thus, it is possible that a negative feedback mechanism exists whereby exposure to supplemental levels of FA and ISO up-regulates the transcription of genes involved in bone resorption (i.e. Ctnnb1 and PTHr1). In conclusion, exposure to adequate FA+ISO or supplemental FA alone improved bone outcomes of female CD-1 mice at adulthood, while exposure to supplemental FA+ISO had a modest effect. These functional outcomes are clearly a reflection of both epigenetic and endocrine changes that need further investigation.

6.2 Introduction

Exposure to nutrients or food components during perinatal life can influence the development of physiological and endocrine systems in ways that affect the prevalence of chronic diseases [207-209]. Our published studies demonstrate that female mice exposed to dietary estrogens (i.e. ISO), at serum levels that mimic human infants fed soy protein formula, for the first 5 or 21 days of life have higher BMD, improved trabecular connectivity and greater resistance to fracture at 4 months of age, which is the time when peak bone mass is established in this mouse model. In addition, CD-1 females treated with ISO have less deterioration of bone tissue after ovariectomy and a lower incidence of fragility fractures

[13]. Taken together, this data provides support that postnatal exposure to ISO induces programming effects that prevent or delay the skeleton from becoming weak and susceptible to fragility fractures. However, the underlying mechanism has not been elucidated.

There is some evidence to suggest that early life exposure to ISO programs epigenetic patterns that persist into adulthood and help to prevent or delay the onset of chronic diseases [15]. For example, offspring of agouti dam's fed a diet rich in genistein (the most abundant ISO in soy protein) during pregnancy and lactation had hypermethylated CpG sites in the promoter of the agouti gene, higher prevalence of pseudoagouti phenotype and a lower body weight at 15 months of age compared to animals fed a diet devoid of genistein [15]. The extent of DNA methylation was similar in endodermal, mesodermal, and ectodermal tissues, which suggested that early life exposure to ISO might have beneficial or adverse effects on development of many tissues. In line with this, we [83, 84] and others [86, 87, 89, 90, 123, 124, 126] have shown that aside from having benefits to bone, exposure to ISO for the first 5, 10 or 21 days of life can disrupt structural development of female reproductive organs (i.e. ovaries and uteri) and reduce fertility. In addition, a recently published study showed that offspring of dams fed a diet rich in ISO during pregnancy and lactation had increased DNA methylation of some repetitive elements that are associated with a marked down-regulation of estrogen-responsive genes (*Grin2d*, *ApoE*, *Cdkn1a*, *MacroD*, *Vegfa*, *Hdac6*, *Tacc1*, *Abcc5*, *Ctsd*, *Ccnd1*, *Pcna*, and *Igf2*) and genes involved in hematopoiesis of bone marrow cells [135]. Based on these findings it is possible that life-long improvements that we previously observed in bone were in part induced by increased DNA methylation of CpG islands at the promoter of protein coding genes that favor bone formation.

From a molecular perspective, DNA methylation is mediated by a group of enzymes called DNA methyltransferases that recognize CpG dinucleotides of palindromic sequences and catalyze the transfer of one-carbon group from a methyl donor to the cytosine residue. For this process to occur, histone acetyltransferases need to add a bulky acetyl group to the N-terminal tail of histones and loosen-up the covalent bonds between histones and DNA. As such, changes in the expression of histone acetyltransferases, DNA methyltransferase or the availability of methyl donors/acceptors can alter DNA methylation patterns. To date, one study showed that through an estrogen receptor- α and - β mediated mechanism, ISO can increase acetylation of core histones in the nucleosome [18], which loosens the DNA near the promoter and allow FA to more easily donate a one-carbon group to the cytosine residue of CpG sites – a hallmark of DNA methylation. Thus, exposure to ISO in combination with a supplemental levels of FA may have additive or synergistic effects on programming of bone health by increasing the methylation of CpG sites in the promoter of protein coding genes in such a way that it promotes the transcription of genes favoring bone formation [15].

During development, human infants can be exposed to elevated levels of ISO and FA. Infants fed commercially-available soy protein formula, which contain 32-47 mg of ISO and 2-4 mg of FA per liter of formula [8, 9], have markedly higher serum ISO (10 fold higher) and FA (2.3 fold higher) levels than infants fed breast milk or cow milk formula [7-9]. In addition, due to mandatory fortification of the food supply and widespread use of FA supplements up to 40% of North Americans have higher than recommended red blood cell folate concentrations (>1360 nmol/L) [10]. If pregnant, the high level of FA is transferred from mother to fetus. Therefore, there is an ongoing need to characterize how early life exposure to supplemental FA and ISO affects growth, development and future health.

In a recently published study, Zhao et al [19] showed that exposure to a supplemental levels of FA (0.7 mg of FA per kg of diet) in combination with ISO (160 mg of ISO per kg of diet) from beginning of pregnancy to 20 days of gestation has greater protective effects on the post-neural tube closure of rats than either treatment alone. This effect may in part be mediated through stimulation of cellular DNA methylation reactions. No studies have investigated how combining supplemental FA and ISO affects female bone development. Thus, the objective of this study was to determine whether early life exposure to a supplemental level of FA in combination with ISO provides greater benefits to female bone development than ISO alone. Illumina gene-chip microarray was performed at the University Health Network to explain tissue effects of female mice treated with adequate or supplemental levels of FA with or without ISO. We hypothesized that exposure to supplemental FA and ISO improves bone outcomes of female mice at adulthood to a greater extent than either treatment alone by permanently changing the expression of genes that regulate the production of enzymes and transcription factors involved in bone metabolism and hormone secretion.

6.3 Methods

6.3.1 Animals and treatment

Seven week-old CD-1 mice, obtained from Charles River Canada, were randomized to an amino acid based diet (Dyets, Bethlehem, PA) devoid of ISO and fortified with 0, 2 or 8 mg of FA per kg of diet (

Figure 6-1, Table 6-1). The amino acid diet was used because it is the only rodent diet that does not contain any natural folate so the level of FA within a diet can be tightly

controlled. The diet containing 0 mg of FA per kg of diet produces progressive FA deficiency of a moderate degree without growth retardation or premature death

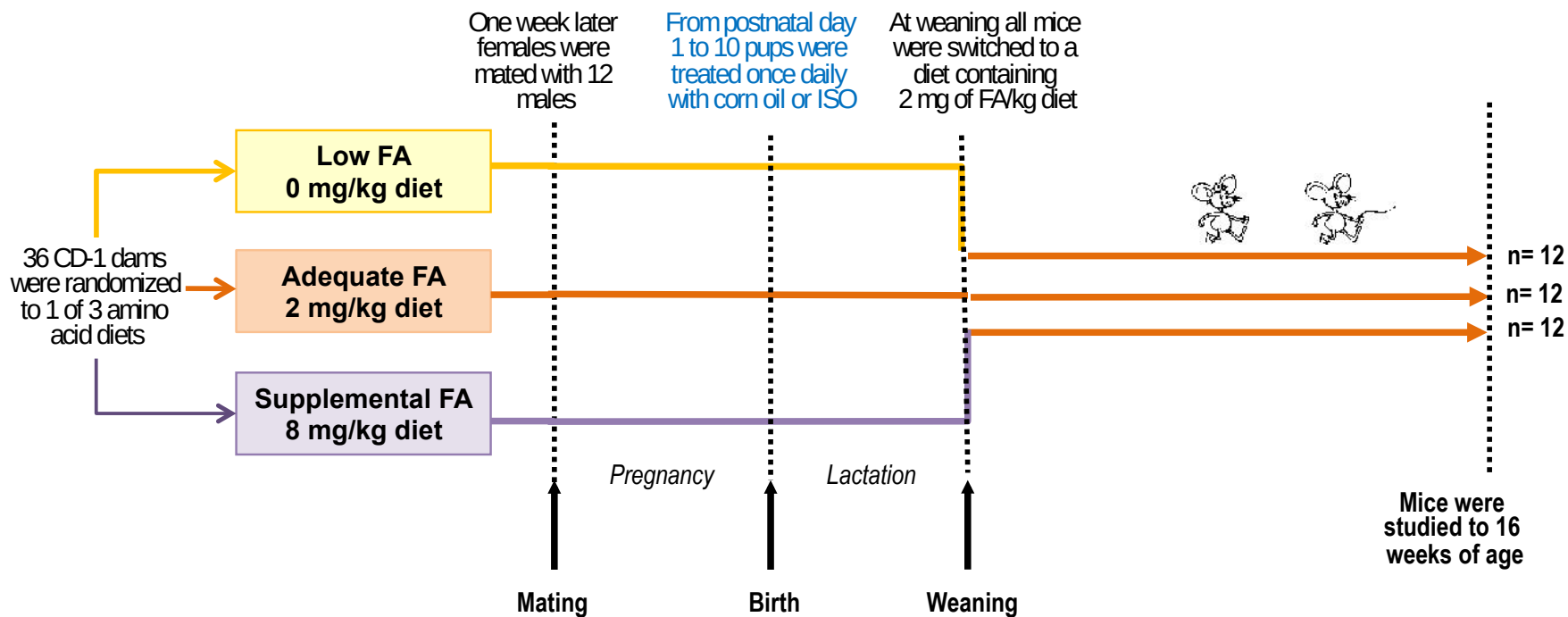


Figure 6-1. Study design.

Abbreviations: FA – folic acid; ISO – soy isoflavones.

Table 6-1. Summary of ingredients in L-amino acid rodent diet

Ingredients	Units	
L-Alanine	3.5	g/kg
L-Arginine (free base)	11.2	g/kg
L-Asparagine	6.0	g/kg
L-Aspartic Acid	3.5	g/kg
L-Cystine	3.5	g/kg
L-Glutamic Acid	35.0	g/kg
Glycine	23.3	g/kg
L-Histidine (free base)	3.3	g/kg
L-Isoleucine	8.2	g/kg
L-Leucine	11.1	g/kg
L-Lysine HCl	14.4	g/kg
L-Methionine	8.2	g/kg
L-Phenylalanine	11.6	g/kg
L-Proline	3.5	g/kg
L-Serine	3.5	g/kg
L-Threonine	8.2	g/kg
L-Tryptophan	1.74	g/kg
L-Tyrosine	3.5	g/kg
L-Valine	8.2	g/kg
Total L-Amino Acid	171.44	g/kg
Dextrin	407	g/kg
Sucrose	193	g/kg
Cellulose	50	g/kg
Corn oil	100	g/kg
Salt Mix # 210006	57.96	g/kg
Vitamin Mix # 310025	10	g/kg
<i>Thiamin HCl</i>	6	mg/kg
<i>Riboflavin</i>	6	mg/kg
<i>Pyndoxine HCL</i>	7	mg/kg
<i>Nicotinc Acid</i>	30	mg/kg
<i>Ca Pantothenate</i>	16	mg/kg
<i>Biotin</i>	0.02	mg/kg
<i>Vitamin B12, 0.1%</i>	50	mg/kg
<i>Menadion Na Bisulfite</i>	0.8	mg/kg
<i>Vitamin A Palmitate</i>	4000	IU
<i>Vitamin E Acetate</i>	50	IU
<i>Vitamin D3</i>	1000	IU
Choline Chloride	2.0	g/kg
Sodium Bicarbonate	6.6	g/kg
Folic Acid	0, 2 or 8	mg/kg

[210, 211] because mice are coprophagous and can obtain some folate that is synthesized by bacteria in the intestine [212]. The diet containing 2 mg of FA per kg of diet was used to represent the basal dietary requirement for rodents, [213] while the 8 mg of FA diet was used to mimic the amount of FA that pregnant women may obtain by consuming FA supplements and FA-fortified foods. This supplemental level of FA has also been consistently shown to provide a degree of chemoprevention against colorectal cancer in rodent models [210, 211, 214, 215].

After one week of adaptation to the environment and diet, mice were mated harem-style by housing one male with three females. Once females were identified as being pregnant they were housed in individual cages under standard environmental conditions (23°C, 50% humidity, 12:12h light-dark cycle), and remained on their respective diets. Water was provided *ad libitum*. Offspring of dams that delivered 8-12 pups on the same day and were fed the same FA diet were cross-fostered by assigning a maximum of one male and one female pup from each dam to a litter. Cross-fostered litters on low, adequate or supplemental FA diet were then randomized to control or ISO treatment. Control pups received corn oil as it is used as the vehicle for ISO [12, 13, 54]. The ISO groups received a daily dose containing 0.010 mg of daidzein and 0.025 mg of genistein per pup. Because the dose of ISO is directly related to a pup's body weight, the dose of daidzein in CD-1 mice ranged from 5.43 mg/kg body weight on postnatal day one to 2.69 mg/kg body weight on postnatal day ten. Similarly, the dose of genistein ranged from 13.6 mg/kg of body weight on postnatal day one to 6.7 mg/kg body weight on postnatal day ten. This dose is similar to the dose previously shown to resemble the concentration of daidzein and genistein in infants fed soy protein formulas [54, 122]. All ISO mixtures were prepared fresh prior to treatment by

being solubilised in 1 ml of dimethyl sulfoxide and suspended in corn oil. Treatments were administered in the morning from postnatal day one to ten via daily subcutaneous injections using a 3/10 cc insulin syringe with a 30 gauge needle and a 12.7 mm needle length. The 10-day duration of exposure was selected to target the stage of development when CD-1 mice have very low concentrations of endogenous sex steroids and a low probability of consuming their mothers' diet. The total volume of treatment was 20 μ l. At weaning (postnatal day 21), all female offspring were housed 3-4 per cage and fed an amino acid diet containing 2 mg of FA per kg of diet. Offspring's body weight was measured bi-monthly using an electronic scale (Denver Instrument XP-1500). At 6 weeks of age, when bones have become more mineralized a subset of mice (n=6/group) were euthanized, by carbon dioxide and cervical dislocation, to extract RNA from femurs and perform microarray analyses. All remaining mice were euthanized at age 4 months of age, which is the time when peak bone mass is established in this mouse model [11]. All protocols for animal use and treatment were approved by the University of Toronto Animal Care Committee and were in compliance with the guidelines of the Canadian Council on Animal Care [216]. This study reports findings from females and Study 4 reports findings from males.

6.3.2 BMD of femurs and LV1-LV3

To measure BMD, femurs and intact lumbar vertebrae (LV1-LV3) removed from the -80°C freezer were analyzed using dual energy x-ray absorptiometry (DXA) (pSabre, Orthometrix, White Plains, NY) and a specialized software program (Host Software Version: 3.9.4; Scanner Software Version: 1.2.0) as previously described [11, 13, 54]. Scans were conducted in air at room temperature using a speed of 2 mm/min and a resolution of 0.01mm X 0.01mm.

6.3.3 Biomechanical strength properties of femurs and LV2

Femurs and LV2 were hydrated in 0.9% saline solution for 3 hours at room temperature. Using an electronic balance (Sartorius AG, Goettingen, Germany) the weight of the femur and LV2 was determined. Femur length was also measured, from the proximal tip of the femoral head to the distal tip of the medial condyle, using electronic precision callipers (Cedarlane Laboratories Ltd. Hornby, ON. Canada). At the femur midpoint, anterior-posterior and medio-lateral width were measured. To determine biomechanical strength properties of the lumbar spine, femur neck and femur midpoint a materials testing system (Model 4442, Instron Corp., Canton, MA) and specialized software (Series IX Automated Materials Tester, Version 8.15.00) were used as previously described [11, 13, 54, 202]. Peak load (the maximal force a bone can withstand before fracture occurs) was identified as the first peak of the load-displacement curve.

6.3.4 Microarchitecture of femur and LV4

The 3D microarchitecture of femur neck and lumbar spine were evaluated using a high resolution (8 μ m) μ CT imaging system (GE Healthcare System, Model # MS0900325-0010). Scans were conducted in water and used an x-ray tube source of 80kV and an x-ray intensity of 80 μ A. During scanning, a 1 mm thick aluminum filter was employed to minimize beam-hardening effects, and a bath was placed over top of the specimen holder to improve image quality and increase overall accuracy by preventing interference of the refracted wavelength of x-rays with unrefracted x-rays. For each scan, 500 radiographic projections were acquired over an angular range of 180° (angular step of 45°) with a fixed exposure time of 3 sec per frame. From 2D radiographic projections, a 3D structural image was reconstructed (voxel size of 0.014 mm³) using a 3D Gaussian filter that partly suppresses the volume noise. Femur neck was analyzed from the bottom edge of the femur head to the

narrowest part of the femur shaft by selecting a fixed region of 1.5 mm. Similarly, LV4 was analyzed by selecting a fixed region of 1 mm at LV4 midpoint. Unbiased 3D microstructural properties of trabecular bone (i.e. trabecular bone volume, BV/TV; trabecular surface area, BS/BV; trabecular thickness, Tb.Th.; trabecular number, Tb.N.; trabecular separation, Tb.Sp.) were calculated using methods based on distance transformation of the binarized images.

6.3.5 Serum measures of bone turnover and metabolism

Serum ACTH, OPG, RANKL, osteocalcin, IGF-I and IGFBP-1,2,3,5,6,7 were measured using Milliplex MAP, based on the Luminex xMAP technology by Millipore (Billerica, MA). The intra-assay coefficient of variation for ACTH was 1.3-3.1%, for OPG was 1.03-2.6%, for RANKL was 2.3-5.7%, for OC was 3.7-5.87%, for IGF-I was 2.5-4.3%, and for IGFBP-1,2,3,5,6,7 ranged from 0.82-6.4%. The OPG/RANKL ratio was determined based on serum levels of OPG and RANKL for each mouse.

6.3.6 Illumina chip-based gene expression

Total RNA was isolated from fresh femurs using Trizol reagent (Invitrogen, San Diego, CA) according to the manufacturer's protocol. RNA integrity of isolated RNA was assessed by Agilent 2100 Bioanalyzer on the RNA 600 Nano kit (Agilent Technologies, Santa Clara, CA, USA), and only samples with an RNA Integrity Number (RIN) of 8 or higher were used for microarray. In total, the RNA of 24 samples, 6 from each of the adequate and supplemental FA groups, was analyzed. The Illumina TotalPrep-96 RNA Amplification kit (Ambion, Wilmington, DE, USA) was used for RNA amplification of each sample according to manufacturer's instructions. In short, 200 ng of RNA sample was reversed transcribed with an oligo(dT) primer with a T7 promoter using ArrayScript reverse transcriptase. cDNA generated from this reaction then underwent a second synthesis and

cleanup to generate a template for in vitro transcription with T7 RNA Polymerase. In vitro transcription amplification generates biotinylated, antisense RNA copies of each mRNA in the sample (cRNA) that was then used for hybridization with Illumina beadchips. For each sample, 1.5 ug of the generated cRNA was hybridized onto the Mouse WG-6 V2 Bead chips (Illumina, Inc, San Diego, USA) according to manufacturer's protocol. All samples passed Illumina's sample dependent and independent quality control metrics. Data were extracted using GenomeStudio Version 2011.1 (Illumina Inc., San Diego, CA, USA). Microarray experiments were performed at the University Health Network Microarray Centre, Toronto, Canada.

6.3.7 Bioanalyses of Illumina chip-based gene expression

Data was processed using the Bioconductor R (v2.14.1) framework and Lumi package (www.bioconductor.org), and was imported into GeneSpring v11.5.1 for analysis. In the Bioconductor R framework, technical outliers were checked using the “Array Quality Metrics” and no technical outliers were identified. As a result, all measurements were used for each probe and were part of the statistical measurement. During import, data was normalized using a standard quantile-based (for Illumina arrays) normalization followed by a “per probe” median-centered normalization. All data analysis and visualization were performed on log₂ transformed data. In this analysis, looking at the dataset as a whole, there were 4 groups with replicate samples in each. A total of 45281 probes are represented on the Mouse WG-6v2r3 array. For statistical tests, data was first filtered to remove the confounding effect of probes that showed no signal. Only probes that were in the upper 80th percentile of the distribution of intensities in 100% of any of the 4 groups were allowed to pass through this filtering. The filtered set contained 30269 probes. A threshold of 1.5 fold change was used to find the probes that were most representative of any one of the 4 groups.

6.3.8 Statistical analyses

Based on our published studies [13, 54] and statistical consults, 12 mice per group is adequate to observe differences in BMD ($\beta=0.88$ females) and peak load of LV2 ($\beta=1.00$ for females) at 4 months of age. This sample size is also sufficient to observe differences for measures in serum markers of metabolism (i.e. OPG, RANKL, osteocalcin, ACTH). Based on our published studies [13, 54], only 6 mice per group are needed to observe differences in bone structure at LV2. Taking that we did not have pilot data for microarray analyses the sample size was estimated by the bioinformatics expert (Carl Virtanen) at the University Health Network Microarray Centre. The estimate of 6 samples per group was based on prior knowledge and experience of running thousands of arrays across various projects and falls in the realm of “best guess”.

Statistical analyses were performed using SigmaStat (Version 3.5, Jandel Scientific). Data are expressed as mean $\pm$ SEM. One-way ANOVA followed by a Student Newman–Keul’s post-hoc test was used to analyze differences in body weight at birth. For microarray analyses, an ANOVA using an FDR Benjamini and Hochberg multiple testing correction threshold of $q < 0.05$ was performed [217]. For all other measures, two-way ANOVA was used with FA and ISO as the main effects. For data that did not follow a normal distribution, a Kruskal-Wallis two-way ANOVA on ranks was used to determine differences among groups. Statistical significance was determined as $p \leq 0.05$.

6.4 Results

6.4.1 Body weight

At birth, female mice exposed to adequate FA had lower ($p < 0.001$) body weight than mice exposed to low or supplemental FA (Table 6-2). However, on postnatal day 10 and

postnatal day 112, mice exposed to low FA had lower ($p<0.001$) body weight than all other groups, and this effect was independent of ISO treatment (Table 6-2). Mice exposed to adequate or supplemental FA had higher body weight ($p<0.001$) on postnatal day 10 if treated with ISO rather than corn oil (Table 6-2). At weaning (postnatal day 21), corn oil-treated mice whose mother's were fed supplemental FA had higher body weight ($p<0.001$) than those of mothers fed low FA (Figure 6-2). Among females exposed to adequate FA, those treated with ISO had higher ($p<0.05$) body weight from 4 through 16 weeks of age than corn oil treated mice (Figure 6-2); however, the body weight of all mice was within the normal growth range (Figure 6-2). ISO exposure did not have an effect on body weight of females exposed to low or supplemental FA (Figure 6-2). Females exposed to low FA+ISO had lower ($p=0.003$) body weight than females exposed to adequate FA+ISO with other groups having intermediate effects.

6.4.2 Organ weight

There were no differences in the weight of uteri or kidney between groups (Table 6-3). Female mice exposed to low FA during pregnancy and lactation had higher ($p=0.021$) ovarian weight than mice exposed to adequate or supplemental FA irrespective of ISO exposure (Table 6-3).

6.4.3 Anthropometry of the femur

Female mice exposed to supplemental FA+ISO had higher ($p=0.012$) femur weight than mice exposed to low FA+ISO (Table 6-4). Females treated with ISO had shorter femurs at adulthood compared to corn oil-treated mice irrespective of FA exposure (Table 6-4). Female mice exposed to adequate FA+ISO had higher ($p=0.028$) anteroposterior width of the femur than all other treatments.

Table 6-2. Body weight and total weight gain of 4 month old female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO

LV2	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
Birth weight (g)	Low	1.90 ± 0.022 ^a	1.95 ± 0.024 ^a	<0.001	0.007	NS
	Adeq	1.81 ± 0.022 ^b	1.85 ± 0.023 ^b			
	Supp	1.88 ± 0.026 ^a	1.95 ± 0.023 ^a			
Post-treatment (g), postnatal day 10	Low	5.02 ± 0.115 ^c	5.69 ± 0.124 ^b	<0.001	<0.001	NS
	Adeq	5.98 ± 0.120 ^b	6.25 ± 0.131 ^{ab}			
	Supp	5.96 ± 0.136 ^b	6.58 ± 0.128 ^a			
Weaning (g), postnatal day 21	Low	9.92 ± 0.88 ^b	11.97 ± 1.10	0.002	NS	NS
	Adeq	12.00 ± 0.95 ^{ab}	12.91 ± 0.95			
	Supp	15.23 ± 1.10 ^a	13.83 ± 0.92			
Final weight (g), postnatal day 118	Low	32.68 ± 1.05 ^b	33.4 ± 1.35 ^b	<0.001	0.033	NS
	Adeq	36.64 ± 1.18 ^a	41.99 ± 1.21 ^{a*}			
	Supp	38.35 ± 1.31 ^a	38.67 ± 1.34 ^a			
Weight gain From postnatal day 21 to 118 (g)	Low	22.76 ± 1.34 ^{ab}	21.36 ± 1.69 ^b	0.003	NS	NS
	Adeq	26.25 ± 1.42 ^{ab}	28.18 ± 1.48 ^a			
	Supp	23.12 ± 1.64 ^{ab}	25.47 ± 1.42 ^{ab}			

Values are means ± SEM, n = 8-18. Labelled means in a column for a variable without a common letter differ, $p < 0.05$. NS, $p > 0.05$. Different from corresponding corn oil-treated females, $p < 0.05$. NS, $p > 0.05$.

Abbreviations: Adeq - adequate; CON - control; FA - folic acid; ISO - soy isoflavones; NS - not significant; PND - postnatal day; Supp – supplemental.

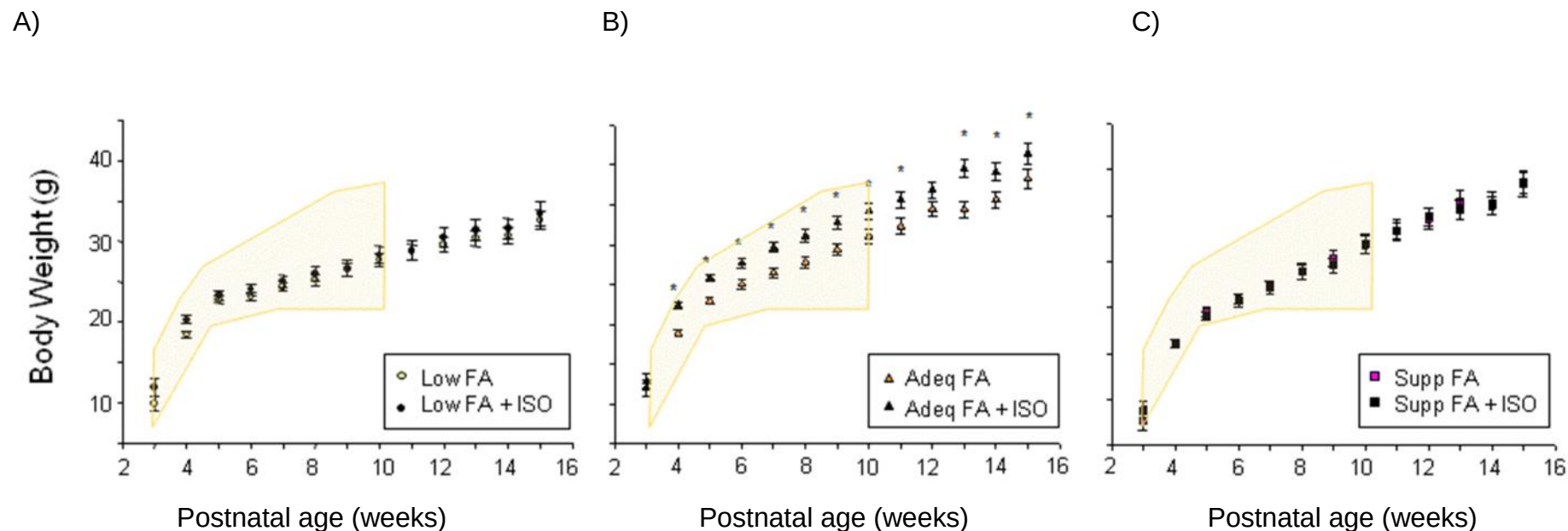


Figure 6-2. Body weight from weaning through 16 weeks of age of female mice whose mothers' were fed low (A), adequate (B) or supplemental (C) FA during pregnancy and lactation, and who from postnatal day 1 to 10 were treated with subcutaneous injections of corn oil or ISO.

The shaded area represents the normal growth range of CD-1 mice.

Values are means $\pm$ SEM, $n = 8-18$. *Different from corresponding corn oil treated females, $P < 0.05$.

Abbreviations: Adeq - adequate; FA - folic acid; ISO - soy isoflavones; NS - not significant; Supp – supplemental.

Table 6-3. Organ weights expressed as percent of body weight from 4 month old female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO

	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
Ovary (%)	Low	0.064 ± 0.004 ^a	0.069 ± 0.005 ^a	0.021	NS	NS
	Adeq	0.059 ± 0.004 ^b	0.055 ± 0.004 ^b			
	Supp	0.057 ± 0.005 ^b	0.053 ± 0.004 ^b			
Uterus (%)	Low	0.393 ± 0.037	0.454 ± 0.046	NS	NS	NS
	Adeq	0.413 ± 0.039	0.367 ± 0.042			
	Supp	0.435 ± 0.045	0.385 ± 0.038			
Kidney (%)	Low	1.119 ± 0.061	1.110 ± 0.077	NS	NS	NS
	Adeq	1.148 ± 0.065	1.109 ± 0.069			
	Supp	0.984 ± 0.075	1.003 ± 0.066			

Values are means ± SEM, n = 8-18. Labelled means in a column for a variable without a common letter differ, $P < 0.05$. NS, $p > 0.05$. Different from corresponding corn-oil treated females, $P < 0.05$. NS, $P > 0.05$.

Abbreviations: Adeq – adequate; CON - control; FA - folic acid; ISO - soy isoflavones; NS - not significant; Supp – supplemental.

Table 6-4. Anthropometry of femur from 4 month old female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO

Femur	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
Weight (mg)	Low	82.30 ± 1.38	80.70 ± 1.86 ^b	0.012	NS	NS
	Adeq	83.40 ± 1.49	85.10 ± 1.65 ^{ab}			
	Supp	86.10 ± 1.65	86.90 ± 1.65 ^a			
Length (mm)	Low	16.35 ± 0.11	16.08 ± 0.14*	NS	0.024	NS
	Adeq	16.23 ± 0.12	16.00 ± 0.13*			
	Supp	16.29 ± 0.13	16.08 ± 0.13*			
Depth† (mm)	Low	1.42 ± 0.02 ^b	1.48 ± 0.03 ^b	0.002	0.028	NS
	Adeq	1.46 ± 0.02 ^b	1.57 ± 0.03 ^{a*}			
	Supp	1.46 ± 0.03 ^b	1.50 ± 0.025 ^b			
Width‡ (mm)	Low	1.73 ± 0.03	1.74 ± 0.04	0.041	NS	NS
	Adeq	1.75 ± 0.03	1.82 ± 0.03			
	Supp	1.75 ± 0.03	1.83 ± 0.03			

Values are means ± SEM, n = 8-18. Labelled means in a column for a variable without a common letter differ, p<0.05. NS, p>0.05.

† Depth refers to the anteroposterior width at the midpoint of the femur.

‡ Width refers to the mediolateral width at the midpoint of the femur.

*Different from corresponding corn-oil treated females, P<0.05. NS, P>0.05.

Abbreviations: Adeq - adequate; CON - control; FA - folic acid; ISO - soy isoflavones; NS - not significant; Supp- supplemental

6.4.4 BMD of intact LV1-3, and peak load of LV2

Exposure to adequate FA resulted in higher ($p<0.05$) peak load of LV2 than low FA (Figure 6-3). Females exposed to low or adequate FA+ISO had higher ($p<0.05$) BMD and peak load of LV2 compared to females exposed to low or adequate FA without ISO. Exposure to supplemental FA with or without ISO resulted in higher LV1-3 BMD than exposure to low or adequate FA alone, and higher ($p=0.002$) LV2 peak load than exposure to low FA alone.

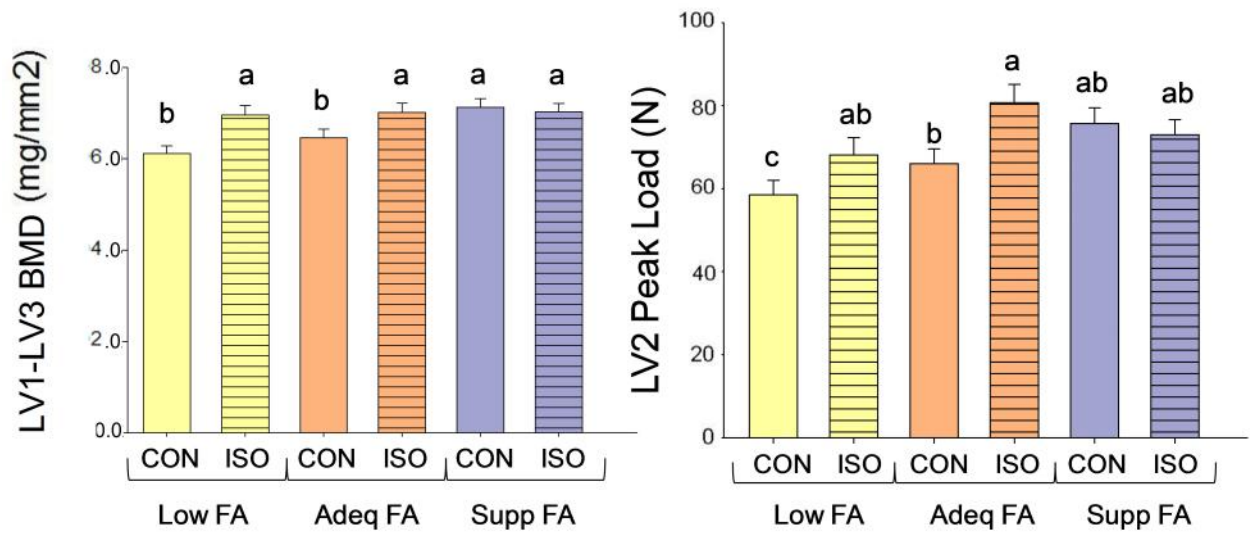
6.4.5 BMD of whole femur, and peak load of femur neck and midpoint

At the femur, ISO-treatment did not provide any benefits to BMD or peak load of females exposed to low or supplemental levels of FA (Figure 6-3). In contrast, exposure to adequate FA+ISO resulted in higher ($p<0.05$) BMD of whole femur, and higher ($p<0.001$) peak load of femur midpoint compared to the low or adequate FA groups (Figure 6-3). At the femoral neck, peak load was not different between groups (data not shown).

6.4.6 Microarchitecture of femur and LV4

Females exposed to supplemental FA or adequate FA+ISO had higher ($p=0.001$) BV/TV of LV4 compared to females exposed to adequate FA, respectively (Table 6-5). There were no differences in BS/BV or Th.Th. at the LV among groups (Table 6-5). Female mice exposed to adequate FA+ISO had higher ($p=0.023$) Tb.N. and lower ($p<0.001$) Tb.Sp. than mice exposed to adequate FA. Exposure to supplemental levels of FA also resulted in Tb.Th. and Tb.N. Female mice exposed to adequate FA+ISO had higher ($p=0.005$) BS/BV, higher Tb.N. and lower ($p=0.010$) Tb.Sp. compared to adequate FA but not the adequate FA+ISO group. There was an overall effect of FA x ISO ($p<0.05$) on femur neck BS/BV, higher ($p=0.003$) Tb.Th and lower ($p=0.012$) Tb.N compared to mice exposed to adequate FA or supplemental FA+ISO.

A)



B)

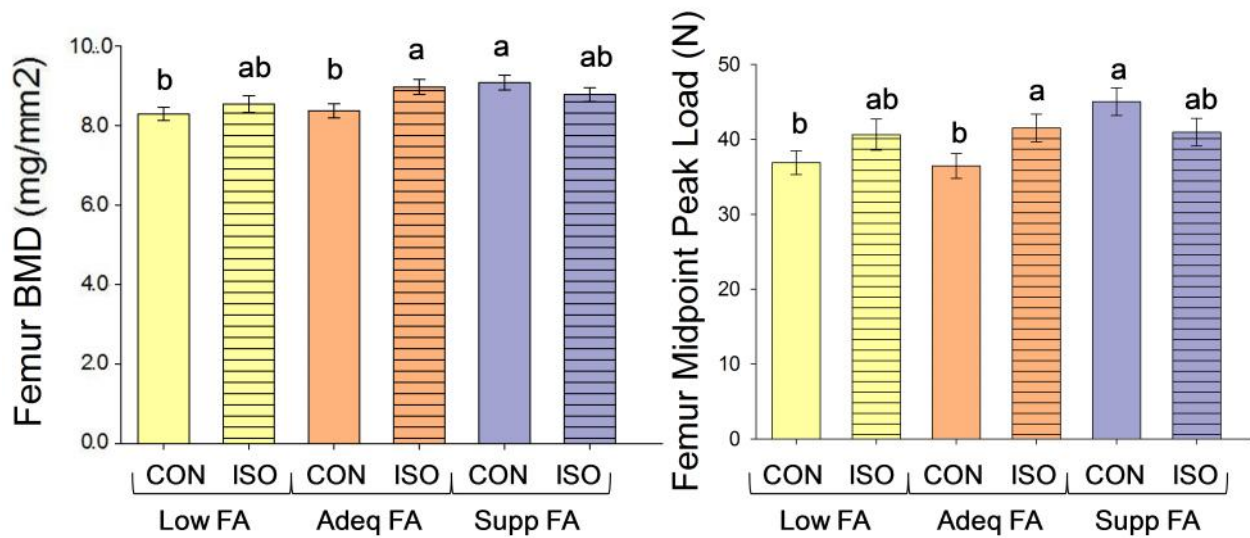


Figure 6-3. BMD and peak load of lumbar vertebrae (A) and femur (B) from 4-month old female CD-1 mice whose mothers' were fed a low, adequate or supplemental FA during pregnancy and lactation, and who from postnatal day 1 to 10 were treated with subcutaneous injections of corn oil or ISO.

Values are expressed as mean $\pm$ SEM, n = 8-18. Bars without a common letter differ, P < 0.05. Abbreviations: Adeq - adequate; CON - control; FA - folic acid; ISO - soy isoflavones; Supp- supplemental

Table 6-5. Trabecular bone parameters of LV4 and femur neck from female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO

	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
LV 4						
BV/TV (%)	Adeq	29.8 ± 1.24 ^b	33.9 ± 1.24 ^{b*}	0.001	<0.001	NS
	Supp	33.4 ± 1.16 ^a	39.1 ± 1.24 ^{a*}			
BS/BV (mm ² /mm ³)	Adeq	39.9 ± 3.25	43.3 ± 3.04	NS	NS	NS
	Supp	44.3 ± 3.25	45.0 ± 3.25			
Tb.Th. (mm)	Adeq	0.051 ± 0.003	0.047 ± 0.003	NS	NS	NS
	Supp	0.047 ± 0.003	0.048 ± 0.003			
Tb.N. (mm)	Adeq	5.94 ± 0.64 ^b	7.52 ± 0.64 ^{a*}	0.060	0.023	NS
	Supp	7.24 ± 0.60 ^a	8.69 ± 0.64 ^{ab}			
Tb.Sp. (mm)	Adeq	0.120 ± 0.007 ^b	0.089 ± 0.007 ^{a*}	0.009	<0.001	NS
	Supp	0.096 ± 0.007 ^a	0.075 ± 0.007 ^{ab*}			
Femur neck						
BV/TV (%)	Adeq	61.4 ± 1.40	59.4 ± 1.29	NS	NS	NS
	Supp	59.4 ± 1.40	59.7 ± 1.21			
BS/BV (mm ² /mm ³)	Adeq	13.9 ± 0.99 ^{ab}	18.5 ± 0.92 ^{a*}	NS	NS	0.005
	Supp	16.0 ± 0.99 ^{ab}	14.7 ± 0.86 ^b			
Tb.Th. (mm)	Adeq	0.144 ± 0.007 ^{ab}	0.112 ± 0.006 ^{b*}	NS	NS	0.003
	Supp	0.127 ± 0.007 ^{ab}	0.138 ± 0.006 ^a			
Tb.N. (mm)	Adeq	4.27 ± 0.32 ^{ab}	5.53 ± 0.29 ^{a*}	NS	NS	0.013
	Supp	4.74 ± 0.32 ^{ab}	4.37 ± 0.27 ^b			
Tb.Sp (mm)	Adeq	0.091 ± 0.006	0.077 ± 0.006	NS	NS	NS
	Supp	0.087 ± 0.006	0.075 ± 0.007			

¹ Values are expressed as mean ± SEM. Means in a row with subscripts without a common letter differ, p < 0.05. NS, p>0.05. *Different from corresponding corn-oil treated females, P<0.05. NS, P>0.05.

Abbreviations: Adeq - adequate; BV/TV – trabecular bone volume; BS/BV – trabecular surface area; CON - control; FA - folic acid; ISO - soy isoflavones; NS - not significant; Supp- supplemental; Tb.Th – trabecular thickness; Tb.N – trabecular number; Tb.Sp – trabecular separation.

6.4.7 Serum measures of bone turnover and metabolism

There was an overall FA x ISO effect on serum OPG ($p=0.043$) concentrations. Female mice exposed to adequate FA+ISO had higher (FA, $p=0.003$; ISO, $p=0.005$) concentrations of OPG than all other treatment groups (Table 6-6). In addition, females exposed to adequate FA+ISO had a two-fold higher (FA, $p=0.004$; ISO, $p=0.026$; FA x ISO, $p=0.010$) ratio of OPG to RANKL compared to all other treatment groups (Figure 6-4). There were no differences in serum osteocalcin concentrations between groups. Female mice exposed to adequate FA+ISO had higher concentrations of ACTH ($p<0.001$) and IGF-I ($p=0.013$) compared to females exposed to adequate FA without ISO (Table 6-6). Exposure to adequate FA resulted in higher ($p<0.001$) serum ACTH levels than exposure to low FA, irrespective of treatment (Table 6-6). Mice exposed to low FA had lower concentrations of serum IGFBP-7 compared to mice exposed to adequate or supplemental FA with or without ISO. Exposure to supplemental FA+ISO had lower ($p=0.036$) levels of serum IGFBP-7 than exposure to supplemental FA alone.

6.4.8 Microarray analyses of the femur

Out of 45281 genes represented on the Mouse WG-6v2r3 array 30269 probes were analyzed on each of the 24 slides with each slide representing one mouse. Out of these probes, 949 genes were identified to be statistically different among groups (Figure 6-5). Out of these genes, 118, 113 and 405 genes were differentially expressed ($p<0.05$) by early life exposure to adequate FA+ISO, supplemental FA alone and supplemental FA+ISO, respectively (Figure 6-6). In addition, 37 genes were differently expressed by all three treatments (Figure 6-6). Out of the 37 genes, one gene (Gdf10) was identified to be an important modulator in skeletal morphogenesis.

Table 6-6. Serum markers of bone turnover and metabolism measured in 4 month old female CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil

	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
OPG (pg/ml)	Low	1298.7 ± 95.5	1451.8 ± 91.4 ^b	0.003	0.005	0.043
	Adeq	1422.6 ± 91.4	1901.7 ± 91.4 ^{a*}			
	Supp	1366.1 ± 91.4	1389.4 ± 91.4 ^b			
RANKL (pg/ml)	Low	111.1 ± 13.4	127.4 ± 14.0	NS	NS	NS
	Adeq	124.0 ± 13.4	90.4 ± 13.4			
	Supp	138.2 ± 13.4	113.2 ± 14.0			
Osteocalcin (ng/ml)	Low	3.31 ± 0.24	3.25 ± 0.23	NS	NS	NS
	Adeq	3.34 ± 0.23	3.18 ± 0.23			
	Supp	3.54 ± 0.23	2.88 ± 0.23			
ACTH (pg/ml)	Low	21.3 ± 7.2 ^b	20.6 ± 7.2 ^b	<0.001	NS	0.019
	Adeq	43.6 ± 7.2 ^a	64.3 ± 6.9 ^{a*}			
	Supp	43.3 ± 6.9 ^a	29.9 ± 7.2 ^b			
IGF-I (ng/ml)	Low	1.60 ± 1.43	1.94 ± 1.43	NS	0.013	NS
	Adeq	1.83 ± 1.43	2.40 ± 1.43*			
	Supp	1.97 ± 1.50	1.94 ± 1.50			
IGFBP-7 (pg/ml)	Low	10.4 ± 1.4 ^b	8.9 ± 1.3 ^b	<0.001	0.036	NS
	Adeq	14.8 ± 1.3 ^a	14.6 ± 1.4 ^a			
	Supp	17.4 ± 1.3 ^a	12.0 ± 1.4 ^{ab*}			

¹ Values are means ± SEM, n = 10-12. Labelled means in a column for a variable without a common letter differ, P < 0.05. NS>0.05. *Different from corresponding corn oil treated females, P<0.05. NS, P>0.05.

Abbreviations: ACTH - adrenocorticotrophic hormone; Adeq – adequate; CON - control; FA - folic acid; ISO - soy isoflavones; IGF - insulin growth factor; IGFBP - IGF binding proteins; NS - not significant; OPG - osteoprotegrin; RANKL - receptor activator of nuclear factor kappa-β ligand; Supp – supplemental.

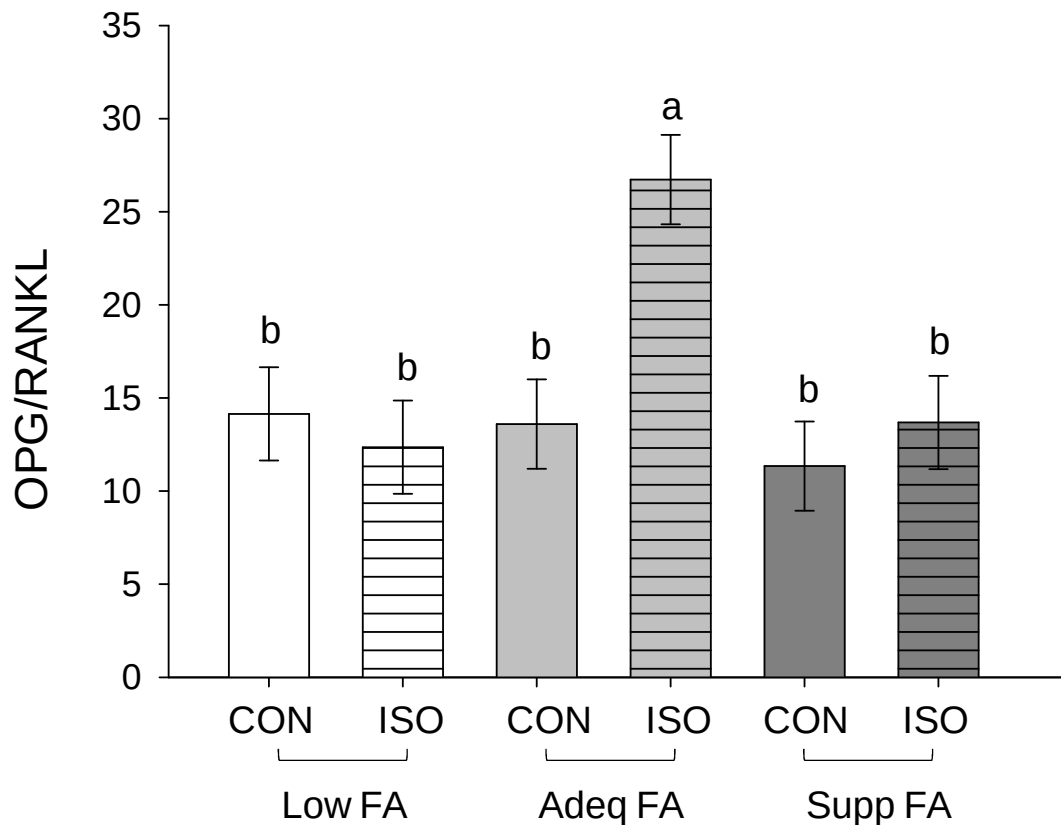


Figure 6-4. The OPG/RANKL ratio measured from serum of 4 month old female CD-1 mice whose mothers' were fed a low, adequate or supplemental FA during pregnancy and lactation, and who from postnatal day 1 to 10 were treated with subcutaneous injections of corn oil or ISO.

Values are given as ratios (n=8-10/group) and variables without a common letter differ, $P < 0.05$. Abbreviations: Adeq – adequate; FA – folic acid; OPG - osteoprotegerin; RANKL - receptor activator of nuclear factor kappa- β ligand; Supp – supplemental.

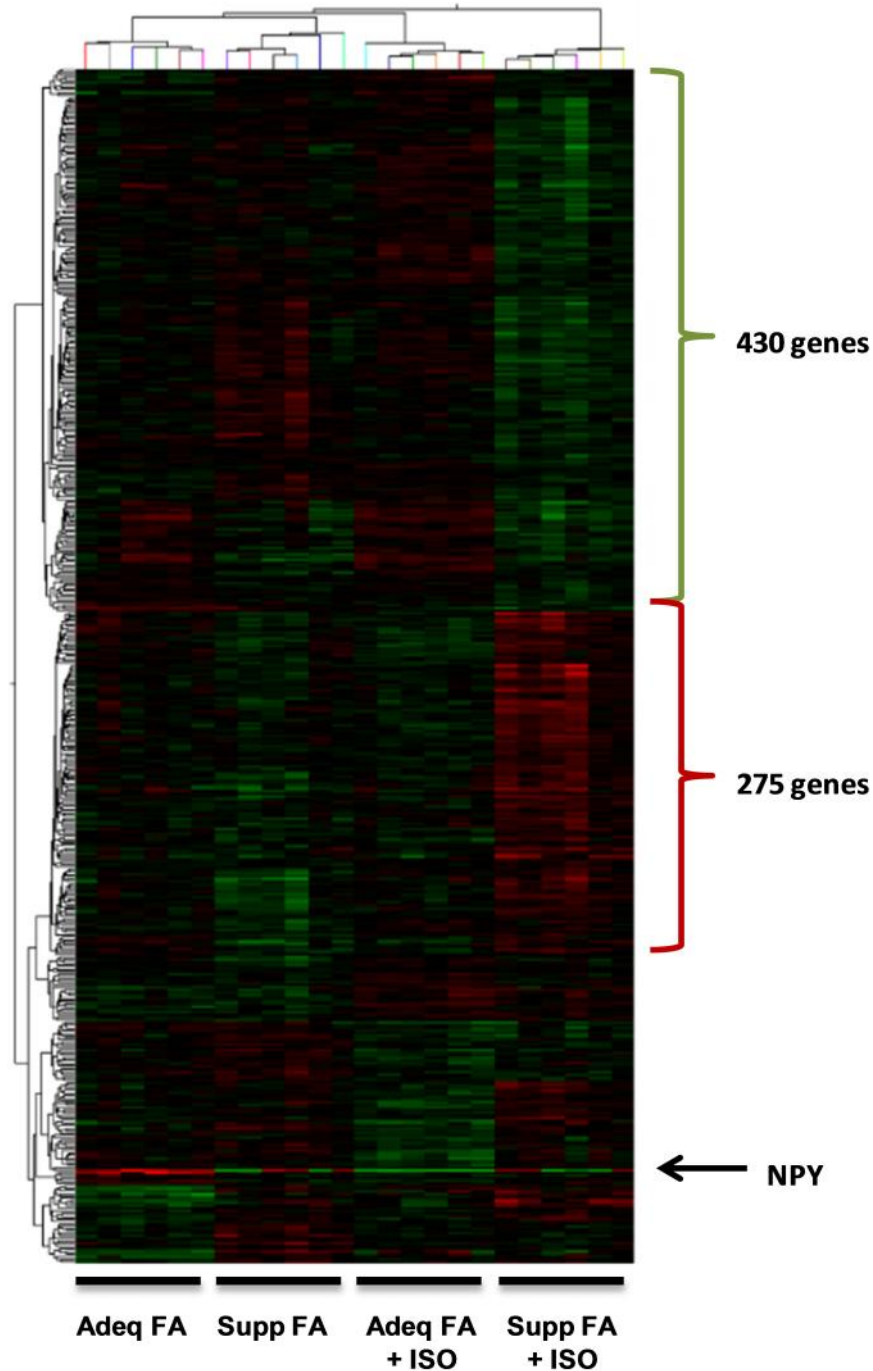


Figure 6-5. Expression profile and clustering of 949 signature genes that were identified to be statistically different between the groups.

The heat map visualizes the pattern of gene expression values ($\log_2$ transformed hybridization intensities). There are 949 rows corresponding to the 949 genes and the displayed intensities are the differences between the groups. The expression value for each difference is normalized across the samples to zero mean and one standard deviation for visualization purposes. Differences with expression levels greater than the mean are colored in red and those below the mean are colored in green.

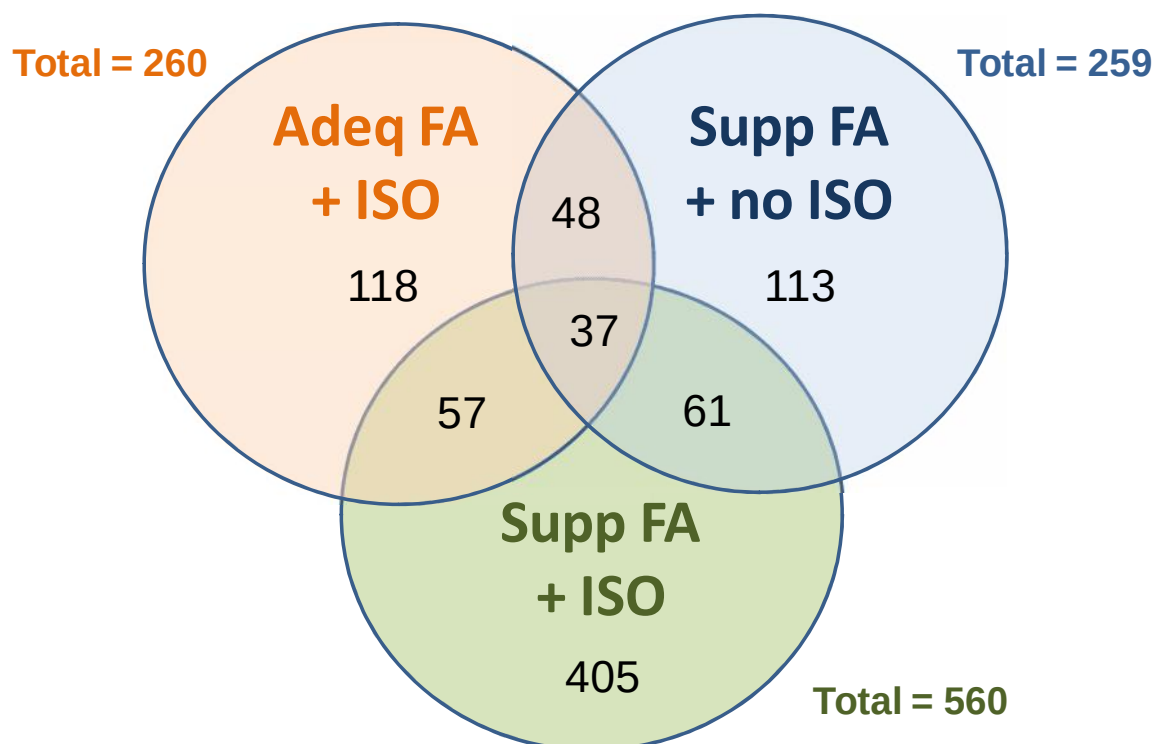


Figure 6-6. Venn Diagram showing the number of genes that are up- or down-regulated in femurs of 6-week old females whose mothers were fed a diet containing either adequate or supplemental levels of FA during pregnancy and lactation and were treated with either corn oil or ISO treatment from postnatal day 1 to 10.

Abbreviations: Adeq – adequate; FA – folic acid; ISO – soy isoflavones; Supp – supplemental.

Gene ontology revealed that exposure to adequate FA+ISO up-regulates the expression of 8 genes in the ‘isomerase activity’ pathway (Table 6-7). These genes are involved in protein folding, release of super-coiling and torsional tension of DNA, rearrangement of disulfide bonds and regulation of different receptors. A corrected hypergeometric test (Benjamini and Yekutieli, $p < 0.1$) revealed that females exposed to supplemental FA+ISO had an enrichment of genes showing over expression in the bone remodeling and resorption gene ontology when compared across all treatment groups. This effect was driven by increased expression of PTHr1 and Ctnnb1 (Table 6-8).

6.5 Discussion

This study is the first to show that ISO induced benefits to bone depend on the level of FA exposure. Female offspring whose mothers were fed an adequate FA diet, and were treated with ISO for the first 10 days of life had higher BMD, more bone volume (BV/TV) and greater trabecular connectivity ($\uparrow$ Tb.N. $\downarrow$ Tb.Sp.) at the femur neck and lumbar spine than females exposed to adequate FA without ISO. In addition, these mice had higher levels of serum OPG, IGF-I and ACTH, and a greater resistance to fracture at the femur and lumbar spine demonstrating that exposure to adequate FA+ISO during development improves overall bone quality and quantity at adulthood. Exposure to supplemental FA alone, at a dose that is four times the dietary requirement for rodents, also provided benefits to female bone but the effects were less pronounced than those induced by adequate FA+ISO as the serum concentrations of OPG and IGF-I, as well as trabecular connectivity at the femur neck were not significantly improved. This less pronounced effect may be because during early stages of development exposure to estrogen-like compounds rather than nutrients has a more profound effect on programming of bone development. In contrast, exposure to a

Table 6-7. List of differentially expressed gene categorized according to gene ontology

Group	Gene Ontology	Number of genes affected	Direction of change
Adequate FA + ISO	Isomerase activity	8	↑ 8
Supplemental FA + no ISO	Ribosome	7	↓ 7
	Organellar	4	↓ 4
	Mitochondrial ribosome	4	↓ 4
	Ribonuclear complex	9	↓ 9
	Mitochondrial matrix	4	↓ 4
Supplemental FA + ISO	DNA recombination	9	↓ 9
	Double strand break repair	6	↓ 6
	DNA repair	12	↓ 12
	Response to DNA damage stimulus	13	↓ 13
	DNA metabolic process	18	↓ 18
	Cellular response to stress	14	↓ 14
	Cellular macromolecule metabolic process	71	↓ 71
	Somatic cell DNA recombination	5	↓ 5
	Somatic diversification of immune receptors via germ-line	5	↓ 5
	Recombination repair	3	↓ 3
	Double-strand break repair via homologous recombination	3	↓ 3
	V(D)J recombination	4	↓ 4
	Somatic diversification of immune receptors	5	↓ 5
	Nuclear part	23	↓ 23
	uracil DNA N-glycosylase activity	2	↓ 2

Abbreviations: FA – folic acid; ISO – isoflavones.

Table 6-8. List of differentially expressed genes in mouse clones that help to explain the effects induced by adequate FA+ISO, supplemental FA and supplemental FA+ISO on bone programming

Gene Symbol	Full Name	Gene Description	Fold Change
Adequate FA+ISO			
Top1mt	DNA topoisomerase I	Releases the super coiling and torsional tension of DNA	1.29
P4hb	Protein disulfide-isomerase	Catalyzes the formation, breakage and rearrangement of disulfide bonds.	1.10
Ppia	Peptidylprolyl isomerase A	Accelerates protein folding	1.10
NPY	Neuropeptide Y	Neurotransmitter that promotes bone formation by increasing osteoblast number	-5.67
Supplemental FA+no ISO			
Irf-5	Interferon regulatory factor	Transcription factor that plays a role in cell growth, differentiation and immune function; modulates IGFBP-7 transcription	1.19
P4hb	Protein disulfide-isomerase	Catalyzes the formation, breakage and rearrangement of disulfide bonds	1.26
Supplemental FA+ISO			
LOC100046770	Histone macroH2A1.2	Regulates stable X chromosome inactivation by inhibiting histone acetylation, interfering with DNA remodeling complexes, and inactivating transcription factor binding	-1.29
Top2a	DNA topoisomerase 2-alpha	Controls and alters the topologic states of DNA during transcription by catalyzing the transient breaking and rejoining of two strands of duplex DNA; involved in chromosome condensation, chromatid separation, and the relief of torsional stress that occurs during DNA transcription and replication.	-1.32
P4hb	Protein disulfide-isomerase	Catalyzes the formation, breakage and rearrangement of disulfide bonds	1.18
PTHr1	Parathyroid hormone	Regulates calcium ion homeostasis through activation of	1.63

Gene Symbol	Full Name	Gene Description	Fold Change
Ctnnb1	receptor 1 β -Catenin	adenylate cyclase and phospholipase C; when activated by PTH stimulates bone resorption Part of a complex of proteins that constitute adherens junctions; it anchors the actin cytoskeleton and play a role in transmitting the contact inhibition signal that causes cells to stop dividing once the epithelial sheet is complete.	1.76
Phospho1	Phosphatase, orphan 1	Involved in the generation of inorganic phosphate for bone mineralization	1.80
Irf-4	Interferon regulatory factor	Transcription factor that plays a role in cell growth, differentiation and immune function; modulates IGFBP-7 transcription	-1.20
Crebl1	cAMP response element binding protein	stimulates transcription and indirectly regulates IGFBP-7	-1.21

Abbreviations: FA – folic acid; ISO – soy isoflavones; PTH – parathyroid hormone.

supplemental level of FA+ISO had a modest effect on bone development with only BMD at the lumbar spine being significantly higher than that of mice treated with adequate FA without ISO. Thus, this study is the first to show that early exposure to supplemental FA stimulates bone development when given alone but not in combination with ISO. Based on these observations we hypothesized that ISO and FA may be inducing their effects through a common mechanism. To better understand the underlying mechanism, we compared the gene expression profiles of femurs extracted from 6-week old female CD-1 mice who during development were exposed to an adequate or a supplemental level of FA with or without ISO. We did not investigate the gene expression profiles of females exposed to a low level of FA with or without ISO because there were no significant changes in BMD or bone strength in this group.

The finding that female mice exposed to adequate FA+ISO, supplemental FA or supplemental FA+ISO had 3% of their genes changed in bone at 7 weeks of age provided evidence that ISO and FA improve bone through an epigenetic mechanism. We originally hypothesized that ISO enhance histone acetylation, which loosens up the DNA and allows FA to more easily transfer the one-carbon unit that is needed for DNA methylation. Illumina gene-chip based microarray data showed that females exposed to adequate or supplemental FA+ISO had lower expression of LOC100046775 gene (a homologue of Histone Macro H2A.1) and higher expression of Top-1mt/2a (topoisomerase) than females exposed to adequate FA without ISO (Figure 6-7). LOC100046775 is a protein that inhibits histone acetylation and transcription factor binding [218], while topoisomerase is an enzyme that releases the super coiling and torsional tension of DNA. Thus, by altering the expression of these genes, ISO and FA may activate histone acetylation and assists in releasing the super

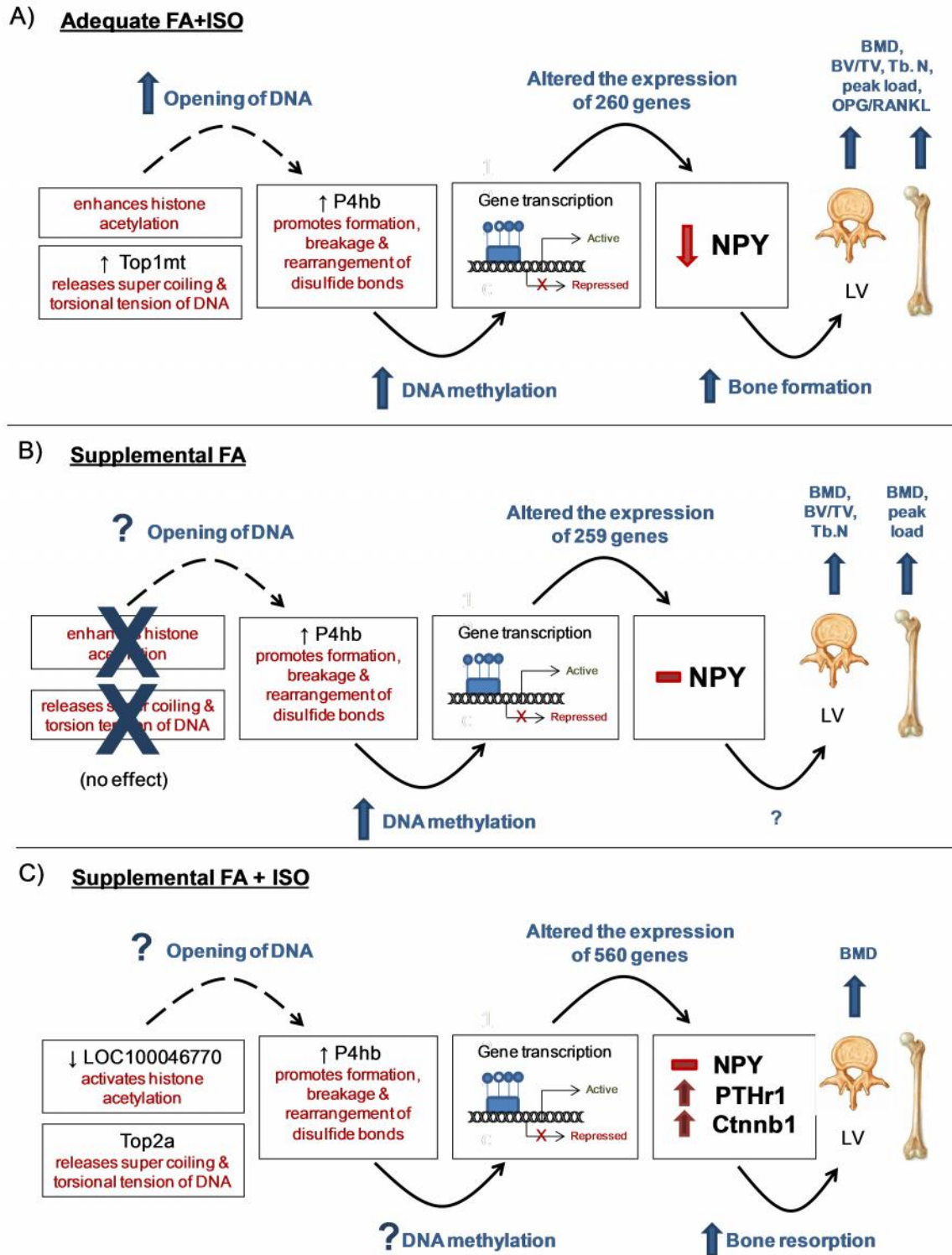


Figure 6-7. Mechanism used to describe how early life exposure to adequate FA+ISO, supplemental FA and supplemental FA+ISO programs bone development of female CD-1 mice at adulthood.

Dotted curved arrows are showing the effects that have not been scientifically proven while solid curved arrows represent the effects for which there is some scientific proof.

coiling and torsional tension of DNA.

Opening of DNA near the promoter allows enzymes, proteins and transcriptions factors to more easily regulate DNA methylation and gene transcription. Accordingly, we identified that females exposed to adequate FA+ISO, supplemental FA or supplemental FA+ISO had higher expression of P4hb gene (Figure 6-7, Table 6-8). The gene P4hb encodes an enzyme that catalyzes the formation, breakage and rearrangement of disulfide bonds. Therefore, all three treatments may enhance DNA methylation. The number of disulfide bonds created in part depends on the amount of available methyl donors, which is why we originally provided different levels of FA to CD-1 mice. As expected, females exposed to supplement FA+ISO had a higher number of differentially expressed genes (560 genes) than those exposed to adequate FA+ISO (260 genes) or supplemental FA alone (259 genes) (Figure 6-6). Importantly, the differentially expressed genes of females exposed to supplemental FA with or without ISO were involved in DNA synthesis, stability, integrity or repair - supporting the principle function of folate. However, based on the difference observed between groups in bone outcome measures (i.e. BMD, bone structure, bone strength and serum marks of bone turnover) we knew that females exposed to supplemental FA+ISO had some underlying changes in gene expression that were not present in mice exposed to adequate FA+ISO or supplemental FA alone. As such, we searched for potential hormones, enzymes, receptors or transcription factors that may be altered when supplemental FA and ISO are given in combination but not alone. Gene ontology⁴ revealed that early life exposure to supplemental FA+ISO up-regulates bone remodeling and bone resorption by increasing the expression of type 1 parathyroid hormone receptor (PTHr1) and beta catenin (Ctnnb1). Therefore, the

⁴ Gene ontology is a bioinformatics tool that can be used to identify groups of genes that are differentially expressed in the same pathway and thus, have the potential to have profound biological effects.

higher rate of bone resorption induced by PTHr1 and Ctnnb1 activation is likely the underlying reason why female mice exposed to supplemental FA+ISO do not have improved bone structure and bone strength at adulthood.

Similarly, to help identify the underlying mechanism by which early life exposure to adequate FA and ISO improves bone development, gene expression profiles were compared. Only females exposed to adequate FA+ISO had a 5.7-fold lower expression of NPY at the femur than females exposed to adequate FA without ISO. The promoter of NPY spans over 246 base pairs and has 3 transcription factor binding sites (NGF-RE, AP-2 and NGFI-A) that contain CpG sites [219], which indicates that imprinting of female bone may in part be mediated by NPY. Out of 949 genes that were identified to be differentially expressed in the mouse genome, NPY was the second most highly altered gene. The most profoundly misexpressed gene was A530055J02Rik, which is a non-coding RNA that was equally suppressed in all three treatment groups. Thus, NPY rather than A530055J02Rik is the more likely candidate responsible for bone improvement of female mice exposed to adequate FA+ISO.

Widely expressed in the central and peripheral nervous system, NPY is an important regulator of many physiological processes, including bone and energy metabolism [220]. In bone, NPY inhibits osteoblast formation [221], decreases osteoblast cell number [222], reduces the expression of late stage genes that promote bone formation (i.e. osteoclastin and DMP-1) [223] and decreases mineral deposition [224]. Thus, the finding that female CD-1 mice exposed to adequate FA+ISO had reduced NPY expression in bone tissue at 6-weeks of age demonstrates that NPY may in part be responsible for the observed improvements in bone tissue (i.e. $\uparrow$ BMD, $\uparrow$ Tb.N, $\uparrow$ OPG/RANKL ratio, $\uparrow$ bone strength). In addition, the

discovery that NPY expression is suppressed 6-week after exposure suggests that adequate FA+ISO may be inducing this effect through an epigenetic mechanism which bringing us back to our original hypothesis that ISO and adequate FA may work in combination to enhance DNA methylation. Lastly, taking that genes are not expressed in isolation but rather in the context of other genes and their products, cells and tissues in a temporal and special dimension, the finding that NPY is down-regulated in bone suggests that NPY gene expression may also be altered in other tissues.

Traditionally bone was studied as an independent organ, but emerging findings indicate that signalling pathways exist between bone, adipose tissue and reproductive organs so studying whole-organism physiology is highly important. From published literature we know that there is a degree of connection between the NPY-mediated changes in bone and energy homeostasis [220]. However, the degree to which these processes are interrelated remains to be elucidated.

In this study, we identified that females exposed to adequate FA+ISO had higher body weight from 4 through 16 weeks of age than females exposed to adequate FA without ISO. Although this finding has previously been shown [85], this study is the first to demonstrate that ISO-induced effects on body weight depend on the amount of FA exposure. Body weight of females exposed to a low or supplemental level of FA in combination with ISO did not differ from females exposed to a low or supplemental level of FA without ISO. In another study, Penza et al [225] showed that C57BL/6 female mice exposed to ISO from 4 to 6 weeks of age had higher deposition of adipose tissue than control animals. To date, no studies have explored the underlying mechanism but it is possible that ISO-induced weight gain and fat deposition is in part mediated by NPY.

According to published literature low concentrations of NPY in bone stimulate osteoblasts to produce osteocalcin [223]. Bone-derived osteocalcin is a well known regulator of glucose metabolism that when carboxylated promotes β -cell proliferation, insulin secretion and insulin sensitivity in muscle, liver and white adipose tissue [226]. Thus, it is possible that coordinated regulation exists between bone and energy metabolism that is in part controlled by NPY and/or osteocalcin. In this study we did not observe significant changes in total serum osteocalcin concentrations but this is likely because we did not measure bone-specific osteocalcin or the carboxylated form of osteocalcin, which would have been better measures of NPY-induced osteocalcin production.

A recently published study highlighted that during stress NPY can signal directly to Y1 receptors in osteoblasts and Y1 and Y2 receptors in adipocytes to suppress NPY in bone and up-regulate NPY in fat tissue (Figure 6-8). Accordingly, female mice exposed to adequate FA+ISO had higher concentrations of serum ACTH, which is a hormone that stimulates cortisol production and activates the stress hormone response. Thus, it is possible that by activating the stress-hormone pathway through an estrogen receptor-mediated mechanism ISO and adequate FA suppress NPY gene expression in bone tissue. Further research is needed to explore this pathway.

In summary, female mice exposed to an adequate level of FA+ISO or a supplemental level of FA without ISO had significant improvements in BMD, trabecular connectivity and resistance to fracture when compared to females exposed to adequate FA without ISO, while improvements induced by low or supplemental FA+ISO were limited to higher BMD at the lumbar spine. Based on the gene expression profiles, we believe that these functional outcomes are in part a reflection of epigenetic changes that drive the expression of endocrine

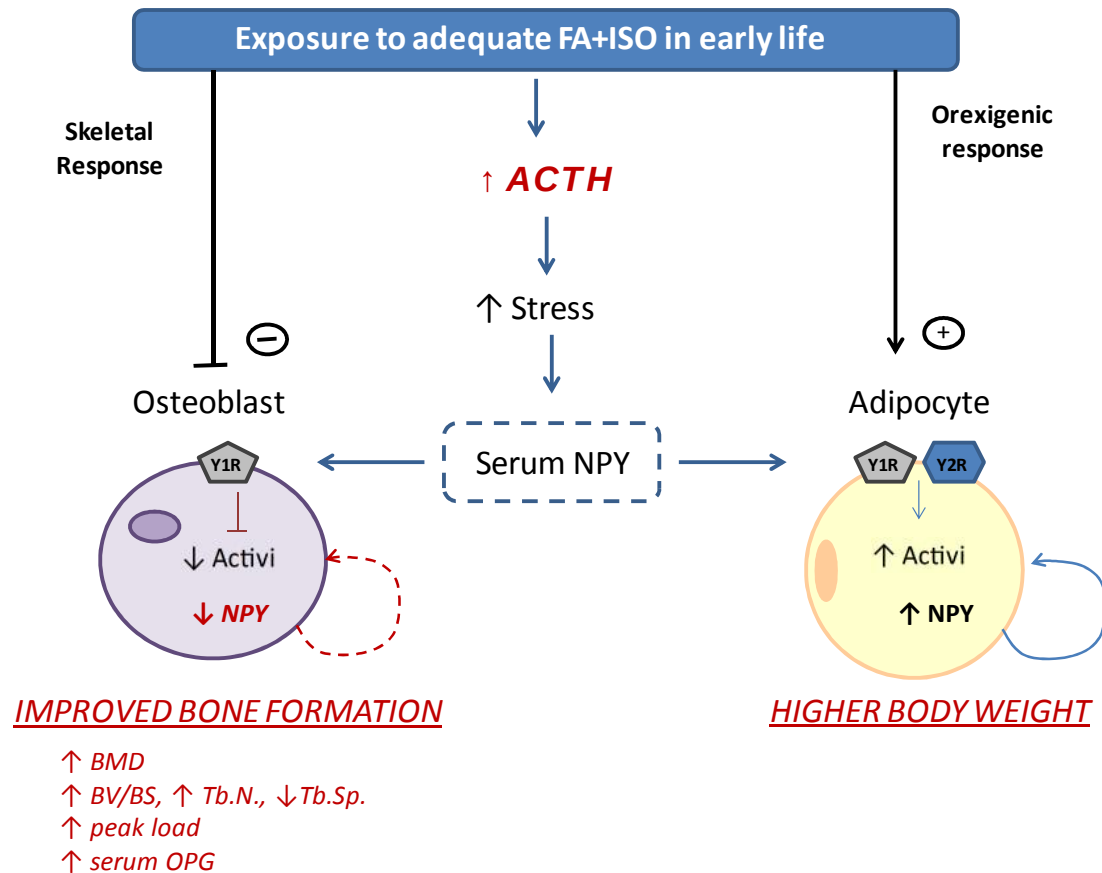


Figure 6-8. Effects of adequate FA+ISO on peripheral regulation of bone and fat mass by NPY.

Although still largely undefined exposure to adequate FA+ISO during early life may regulate both bone formation and body weight regulation by activating NPY through the stress hormone pathway. In this study, early life exposure to adequate FA+ISO resulted in higher ACTH concentrations. Elevated levels of ACTH stimulate the stress hormone pathway to activate NPY secretion. Circulating NPY can then signal directly to Y1 receptors in bone cells and/or Y1 and Y2 receptors in fat cells. Activation of Y1 receptors in osteoblasts suppresses bone-specific NPY expression, which directly improves bone formation resulting in higher BMD, trabecular connectivity, bone strength and higher serum OPG. In contrast, activation of Y1 or Y2 receptor in adipocytes stimulates NPY expression that in turn promotes lipid accumulation. Note: the measures that are italicized and shown in red have been analyzed in this study. This figure was modified from Shi and Baldock, 2012 [220].

factors. For example, the finding that early life exposure to adequate FA+ISO up-regulates the expression of genes involved in the isomerase activity pathway versus exposure to adequate FA + corn oil suggests that ISO regulate gene transcription through epigenetic regulation. In this study, the expression of NPY was most profoundly suppressed in bone tissue of females exposed to adequate FA+ISO during development. Since low levels of NPY promote bone formation, it is possible that observed improvements in BMD, bone structure and bone strength observed in this group were in part mediated by NPY. In contrast, the finding that early life exposure to a supplemental level of FA+ISO activates bone resorption by up-regulating the expression of PTHr1 and Ctbbn1 genes in bone provides a potential mechanism to explain the lack of bone improvements observed in this group compared to adequate FA + corn oil. Ongoing studies are needed to confirm that changes in gene expression profiles of PTHr1, Ctnnb1, NPY are present at both the gene and protein level when analyzed via real-time PCR. For the foreseeable future, animal models provide the most powerful tool to map out and to study the effects of ISO and FA on programming of bone and all the unidentified inter-organ connections.

Chapter Seven

STUDY 4

**ADEQUATE BUT NOT SUPPLEMENTAL FOLATE COMBINED WITH SOY
ISOFLAVONES DURING EARLY LIFE IMPROVES BONE HEALTH AT
ADULTHOOD IN MALE MICE**

7.0 STUDY 4: ADEQUATE BUT NOT SUPPLEMENTAL FOLATE COMBINED WITH SOY ISOFLAVONES DURING EARLY LIFE IMPROVES BONE HEALTH AT ADULTHOOD IN MALE MICE

7.1 Abstract

Previous investigations from our laboratory have demonstrated that neonatal exposure to ISO improves bone outcomes in CD-1 mice at adulthood with greater benefits in female than males. This study determined whether exposure to a supplemental level of FA during pregnancy and lactation – that may enhance DNA methylation of CpG sites upstream of a gene promoter - in combination with neonatal exposure to ISO provides greater benefits to male bone development than ISO alone. CD-1 dams (n=36) were randomized to a low (0 mg/kg), adequate (2 mg/kg) or supplemental (8 mg/kg) level of FA during pregnancy and lactation. Offspring received daily subcutaneous injections of corn oil (vehicle) or ISO (7 mg/kg body weight/day) from postnatal day 1 to 10. From weaning, all male pups were fed an adequate FA diet and studied to age 4 months, which is the time when peak bone mass is established in this mouse model. The benefit of exposure to low FA+ISO was limited to a higher ($p<0.05$) femur BMD than mice exposed to low FA alone. In contrast, offspring exposed to adequate FA+ISO had multiple benefits to bone health: higher ($p<0.05$) BMD and greater ($p<0.05$) resistance to fracture at the femur and lumbar spine than mice exposed to adequate FA without ISO. Exposure to a supplemental level of FA+ISO resulted in higher ($p<0.05$) serum OPG and higher ratio of OPG to RANKL but did not result in greater BMD or strength at the femur or lumbar spine than supplemental FA alone. In conclusion, early life exposure to adequate FA+ISO provided functional benefits to male bone development, while improvements induced by supplemental FA+ISO were limited to a higher level of serum OPG and had no functional benefits to bone. Further investigation is needed to determine

whether the combination of supplemental FA and ISO activates a negative feedback mechanism that in turn attenuates bone formation.

7.2 Introduction

Many foods contain an array of nutrients and food components that can program an individual's risk of developing a chronic disease during adulthood [207-209]. These effects are more pronounced if exposure occurs during critical stages of development when tissues are sensitive to epigenetic programming [207-209]. For example, when larvae of genetically identical female clones are exposed to royal jelly (an incompletely defined mixture of proteins, amino acids, vitamins, lipids and other nutrients) for the first three days of life, the larvae develop into worker bees [208, 227, 228]. In contrast, if larvae are provided royal jelly throughout development, they mature into queen bees. Workers and queens differ in their DNA methylation profiles, morphology, capacity to reproduce, behaviour and longevity. These findings highlight that the timing of exposure to a nutrient or bioactive has profound effects on structural and functional programming of an organism. More recently it was demonstrated that exposure to royal jelly during development altered the expression of 35% of the honeybee genome by stimulating DNA methylation [227, 229]. In vertebrates, ISO and FA have the potential to permanently change gene transcription by enhancing DNA methylation [15, 17, 91].

Human infants can be exposed to elevated levels of ISO and FA during the earliest stages of life. Commercially available soy protein formulas are natural sources of ISO (32-47 mg/L of formula) and are fortified with FA (2-4 mg/L of formula) [8, 9]. Thus, infants fed this type of formula have markedly higher serum ISO (10 fold higher) and FA (2.3 fold higher) levels than infants fed breast milk or cow milk formula [7-9]. Moreover, a portion of

North American infants fed soy protein formula are also exposed to high levels of FA in utero because up to 40% of the population has high red blood cell folate concentrations (>1360 nmol/L) [10] due to mandatory fortification of the food supply and widespread use of FA supplements. Therefore, there is an ongoing need to characterize how early life exposure to supplemental FA and ISO affects growth, development and future health.

ISO are plant-derived phytochemicals that share a common phenolic ring and a 4'-hydroxyl group with 17β -estradiol and can bind to estrogen receptors to selectively stimulate or inhibit estrogen-like responses [2]. Reports from men with aromatase and/or estrogen receptor deficiency have revealed that early life exposure to estrogen-like compounds is crucial for male bone growth and development [106-108, 110]. This is because estrogen withdrawal prevents the epiphyseal fusion that stops bones from lengthening, up-regulates bone resorption, reduces mineral distribution and decreases BMD [110]. Conversely, estrogen therapy or exposure to selective estrogen receptor modulators (i.e. tamoxifen, raloxifene) promotes epiphyseal closer and restores BMD within several months of treatment, but only if exposure occurs during development [110, 111]. Thus, timing of estrogen exposure is crucial for healthy skeletal development in males.

Using the CD-1 mouse model, we have previously shown that short-term neonatal exposure to dietary estrogens (i.e. ISO) provides modest benefits to male bone development [12, 13, 54, 85]. Specifically, male mice treated with genistein for the first 5 days of life, at a dose that mimicked the amount of genistein consumed by infants fed soy protein formula, had higher lumbar spine BMD and strength at 4 months of age compared to mice treated with the corn oil vehicle [12]. In contrast to females, the effects induced by genistein in males were modest, and did not mimic those of the positive control (i.e. DES) suggesting that

genistein may enhance bone development in males through an estrogen-independent mechanism. Assessment of serum bone biomarkers (i.e. osteocalcin and collagen type I C-telopeptide cross-links) showed that genistein-treated males have lower bone turnover [12]; a phenomenon well known to be associated with a lower rate of bone tissue deterioration and a lower incidence of fragility fractures [230]. This finding suggested that early life exposure to genistein may reduce the risk of developing osteoporosis and associated fragility fractures. A follow-up study, which used a similar experimental design but included daidzein and the combination of genistein and daidzein, similarly showed that the male skeleton is less responsive to early ISO exposure than the female skeleton [54]. Unlike female offspring, males exposed to daidzein, genistein or the combination did not exhibit significant improvements in BMD or strength at the femur or lumbar spine at 4 months of age. However, when these two studies are compared, male mice exposed to ISO (i.e. genistein, daidzein or genistein + daidzein) during early development had similar improvements in bone outcomes at adulthood [12, 54]. In the first study, genistein-treated males had a 9.8% higher lumbar spine BMD than corn oil treated mice [12] while, in the follow-up study, genistein exposure resulted in a 8% higher lumbar spine BMD [54]. In support of these findings, Chen et al showed that male piglets fed with soy protein formula, rich in ISO, for the first 21 or 35 days of life have a lower concentration of serum collagen type I C-telopeptide cross-links and a higher ratio of osteoblasts to osteoclasts than piglets fed with breast milk or cow milk formula [98]. In summary, while ISO have some positive effects on male bone development, the male skeleton is less responsive to ISO exposure than the female skeleton. However, because some nutrients or food components have additive or synergistic effects on bone, it is possible that neonatal exposure to ISO in combination with another

bioactive food component may provide greater benefits to male bone development than exposure to ISO alone.

Less studied is the effect of exposure to differing levels of maternal FA. However, the Avon longitudinal study of parents and children from southwest England revealed that maternal FA status has an independent positive effect on bone development of offspring [231]. The authors proposed that the underlying mechanism may involve altering the methylation status of CpG sites or islands that are upstream or near the promoters of bone-specific genes. The viable yellow agouti (A^{vy}) mouse is a commonly used animal model to study the effects of nutrients on DNA methylation. Indeed, studies using this mouse model have demonstrated that offspring of mothers' fed a diet rich in FA as well as other methyl donors during pregnancy and lactation have a shift in coat color from yellow to dark brown and lower body weight due to increased methylation of CpG sites in the agouti genome [15-17]. These changes in coat color and body weight are also imprinted (meaning that the responses are expressed independent of the classical Mendelian inheritance) by early life exposure to genistein – the most abundant ISO in soy protein [15]. According to *in vitro* data, through an estrogen receptor- α and - β mediated mechanism, ISO enhance acetylation of core histones [18], which loosens up the covalent bonds between the histones and DNA and allow FA to more easily donate a one-carbon group to the cytosine residue – a hallmark of DNA methylation. Thus, it is possible that exposure to ISO and FA in combination may have additive or synergistic effects on programming of health including bone growth and development. In a recently published rat study, maternal diet fortified with ISO and FA had greater protective effects on post-neural tube closure in male and female offspring than either treatment alone [19]. This effect was in part due to reduced DNA damage and neuron

apoptosis. No studies have investigated how combining supplemental FA and ISO affects bone development. Therefore, the objective of this study was to determine whether exposure to supplemental FA during pregnancy and lactation in combination with neonatal exposure to ISO results in higher BMD and greater bone strength at femur and lumbar vertebrae than ISO alone in male CD-1 mice at adulthood.

7.3 Methods

7.3.1 Animals and treatment

Seven week old CD-1 mice (Charles River Laboratories, St. Constant, QC, Canada), housed in the Department of Comparative Medicine at the University of Toronto under standard environmental conditions (23°C, 50% humidity, 12:12h light-dark cycle), were randomized to an amino acid based diet (Dyets, Bethlehem, PA) devoid of ISO and fortified with 0, 2 or 8 mg of FA per kg of diet (

Figure 6-1, Table 6-1). Water was provided *ad libitum*. The amino acid diet was used because it is the only rodent diet that does not contain any natural folate, allowing investigators to more tightly control the level of FA within the a diet. Mice consuming the 0 mg FA diet had progressive FA deficiency of a moderate degree without growth retardation or premature death [210, 211] because mice are coprophagous and can obtain some folate that is synthesized by bacteria in the intestine [212]. The diet containing 2 mg of FA was used to represent the basal dietary requirement for rodents while [213], the 8 mg of FA diet was used to mimic the amount of FA that pregnant women may obtain by consuming FA supplements and FA-fortified foods. This supplemental level of FA has also been consistently shown to provide a degree of chemoprevention against colorectal cancer in rodent models [210, 211, 214, 215].

After a week of adaptation to the environment and diet, mice were mated harem style. Offspring of dams that delivered 8-12 pups on the same day and were fed the same FA diet were cross-fostered by assigning a maximum of one male and one female pup from each dam to a litter. Cross-fostered litters on low, adequate or supplemental FA diet were then randomized to corn oil or ISO treatment. Control pups received corn oil [12, 13, 54], while pups randomized to ISO received 0.010 mg of daidzein and 0.025 mg of genistein solubilized in 1 mL of dimethyl sulfoxide and suspended in corn oil [232]. All treatments were administered from postnatal day 1 to 10 via subcutaneous injection using a total volume of 20 μ L/pup/day. Because the ISO concentration is directly related to a pup's body weight, the concentration of daidzein in CD-1 mice ranged from 5.43 mg/kg of body weight on postnatal day one to 2.69 mg/kg of body weight on postnatal day ten. Similarly, genistein concentrations ranged from 13.6 mg/kg of body weight on postnatal day one to 6.7 mg/kg body weight on postnatal day ten. This dose is similar to the dose previously shown to resemble the concentration of daidzein and genistein in infants fed soy protein formula [54, 122]. On postnatal day 21, male sibling pairs of females reported in Chapter 6 (Study 3) were weaned, housed 3-4 per cage and fed an amino acid diet fortified with 2 mg of FA per kg of diet. Body weight of mice was measured daily from birth to postnatal day 10, at weaning (postnatal day 21) and every 4 weeks thereafter until necropsy was performed on postnatal day 120 via carbon dioxide inhalation and cervical dislocation. At necropsy, serum and tissue samples were collected. Organ weight was used to monitor for gross adverse effects due to ISO and/or different levels of FA. All protocols for animal use and treatment were approved by the University of Toronto Animal Care Committee and were in compliance with the guidelines of the Canadian Council on Animal Care [216].

7.3.2 BMD of femurs and LV1-LV3

Intact lumbar vertebrae (LV1-LV3) and whole femur were placed flat on the densitometer and scanned in air at room temperature using DXA (pSabre, Orthometrix, White Plains, NY) and a specialized software program (Host Software Version: 3.9.4; Scanner Software Version: 1.2.0) [11, 13, 54]. Whole femurs and intact LV1-LV3 were scanned at a speed of 2 mm/min and a resolution of 0.01 mm X 0.01 mm.

7.3.3 Biomechanical strength properties of femur and LV2

Biomechanical strength properties of LV2, femur midpoint and femur neck were determined using a materials testing system (Model 4442 Universal Testing System, Instron Corp., Canton, MA) and a specialized software program (Instron Series IX Automated Materials Tester, Version 8.15.00; Instron Corp.) [13, 54, 85]. Prior to biomechanical testing, LV2 and a femur from each mouse were placed in 0.9% saline solution for 3 hrs at room temperature to mimic physiological conditions. Electronic precision calipers (Cedarlane Laboratories Ltd. Hornby, Canada) and a balance (Sartorius AG, Goettingen, Germany) were used to measure the dimensions and weight of femurs as previously described [13, 54, 85].

To determine the strength properties of skeletal sites rich in trabecular bone, each LV2 was placed in the center of a stainless steel plate and a compression force was applied to the vertebrae by lowering a suspended stainless steel plate at a constant rate of 2 mm/min until a compression fracture was achieved [12, 85, 202]. Alternatively, individual femurs were placed vertically in a customized holder and a stainless crosshead was lowered at a constant rate of 2 mm/min until the femur neck fractured. For both sites, peak load (i.e. the maximum force a bone can withstand before fracture occurs) was identified as the first peak of the load displacement curve. To determine the strength properties of a skeletal site rich in cortical bone, three-point bending was performed at the femur midpoint as previously described [11,

13, 54, 202]. The posterior surface of the right femur was placed on two 1-mm wide base supports with a jig span width of 15 mm so that the midpoint was positioned directly under the crosshead. The crosshead was lowered at a speed of 2 mm/min until fracture occurred.

7.3.4 Biochemical Analyses

Serum OPG, RANKL and ACTH were measured using Milliplex MAP, based on the Luminex xMAP technology by Millipore (Billerica, MA). The intra-assay coefficient of variation for ACTH was 1.3-3.1%, for OPG was 1.0-2.6% and for RANKL was 2.3-5.7%. The OPG/RANKL ratio was determined based on serum levels of OPG and RANKL for each mouse.

7.3.5 Statistical analyses

Statistical analyses were performed using SigmaStat (Version 3.5, Jandel Scientific). Data are expressed as mean $\pm$ SEM. One-way ANOVA followed by a Student Newman-Keul's post-hoc test was used to analyze differences in body weight at birth. For all other measures, two-way ANOVA was used with FA and ISO intervention as the main effects, and interaction (FA x ISO) was assessed. For data that did not follow a normal distribution (i.e. lumbar spine BMC and peak load; femur neck peak load; serum OPG and ACTH concentrations; and weight of testes, kidney and liver), a Kruskal-Wallis two-way ANOVA on ranks was used to determine differences among groups. Statistical significance was determined as $p \leq 0.05$.

7.4 Results

7.4.1 Body weight

At birth, male mice exposed to adequate FA had lower ($p < 0.001$) body weight compared to those exposed to low or supplemental levels of FA (Table 7-1). However, on

Table 7-1. Body weight and total weight gain of 4-month old CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO

LV2	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
Birth weight (g)	Low	1.90 ± 0.022 ^a	1.95 ± 0.024 ^a	<0.001	0.007	NS
	Adeq	1.81 ± 0.022 ^b	1.85 ± 0.023 ^b			
	Supp	1.88 ± 0.026 ^a	1.95 ± 0.023 ^a			
Post-treatment (g), postnatal day 10	Low	5.02 ± 0.115 ^b	5.69 ± 0.124 ^b	<0.001	<0.001	NS
	Adeq	5.98 ± 0.120 ^a	6.25 ± 0.131 ^{a*}			
	Supp	5.96 ± 0.136 ^a	6.58 ± 0.128 ^{a*}			
Weaning (g), postnatal day 21	Low	9.82 ± 0.403 ^b	12.3 ± 0.433 ^{b*}	<0.001	<0.001	0.004
	Adeq	13.0 ± 0.482 ^a	12.8 ± 0.535 ^b			
	Supp	12.0 ± 0.637 ^a	15.1 ± 0.460 ^{a*}			
Final weight (g), postnatal day 118	Low	43.1 ± 1.06 ^b	44.9 ± 1.16 ^{ab}	<0.001	NS	NS
	Adeq	48.4 ± 1.19 ^a	48.4 ± 1.48 ^{ab}			
	Supp	47.4 ± 1.71 ^a	47.5 ± 1.16 ^{ab}			
Weight gain	Low	33.6 ± 1.33	32.6 ± 1.45	NS	NS	NS
From postnatal day 21 to 118 (g)	Adeq	37.5 ± 1.48	35.7 ± 1.85			
	Supp	35.4 ± 2.14	34.8 ± 1.45			

Values are means ± SEM, n = 8-18. Labeled means in a column for a variable without a common letter differ, P < 0.05. NS > 0.05. The star (*) denotes that the measured value is different from the corresponding corn oil control male, p<0.05. NS, p>0.05.

Abbreviations: Adeq – adequate; CON - control; FA - folic acid; ISO - soy isoflavones; NS - not significant; PND - postnatal day; Supp - supplemental.

postnatal day 10, mice exposed to a low FA had lower ($p<0.001$) body weight than all other groups, and this effect was independent of ISO treatment. Mice exposed to adequate or supplemental FA had higher body weight ($p<0.001$) on postnatal day 10 if treated with ISO than corn oil. At weaning (postnatal day 21), there was an effect of FA x ISO ($p=0.004$) on offspring's body weight. Corn oil-treated mice whose mother's were fed a low FA diet had lower body weight ($p<0.001$) than all other treatment groups. ISO-treated mice had higher body weight ($p<0.001$) at weaning if exposed to low or supplemental FA during pregnancy and lactation. On postnatal day 112, only maternal FA intake had an effect on body weight. Control mice exposed to low FA had lower ($p<0.001$) body weight than control mice exposed to adequate or supplemental FA. There were no differences in weight gain from postnatal day 21 to 112.

7.4.2 Organ weight

There were no differences in weight of testes, seminal vesicles and adipose tissue among groups (Table 7-2). There was an overall effect of FA x ISO ($p=0.043$) on kidney weight. Control mice exposed to supplemental FA had higher ($p=0.010$) kidney weight compared to all other groups. In contrast, ISO-treated mice exposed to adequate FA had higher ($p<0.001$) liver weight than mice exposed to low or supplemental FA.

7.4.3 BMD of intact LV1-LV3 and peak load of LV2

There were no differences in BMC and BMD of LV1-LV3 or peak load of LV2 among FA groups (Table 7-3). Males exposed to adequate FA+ISO had higher ($p<0.05$) BMC and BMD of LV1-LV3, and higher ($p<0.001$) peak load of LV2 compared to males exposed to adequate FA alone.

7.4.4 BMD of whole femur, and peak load of femur neck and femur midpoint

Femoral BMC ($p=0.002$) and BMD ($p=0.035$) were greater among ISO-treated mice

Table 7-2. Organ weights expressed as percent of body weight from 4-month old CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO

	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
Testes (%)	Low	0.532 ± 0.036	0.460 ± 0.037	NS	NS	NS
	Adeq	0.495 ± 0.040	0.460 ± 0.049			
	Supp	0.510 ± 0.057	0.433 ± 0.039			
Seminal Vesicles (%)	Low	0.859 ± 0.042	0.855 ± 0.044	NS	NS	NS
	Adeq	0.834 ± 0.047	0.841 ± 0.058			
	Supp	0.794 ± 0.070	0.858 ± 0.046			
Kidney (%)	Low	1.30 ± 0.107 ^b	1.30 ± 0.110 ^{ab}	0.010	NS	0.043
	Adeq	1.30 ± 0.119 ^b	1.35 ± 0.146 ^{ab}			
	Supp	1.96 ± 0.169 ^a	1.38 ± 0.115 ^{ab*}			
Liver (%)	Low	4.14 ± 0.133 ^{ab}	4.13 ± 0.138 ^b	<0.001	NS	NS
	Adeq	4.59 ± 0.149 ^{ab}	4.99 ± 0.183 ^a			
	Supp	4.03 ± 0.220 ^{ab}	4.36 ± 0.146 ^b			
Adipose (%)	Low	5.64 ± 0.355	5.80 ± 0.368	NS	NS	NS
	Adeq	6.01 ± 0.397	5.72 ± 0.487			
	Supp	6.45 ± 0.587	6.40 ± 0.382			

1 Values are means ± SEM, n = 8-18. Labelled means in a column for a variable without a common letter differ, P < 0.05. NS>0.05. The star (*) denotes that the measured value is different from the corresponding corn oil control male, p<0.05. NS, p>0.05.

Abbreviations: Adeq- adequate; CON - control; FA - folic acid; ISO - soy isoflavones; NS - not significant; Supp - supplemental.

Table 7-3. BMC, BMD and peak load of lumbar vertebrae and femur excised from 4-month old male CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO

Lumbar vertebrae	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
LV1-3 BMC (mg)	Low	21.2 ± 0.794	21.8 ± 0.756	NS	0.025	NS
	Adeq	21.0 ± 0.866	24.3 ± 0.866*			
	Supp	21.8 ± 0.109	22.8 ± 0.738			
LV1-3 BMD (mg/cm ²)	Low	57.5 ± 0.157	60.8 ± 0.145	NS	0.016	NS
	Adeq	56.7 ± 0.171	64.3 ± 0.171*			
	Supp	60.4 ± 0.216	59.7 ± 0.142			
LV2 peak load (N)	Low	53.6 ± 2.90	64.6 ± 2.67	NS	<0.001	NS
	Adeq	51.8 ± 2.99	69.1 ± 3.31*			
	Supp	55.1 ± 3.78	59.5 ± 2.90			
Femur						
Whole femur BMC (g)	Low	33.1 ± 0.873 ^{ab}	35.5 ± 0.854 ^b	0.002	0.002	NS
	Adeq	35.0 ± 0.986 ^{ab}	39.3 ± 1.12 ^{a*}			
	Supp	36.9 ± 1.32 ^{ab}	38.2 ± 0.854 ^a			
Whole femur BMD (mg/cm ²)	Low	77.7 ± 1.41	82.5 ± 1.47 *	0.035	0.002	NS
	Adeq	79.1 ± 1.70	87.0 ± 2.00 *			
	Supp	83.9 ± 2.28	84.8 ± 1.47			
Femur neck peak load (N)	Low	24.3 ± 1.50	26.3 ± 1.57	NS	0.012	NS
	Adeq	25.2 ± 1.69	32.0 ± 1.86 *			
	Supp	25.7 ± 2.27	28.2 ± 1.80			
Femur midpoint peak load (N)	Low	39.4 ± 1.42	42.1 ± 1.45	NS	<0.001	NS
	Adeq	36.4 ± 1.56	43.3 ± 1.75*			
	Supp	37.4 ± 2.05	41.8 ± 1.60			

¹ Values are means ± SEM, n = 8-18. Labelled means in a column for a variable without a common letter differ, P < 0.05. NS>0.05. The star (*) denotes that the measured value is different from the corresponding corn oil control male, p<0.05. NS, p>0.05.

Abbreviations: Adeq – adequate; BMC – bone mineral content; BMD – bone mineral density; CON - control; FA - folic acid; ISO - soy isoflavones; LV – lumbar vertebrae; NS - not significant; Supp - supplemental.

exposed to adequate or supplemented FA than those exposed to low FA (Table 7-3). Among mice exposed to adequate FA, those treated with ISO had higher femoral BMC ($p=0.002$) and BMD ($p=0.002$), and higher peak load of femur neck ($p=0.012$) and femur midpoint ($p<0.001$) compared to those treated with corn oil.

7.4.5 Serum measures of bone turnover

There was an overall effect of FA x ISO on OPG and RANKL concentrations (Table 7-4). Among ISO-treated mice, those exposed to supplemental FA had higher ($p=0.027$) OPG concentrations than all other groups. Exposure to adequate or supplemental FA+ISO resulted in lower ($p<0.001$) RANKL concentrations than exposure to low FA+ISO. Mice exposed to supplemental FA+ISO had a two-fold higher serum OPG/RANKL ratio than all other groups (Figure 7-1).

7.5 Discussion

Although studies have shown that neonatal exposure to ISO can improve BMD and bone strength of males at adulthood [12, 98], this study is the first to show that ISO-induced benefits to bone depend on the level of FA exposure. Male offspring exposed to low FA+ISO had higher femur BMD than mice exposed to low FA alone, with no other benefits to bone outcomes measured. In contrast, males exposed to adequate FA+ISO had higher BMC and BMD, and greater resistance to fracture at the femur and lumbar spine compared to males given adequate FA alone. These findings suggest that ISO and FA may work together to enhance DNA methylation because the availability of FA in part dictates whether ISO improve bone development. Thus, epigenetic regulation of gene transcription is a strong candidate mechanism by which ISO and FA may program bone development. Interestingly, the positive effect of adequate FA+ISO on bone development has previously been reported in

Table 7-4. Serum markers of metabolism measured in 4-month old male CD-1 mice whose mothers' were fed low, adequate or supplemental FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO

	FA (mg)	Treatment		Two-way ANOVA		
		CON	ISO	FA	ISO	FA x ISO
OPG (pg/ml)	Low	1413.7 ± 151.7 ^{ab}	1383.0 ± 145.3 ^b	0.027	NS	0.013
	Adeq	1582.6 ± 159.2 ^{ab}	1503.1 ± 159.2 ^b			
	Supp	1429.8 ± 167.8 ^{ab}	2228.4 ± 159.2 ^{a*}			
RANKL (pg/ml)	Low	116.6 ± 11.6 ^{ab}	128.3 ± 10.6 ^b	<0.001	NS	NS
	Adeq	87.1 ± 9.8 ^{ab}	80.6 ± 11.1 ^a			
	Supp	85.9 ± 12.2 ^{ab}	73.3 ± 11.1 ^a			
ACTH (pg/ml)	Low	34.7 ± 14.0	42.5 ± 14.0	0.050	NS	NS
	Adeq	50.0 ± 13.4	64.9 ± 12.8			
	Supp	62.1 ± 14.0	84.4 ± 13.4			

¹ Values are means ± SEM, n = 10-12. Labelled means in a column for a variable without a common letter differ, P < 0.05. NS>0.05. The star (*) denotes that the measured value is different from the corresponding corn oil control male, p<0.05. NS, p>0.05.

Abbreviations: ACTH - adrenocorticotrophic hormone; Adeq; adequate; CON - control; FA - folic acid; ISO - soy isoflavones; NS - not significant; OPG - osteoprotegrin; RANKL – receptor activator of nuclear factor kappa-β ligand; Supp - supplemental.

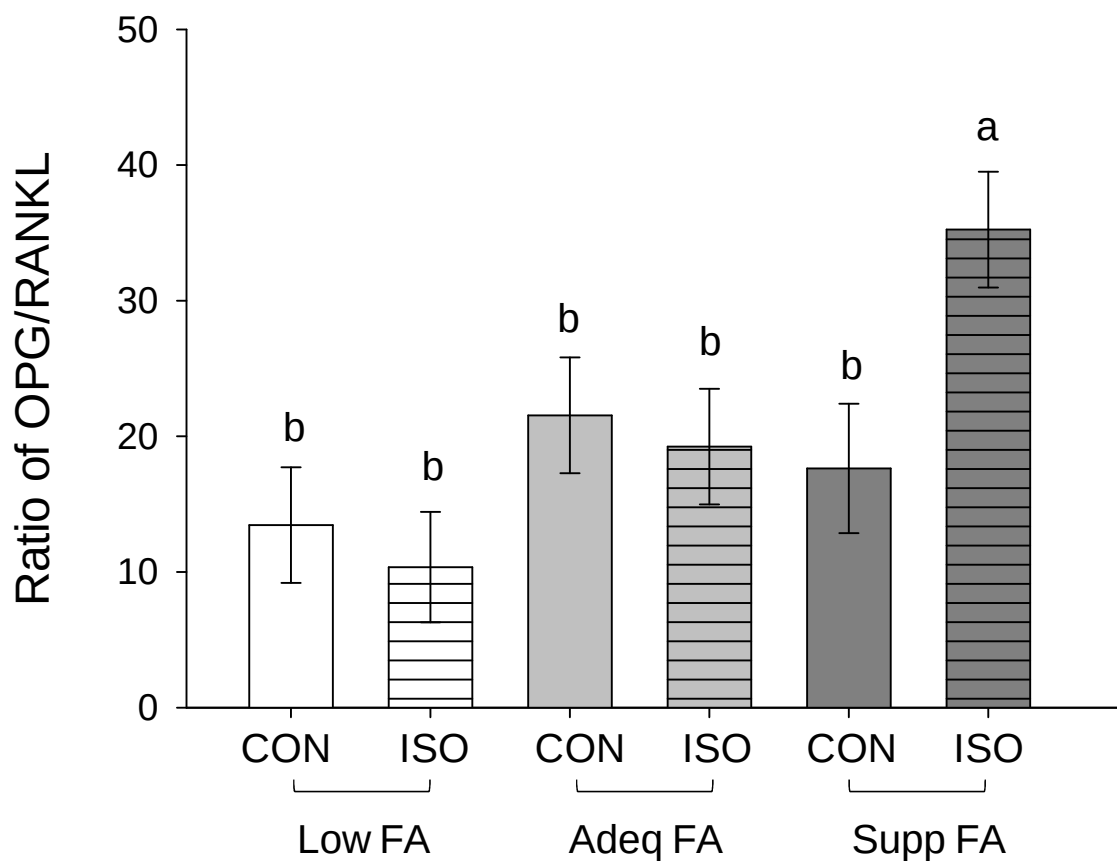


Figure 7-1. The ratio of OPG to RANKL measured in serum of 4 month old male CD-1 mice whose mothers' were fed a low, adequate or supplemental level of FA during pregnancy and lactation, and who during suckling were treated with subcutaneous injections of corn oil or ISO.

Values are given as ratios (n=8-10/group) and variables without a common letter differ, $p < 0.05$.

male and female CD-1 mice because this is the level of FA (2 mg/kg diet) in the AIN93G diet [12, 54]. In comparing across these studies, male CD-1 mice treated with genistein for the first 5 days of life [12], or a combination of genistein and daidzein for the first 10 days of life had similar improvements in lumbar spine quality, including a 10-13% higher lumbar spine BMD and a 29-33% higher lumbar spine peak load. However, only mice exposed to a combination of genistein and daidzein for the first 10 days of life had improved femur outcomes, including a 10% higher femur BMD and a 27% greater resistance to femur neck fracture. Taken together, these findings suggest that in males the first 5-days of life represents a critical window of development during which ISO program lumbar spine (a site rich in trabecular tissue that has a high surface-to volume ratio and is metabolically active) [12, 13, 54]. In contrast, the first 10-days are important for programming of long bones (i.e. femur). This finding is consistent with female data [85], providing support that in both genders ISO induced effects on bone are site specific.

The finding that exposure of male offspring to supplemental levels of FA+ISO did not have significant effects on BMD or strength at the femur or lumbar spine at adulthood compared to corn oil treated mice receiving the same level of FA was unexpected. This paradoxical effect of FA supplementation and ISO may be due to a compensatory mechanism that activates a negative feedback loop and prevents DNA hypermethylation that may repress the expression of many genes. To date, studies have shown that maternal FA supplementation (5 mg/kg of diet) from preconception through lactation stimulates DNA methylation and rescues the normal phenotype when mice are exposed to hypomethylating factors (i.e. protein restriction or bisphenol A) [233]. However, to our knowledge no studies have examined how early life exposure to two hypermethylating factors – FA and ISO –

affects growth and development. It is generally accepted that the epigenome is very reactionary but, its liable nature also allows it to sense and respond to environmental perturbations that have the potential to disrupt fetal growth or survival [234]. Thus, it is possible that exposure to supplemental levels of FA and ISO activates a negative feedback loop that inhibits DNA methylation. A recently published study showed that when HEK-293 cells are treated with the DNA demethylating agent, 5-azadeoxycytidine, there is a 20-fold induction in OPG mRNA expression [235]. In line with this evidence, male mice exposed to supplemental FA+ISO had 1.5-fold higher concentrations of OPG than mice exposed to either treatment alone suggesting that the combination of supplemental FA+ISO suppresses DNA methylation. As a result of higher OPG levels, male mice exposed to supplemental FA+ISO had a two-fold higher ratio of OPG to RANKL than all other treatment groups that suggests a greater rate of bone formation [236].

The exact mechanism by which early life exposure to ISO improves bone development has not been elucidated. Recent progress in defining the estrogen-signalling pathways and modes of epigenetic programming has provided some insight into the potential mechanisms of action. By binding and activating nuclear receptors such as estrogen receptor- α , ISO may interfere with hormonal signalling and/or the production of enzymes and transcription factors [3, 4]. These changes may induce irreversible effects on many physiological processes including bone metabolism if exposure occurs during sensitive stages of development. During development, endocrine hormones and enzymes can alter epigenetic regulation as well as program long-lasting changes in hormone secretion and tissue hormone sensitivity [14]. That being said it is important to note that epigenetic and endocrine processes are not mutually exclusive, so a change to one may inherently affect the other. The finding that ISO

have benefits to bone only in the presence of adequate levels of FA suggests that the observed bone improvements may be a consequence of epigenetic programming. It is generally accepted that in the early embryo, epigenetic patterns are reset so that the developing organism is best programmed to survive in its given environment [208, 237]. In this process the genome is entirely demethylated, and then, through successive cell divisions, one-carbon groups are transferred from methyl donors (i.e. FA) to cytosine residues. Lack of FA during development may lead to hypomethylation, which is linked to chromosomal instability and loss of imprinting. Alternatively, high amounts of FA may lead to hypermethylation of CpG sites in the promoters of genes, which is associated with transcriptional silencing that can be inherited by daughter cells following subsequent cell divisions, or if combined with ISO may inhibit DNA methylation by activating a negative feedback loop. Future studies should investigate the mechanism(s) by which ISO and adequate FA improve bone outcomes in males at young adulthood.

Soy protein formulas have been used for decades and are thought to be safe for human consumption [23, 32]. However, the abundance of ISO in soy protein formula, with potential to alter epigenetic programming, suggests that exposure during developmentally plastic period may influence the development of physiological and endocrine systems in ways that at adulthood improve bone outcomes. If confirmed in humans, this research may have significant public health implications because there is an ongoing need to develop strategies and recommendations not only for treatment but, also for prevention of osteoporosis. There are still many areas of uncertainty around early growth and nutrition that require clarification before clinical guidelines can be developed and implemented. These include the effects of dietary composition and food interactions on programming of gene expression and endocrine

regulation during early life. To date, more studies have examined the effect of early life exposure to ISO on bone development in females [12, 54, 97, 114, 115] than males [12, 54, 98]. Thus, findings from this study contribute to the present understanding of how ISO modulate male bone development.

In summary, early life exposure to adequate FA+ISO had a positive but modest effect on bone development in male mice. Mechanistic studies are needed to decipher why a supplemental level of FA in combination with ISO did not induce functional changes in bone tissue. The findings from this study provide a rationale for prospective studies investigating the role of soy protein formula on programming of growth, development and suggest that FA intake of mothers and their male infants should be considered in such studies.

Chapter Eight

STUDY 5

EARLY LIFE EXPOSURE TO GENISTEIN AND DAIDZEIN DISRUPTS STRUCTURAL DEVELOPMENT OF REPRODUCTIVE ORGANS IN FEMALE MICE

Modified from:

Kaludjerovic J, Chen J, Ward WE. Early life exposure to genistein and daidzein disrupts structural development of reproductive organs in female mice. *J Toxicol Environ Health A*. 2012; 75:1-12. (doi: 10.1080/15287394.2012.688482)

8.0 STUDY 5 - EARLY LIFE EXPOSURE TO GENISTEIN AND DAIDZEIN DISRUPTS STRUCTURAL DEVELOPMENT OF REPRODUCTIVE ORGANS IN FEMALE MICE

8.1 Abstract

In mice, exposure to ISO, abundant in soy infant formula, during the first 5 days of life alters structural and functional development of reproductive organs. Effects of longer exposures are unknown. Study objective was to evaluate if exposure to a combination of daidzein and genistein in the first 10 compared to 5 days of life results in greater adverse effects on ovarian and uterine structure in adult mice. Thirteen litters of 8-12 pups were cross-fostered and randomized to corn oil or ISO (2 mg daidzein + 5 mg genistein/kg body weight/day) for the first 5 or 10-days of life. The 10-day protocol mimicked the period when infants are fed soy protein formula but avoids the time when suckling pups can consume mother's diet. Body and organ weights, and histology of ovaries and uterus were analyzed. There were no differences in the ovary or uterus weight, number of ovarian follicles, number of multiple oocyte follicle or percent of ovarian cysts with 5 or 10-day ISO intervention compared to respective controls. Ten-day ISO group had higher body weight from 6-days to 4 months of age and a higher percent of hyperplasia in the oviduct than the respective control. Lower number of ovarian corpora lutea and a higher incidence of abnormal changes were reported in the uteri of both ISO groups compared to their respective controls. Five and 10-day exposure to ISO had similar long-lasting adverse effects on the structure of ovaries and uterus in adult mice. Only the 10-day ISO exposure resulted in greater body weight gain at adulthood.

8.2 Introduction

Foods, water, soil, cleaning reagents, plastics and pharmacological agents can contain estrogen-like compounds, referred to as environmental estrogens, that imitate the natural activity of estrogen. ISO, such as daidzein, genistein and glycitein, are a form of food estrogens that human infants fed soy protein formula consume at markedly higher levels than infants fed breast milk or cow milk based formula. Such levels may have biological effects [38, 166]. Although exposure to ISO can induce biological effects at any stage of the life cycle, the neonatal period is a particularly vulnerable stage of life because endogenous estrogen production is low allowing ISO to more freely bind to estrogen receptors in estrogen-sensitive tissues and thus, exert their maximal estrogen receptor-mediated effect [38]. Moreover, developing organisms are sensitive to epigenetic programming [238, 239], have an immature immune system [240, 241], poor liver metabolism [242], an increased metabolic rate [243] and a small body size, which are some of the reasons why adverse effects can occur in developing organisms at concentrations that are far below the levels deemed harmful in adults [62, 244]. Consequently, questions have been raised about the safety of ISO and the potential long-term adverse health effects among adults who were exposed to soy protein formula during infancy [166], and as such, long-term prospective trials evaluating safety are ongoing [74].

The sole retrospective cohort study of young adults found no differences in pubertal maturation, growth or a wide range of reproductive measures between those who were fed soy protein formula and cow milk formula during infancy [77]. The only reported difference was that women fed soy protein formula had on average an 8 hour longer duration of menstrual bleeding and greater discomfort during menstruation compared to women fed cow milk formula. No clinical studies have evaluated the potential effects of soy protein formula on

ovarian follicle development but, few studies have examined the development of reproductive organs of infants fed soy protein formula in the first few months or years of life [75, 76, 82]. One study reported no difference in reproductive organ size, as measured by ultrasound, between infants fed soy protein formula, breast milk or cow milk formula at 4 months of age [82]. In contrast, prospective studies of healthy infant fed breast milk, cow milk or soy protein formula showed that females fed soy protein formula have enhanced vaginal wall cell maturation at 6 months of age [75] and more developed breast tissue at 2 years of age than infants fed breast milk or cow milk formula [76]. Thus, despite its long history of use there is some concern that exposure to ISO present in soy protein formula may have adverse effects on developing infants. In 2010, the United States National Toxicology Program Center for the Evaluation of Risks to Human Health Reproduction concluded there was minimal concern (level 2) that soy protein formula, containing ISO, cause adverse reproductive and/or developmental effects in exposed humans, but acknowledged the paucity of well-designed clinical studies to fully address concerns [32]. In contrast, the European Society for Paediatric Gastroenterology Hepatology and Nutrition has taken a more cautious approach and is advising the public to avoid the use of soy protein formula, especially for infants less than 6 months of age, because of uncertainties regarding safety in infants and young children [26].

The CD-1 mouse model, a common animal model for studying environmental estrogens, is useful to predict effects in humans and explore their mechanism of action [86-90, 123, 124, 126]. To date, studies have reported that short-term exposure (i.e. first 5-days of life) to genistein can irreversibly disrupt development by increasing the risk of obesity, metabolic dysfunction, tumours (i.e. uterine fibroids, ovarian and mammary gland cancer), and reproductive problems including infertility/subfertility, endometriosis and multiple oocyte

follicles several weeks or months post-treatment [84, 87-90, 123, 124, 126]. *A recent study showed that F1 females exposed to ISO for the first 10 or 21-days of life have reduced fertility by 55 and 60%, respectively; while fertility of F2 females is not compromised [84].* In contrast, exposure to daidzein and genistein during the first 5 days of life promotes bone development and attenuate deterioration of bone tissue during aging by stimulating bone calcification and bone matrix formation [13, 54, 125, 245, 246]. Because timing of ISO exposure may modulate metabolic regulation and in turn, the risk of adult-onset diseases, it is important to investigate how duration of exposure during early life influences structural development of reproductive organs, timing of puberty, sexual behaviour and fertility of sexually mature animals. The objective of this study was to determine how 5 versus 10-day exposure to a combination of daidzein and genistein – the most abundant ISO present in soy protein formula - modulates weight and structural development of ovaries and uterus of female CD-1 mice at adulthood.

8.3 Methods

8.3.1 Animals and treatment

Six week-old CD-1 mice (n=5 males, n=13 females) obtained from Charles River Laboratories Canada (St. Constant, QC,) were housed in the Department of Comparative Medicine at the University of Toronto. Mice were housed at 23°C and 50% humidity on a 12:12 hr light-dark cycle, fed a semi-purified diet (AIN93G) devoid of estrogenic compounds and provided water *ad libitum*. After two weeks of adaptation to the environment, mice were mated harem style. Thirteen litters with 8-12 pups were cross-fostered and randomized to corn oil or ISO (2 mg daidzein + 5 mg genistein/kg body weight/day) for the first 5 or 10-days of life. The 5-day protocol was selected to mimic previously published studies investigating

environmental and dietary estrogens [87-89, 123, 124, 126] and the 10-day protocol was chosen to more closely represent the period in which human infants fed soy protein formula may be exposed to ISO but avoid the time when suckling pups may start to consume mother's diet. Purified daidzein and genistein powder, purchased from Sigma-Aldrich (Mississauga, ON), was solubilized in 1 mL dimethyl sulfoxide and suspended in corn oil [232]. Each morning, treatments were administered via subcutaneous injection using a total volume of 20 μ L/pup/day. We previously showed that subcutaneous delivery versus oral feeding, and daily versus multiple oral doses does not result in significantly different levels of total serum daidzein, genistein, equol and O-DMA in the developing CD-1 mice [122]. However, oral feeding in the first week of life is very difficult because of the pup's small body size. Oral feeding also results in higher variability of serum ISO concentrations, marked stress and a higher risk of death due to aspiration or perforation of tissues than administering ISO by subcutaneous injection [122]. The administered dose results in serum ISO levels that are similar to those of human infants fed soy protein formula [122, 232]. On postnatal day 21, mice were weaned and thereafter, only female mice were studied because our previous findings have shown that female mice have a greater response to ISO exposure [13, 54]. Body weight of mice was measured daily from birth to postnatal day 10, at weaning (postnatal day 21) and every 4 weeks thereafter until postnatal day 120, representing young adulthood [11]. Necropsy occurred at 4 months of age, at which time organs were collected and stage of estrous cycle was not established. Organ weight was used to monitor for gross adverse effects due to ISO. All procedures involving live animals were reviewed and approved by the University of Toronto Animal Care Committee and compliant with the Canadian Council on Animal Care [216].

8.3.2 Histology

Ovaries and uteri were excised and fixed in 10% buffered formalin. Samples were embedded in paraffin and sectioned using the method developed by Jefferson's group [88, 247] such that 18 sections per mouse were analyzed with a maximum interval of 450 μm . Tissue sections were stained with hematoxylin and eosin (H&E), and evaluated by light microscopy. Both uterine horns were cut 1 mm from the uterine body. The cervix (10-day treatment only) was obtained from the caudal portion of uterine body. Six serial sections (each at an interval of 100 μm) were evaluated for each mouse. The ovary, oviduct, uterus and cervix were analyzed by a blinded observer (J.C.) to determine the presence of abnormalities. Secondary follicle is defined as pre-antral follicle that represents theca cells, multiple layers of granulosa cells, and the zona pellucid; while, tertiary follicle is defined as antral follicle that contains antrum (fluid filled cavity) [248, 249].

8.3.3 Statistical analyses

The number of follicles (secondary to tertiary follicles) and corpora lutea per mouse are presented as mean $\pm$ standard error of mean. All other data are presented as a proportion of mice within the group having the abnormality. Student's t-test was used to compare litter size, body weight, uterus and ovary weights, as well as the number of follicles between the control and ISO treatment group for the 5 and 10-day interventions, respectively. All other frequency were evaluated by Chi-Square (Fisher exact) test. All analyses were performed using Sigma Stat. Statistically significant differences were defined as $p < 0.05$.

8.4 Results

8.4.1 Litter size, body weight and relative organ weight

There were no differences in litter size and birth weight among the respective control and ISO groups (data not shown). Body weight from birth to 4 months of age was not significantly different between the 5-day ISO group and its respective control (Figure 8-1). In contrast, 10-day treated females had significantly higher body weight from postnatal day 6 to 4 months of age compared to its respective control group (Figure 8-1). The 5 or 10-day ISO exposure had no effect on the weight of ovaries, uterus, kidneys and liver when expressed as percent of body weight (Figure 8-1). Absolute liver weight of 5 and 10-day treated ISO groups was significantly higher than that of its respective control group with no differences in absolute weight of other organs with either 5 or 10-day ISO exposure

8.4.2 Ovary and oviduct

There was no significant difference in the number of follicles, from secondary to preovulatory follicles, between the respective control and ISO groups (Table 8-2). The number of corpora lutea was significantly lower in the ISO groups compared to their respective controls, of which five mice in the 5-day ISO (41.7%) and one mouse in the 10-day ISO (7.7%) groups had no corpus luteum (Table 8-2, Figure 8-2B). Ovaries without corpus luteum were filled with interstitial cells in the stroma (Figure 8-2B). Three mice from the 5-day ISO (25.0%) and one mouse from the 10-day ISO (7.7%) group showed abnormal cyst-like structure in the ovaries (Figure 8-2C). One mouse from each 10-day group, CON and ISO, had one multiple oocyte follicles (Table 8-2; Figure 8-2D). ISO treatment induced hyperplasia, mucosal folds extending to serosa, in the oviduct of 3 out of 12 (25.0 %) and 5 out of 13 (38.5 %) mice in the 5 and 10-day ISO groups, respectively (Table 8-2; Figure 8-2F).

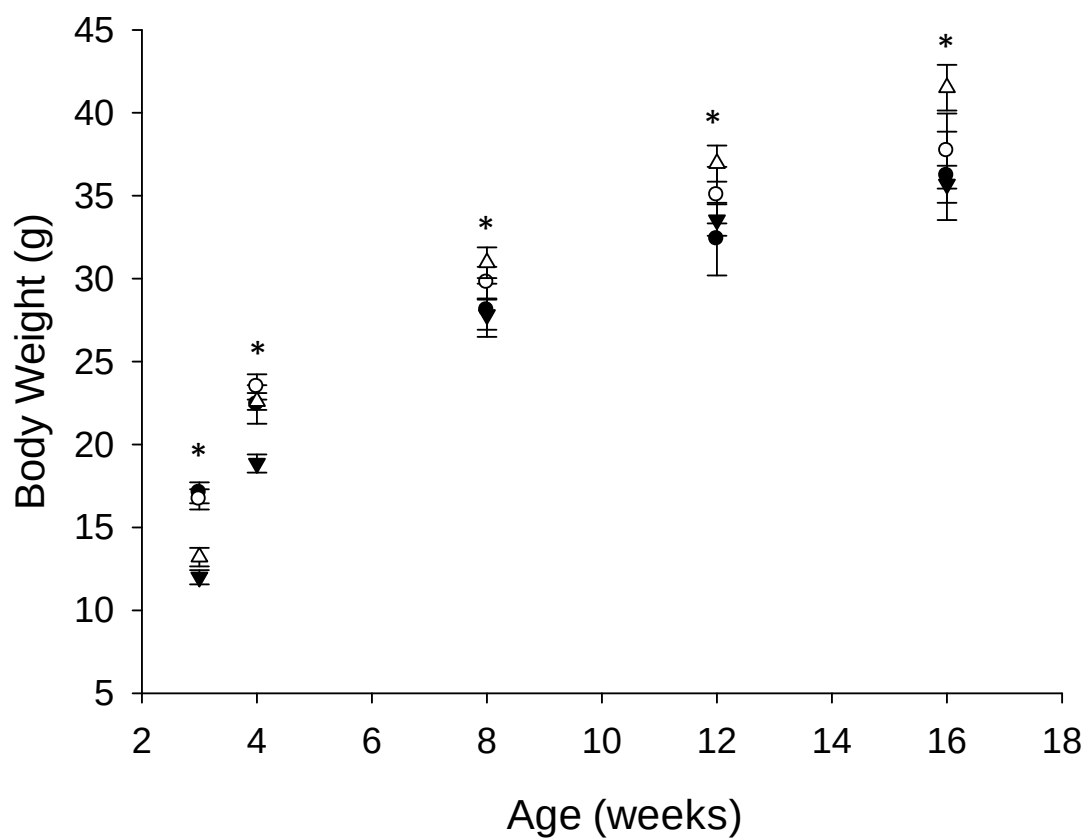


Figure 8-1. Body weight from weaning to 4 months of age of mice treated for the first 5 or 10-days of life with corn oil or ISO.

◦ denotes 5 day ISO group; • denotes 5 day corn oil group; Δ denotes 10 day ISO group; ▲ denotes 10 day corn oil group; * asterisk indicates significant difference, 10 day ISO group > 10 day corn oil group, $p < 0.05$.

Table 8-1. Organ weights from 4-month old female mice expressed both as percent of body weight and as absolute

	5-day CON	5-day ISO	10-day CON	10-day ISO
Organ weight expressed as percent of body weight (%)				
Ovaries	0.051±0.004	0.054±0.004	0.059±0.004	0.117±0.034
Uterus	0.397±0.053	0.437±0.050	0.413±0.038	0.357±0.045
Kidney	1.055±0.041	1.028±0.044	1.148±0.125	1.109±0.070
Liver	4.080±0.113	3.759±0.134	4.425 ±0.206	4.636±0.153
Absolute Organ Weight (mg)				
Ovaries	19.1±1.6	20.6±1.6	22.0±1.31	48.5±14.3
Uterus	139.0±14.0	163.0±18.7	153.0±11.9	151.0±1.87
Kidney	390.0 ±20.8	387.0±16.1	430.0±44.2	454.0±27.0
Liver	1560.0±139.0	1450.0±94.7	1729.0±154.0 ^b	1947.0±107.0 ^a

Data are presented as mean ± standard error of mean. Different superscripts in a row denote significant differences among groups, P≤0.05.

Table 8-2. Histological abnormalities of ovaries, oviduct, uteri and cervix of CD-1 mice at 4 months of age

	5-day CON	5-day ISO	10-day CON	10-day ISO
Ovary				
Follicles, <i>n/ovary</i> †	9.9±1.1	10 ±1.1	13±1.4	11±1.3
Corpora lutea, <i>n/ovary</i> †	9.1±1.2 ^a	4.3±1.7 ^b	11±1.3 ^a	5.1±0.68 ^b
Absence of corpora lutea, <i>n/n total</i> (%)	0/11 (0) ^a	5/12 (41.7) ^b	0/12 (0)	1/13 (8)
Cysts, <i>n/n total</i> (%)	0/11 (0)	3/12 (25.0)	0/12 (0)	1/13 (8)
Multiple oocyte follicles, <i>n/n total</i> (%)	0/11 (0)	0/12 (0)	1/12 (8)	1/13 (8)
Oviduct				
Hyperplasia, <i>n/n total</i> (%)	0/11 (0)	3/12 (25)	0/12 (0) ^a	5/13 (39) ^b
Uterus				
Abnormal, <i>n/n total</i> (%)	0/11 (0) ^a	7/12 (58) ^b	1/12 (8) ^a	8/13 (69) ^b
Hyperplasia, <i>n/n total</i> (%)	0/11 (0) ^a	6/12 (50) ^b	1/12 (8)	5/13 (39)
Atypia, <i>n/n total</i> (%)	0/11 (0)	3/12 (25)	0/12 (0)	2/13 (15)
Polyps, <i>n/n total</i> (%)	0/11 (0)	1/12 (8)	0/12 (0)	1/13 (8)
Cyst, <i>n/n total</i> (%)	0/11 (0)	2/12 (17)	0/12 (0)	1/13 (8)
Edema, <i>n/n total</i> (%)	0/11 (0)	1/12 (8)	0/12 (0)	4/13 (31)
Cervix				
Microglandular structures, <i>n/n total</i> (%)	not tested	not tested	0/12 (0)	4/13 (31)

Data are presented as mean ± standard error of mean or the proportion of mice within the group having abnormality.

Different superscripts in a row denote significant differences among groups, $P \leq 0.05$. Numbers in brackets represent the percent of mice, out of the total number of mice studied, for which the effect was observed.

Abbreviations: control, CON; soy isoflavones, ISO.

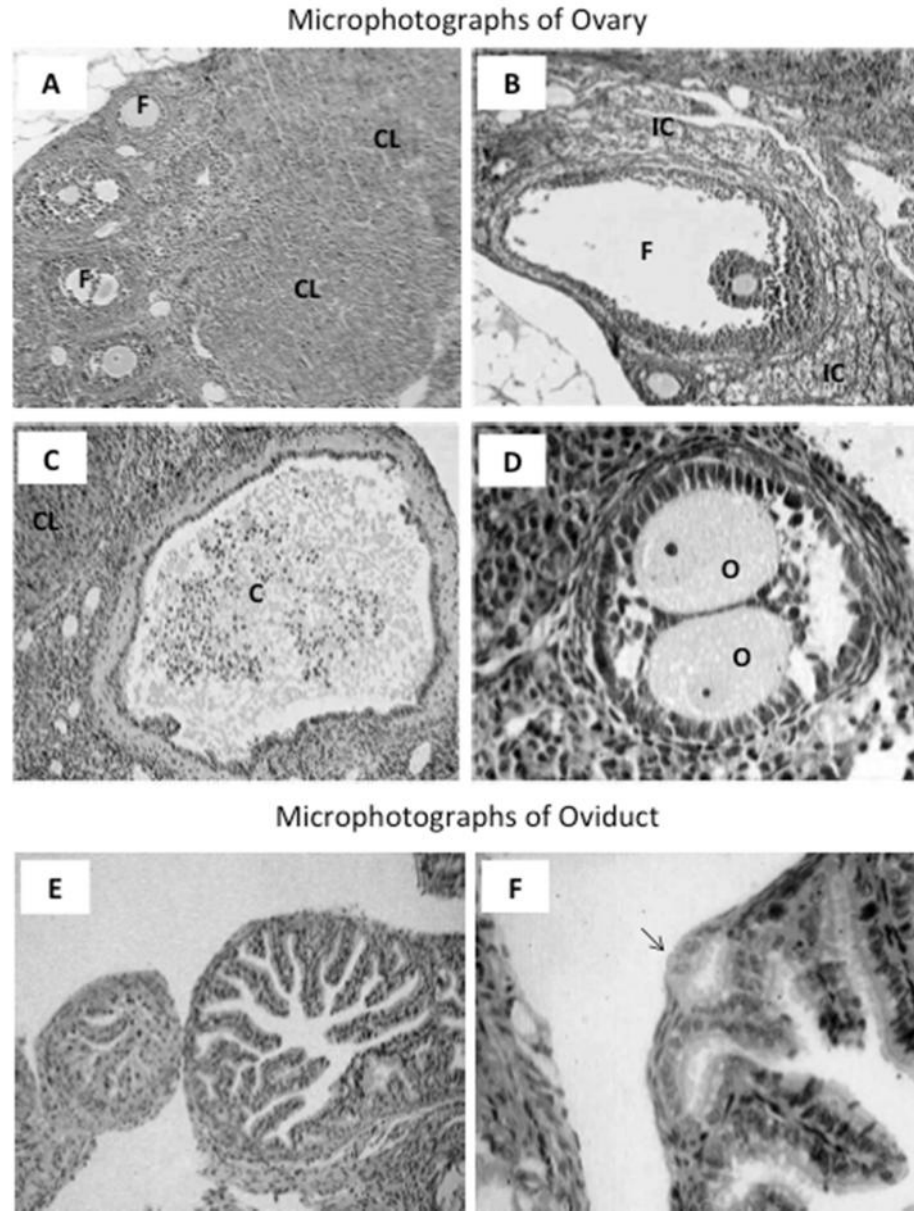


Figure 8-2. Representative microphotographs of ovary and oviduct from 4 month old mice treated with corn oil or ISO.

- A. Ovary from a 5-day corn oil control mouse; follicle (F); corpus luteum (CL); H&E, 100X.
- B. Stroma of ovary from a 5-day ISO treated mouse that lacks corpus luteum (CL) and is filled with interstitial cells (IC). Note a big atrophic cyst-like follicle (F); H&E, 100X.
- C. A big cyst (C) with degenerating cells in the ovary from a 5-day ISO treated mouse; corpus luteum (CL); H&E, 100X.
- D. A multiple oocyte follicle with two oocytes (O) from a 10-day ISO treated mouse; H&E, 400X.
- E. Oviduct from a 10-day corn oil control mouse; H&E, 100X.
- F. Hyperplastic mucosal folds in the oviduct have extended to the serosa (arrow) in a 10-day ISO treated mouse; H&E, 400X.

8.4.3 Uterus and cervix

A significantly higher frequency of abnormal changes was observed in the ISO groups, 7 out of 12 (58.3%) and 8 out of 13 (69.2%) in the 5 and 10-day ISO groups, respectively, compared to 0 out of 11 in the control group (Table 8-2). There are two types of alterations observed in the uterus: endometrial hyperplasia and edema in the stroma. Hyperplasia was the major histological change in the mice treated with ISO and only one mouse in the 10-day control group had hyperplasia (Table 8-2). Hyperplasia with endometrial gland complex, consisting of multiple glands or glandular crowding with glands interconnecting (Figure 8-3B and C), was observed only in the ISO treated mice. Moreover, 3 out of 12 (25.0%) and 2 out of 13 (14.4%) mice from the 5 and 10-day ISO groups, respectively, showed hyperplasia with atypia. One mouse from each ISO group developed endometrial polyps, abnormal projections of endometrium into lumen (8.3% and 7.7%, for 5 and 10-day groups, respectively) (Figure 8-3B). In addition, cystic hyperplasia was presented in the ISO groups with 2 out of 12 (16.7%) and 1 out of 13 (7.7%) for 5 and 10-day ISO groups, respectively (Table 1; Figure 8-3C). Endometrial edema existed in the ISO groups with 1 out of 12 (8.3%) and 4 out of 13 (30.8%) for 5 and 10-day ISO groups, respectively (Table 8-2; Figure 8-3D). In the cervix, the microglandular structures in the epithelium were observed in mice treated with 10-day exposure to ISO at incidence 4 out of 13 (30.8%) (Table 8-2, Figure 8-3F).

8.5 Discussion

This study is the first to show that 5 and 10-day exposure to ISO have similar adverse effects on structural development of ovaries and uterus in sexually mature female mice. Both 5 and 10-day ISO exposure resulted in a lower number of ovarian corpora lutea and a higher incidence of oviduct hyperplasia, as well as a higher occurrence of hyperplasia, atypia,

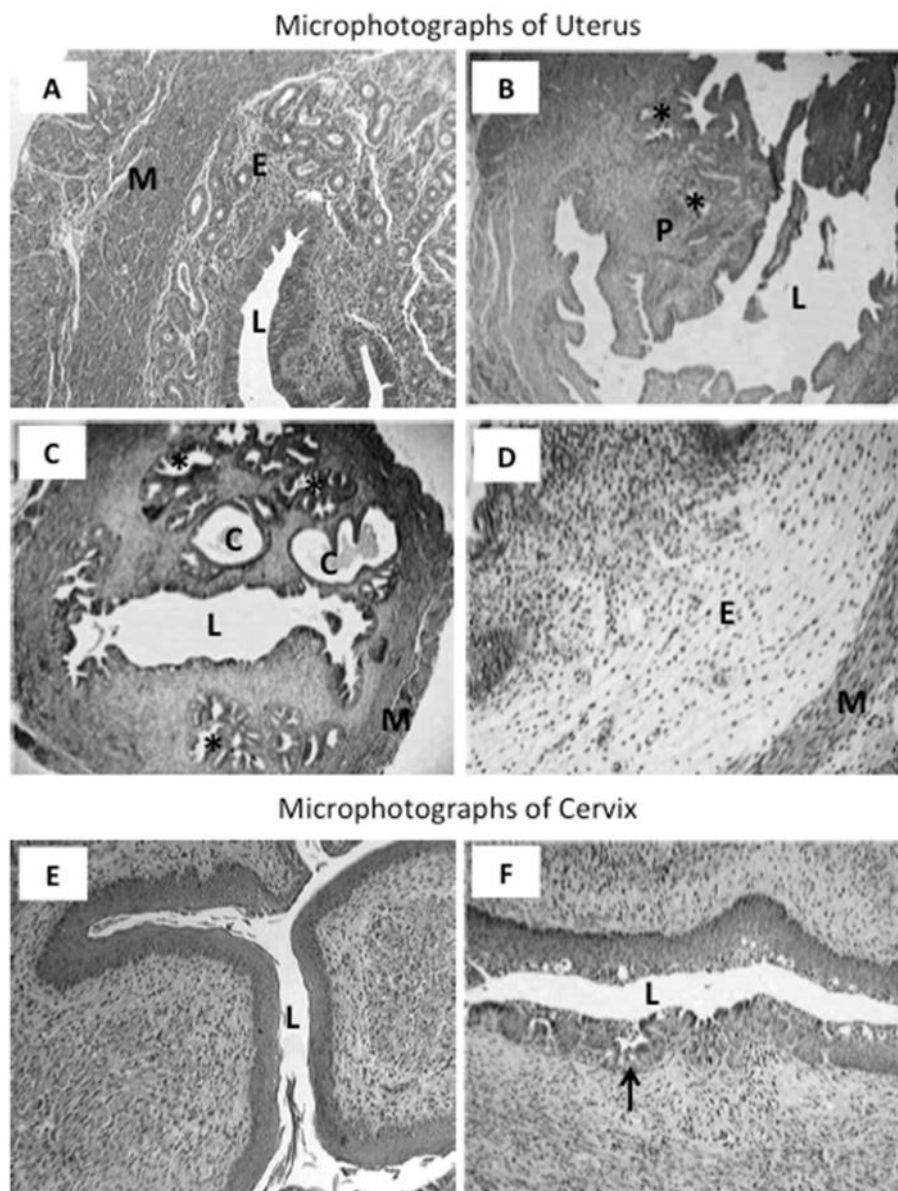


Figure 8-3. Representative microphotographs of uterus and cervix from 4 month old mice treated with corn oil or ISO.

- A. Uterine structure from a 10-day corn oil control mouse; uterine lumen (L); endometrium (E); myometrium (M); H&E, 100X.
- B. Polyp (P) with hyperplastic glands (*) from a 5-day ISO treated mouse; uterine lumen (L); H&E, 100X.
- C. Cystic (C) and hyperplastic glands (*) in the uterus from a 5-day ISO treated mouse; uterine lumen (L); myometrium (M); H&E, 50X.
- D. Edema in the endometrium (E) from a 10-day treated mouse; myometrium (M); H&E, 100X.
- E. Cervix from a 10-day corn oil control mouse; lumen (L); H&E, 100X.
- F. Microglandular structures (arrow) in the cervix from a 10-day ISO treated mouse; lumen (L); H&E, 100X.

polyps and cysts in the uterine tissue structure. These findings are comparable to changes observed in mice with neonatal exposure to potent environmental estrogens (i.e. DES and bisphenol A) and thus, support the hypothesis that ISO bind to both types of estrogen receptors to induce estrogen-like effects in reproductive tissue [126, 128, 250]. It is well documented that the unique biological response of a tissue to ISO involves three inter-related factors, including: the type and level of estrogen receptor- α and estrogen receptor- β expression, the nature of conformational change that occurs in the estrogen receptor upon ligand binding, and the type and expression level of co-regulatory proteins [251]. Both estrogen receptor- α and estrogen receptor- β are expressed in the female reproductive tissue during pregnancy [90, 178, 252, 253]. But in late gestation and early postnatal life, estrogen receptor- α is localized to the stromal and epithelial cells of the uterus and the interstitium of the ovary, while estrogen receptor- β is limited to the ovarian granulosa cells with minimal expression in the uterus [90, 178, 252, 254]. How this distribution of estrogen receptor- α and estrogen receptor- β expression affects reproductive function is currently not well understood but, it is generally accepted that changes in estrogen receptor expression can alter hormone production, non-genomic signalling and the transcription of various genes including steroid receptors, enzymes and transcription factors. These changes have the potential to be inherited during cell division, resulting in permanent maintenance of the acquired phenotype [132, 255]. In one study, rat pups, whose lactating mothers were treated with ISO (100-200 mg/kg body weight/day) from postnatal day 5 to 10, had higher expression of estrogen receptor- β on postnatal day 11 in the ovaries but lower expression in the uterus when compared to the control group [256]. In a different study, continuous exposure to genistein, beginning prenatally to 21 or 97 days of life, resulted in lower mRNA expression of estrogen receptor-

α , estrogen receptor- β , complement component 3, clusterin, IGF-1 and IGF-1 receptor in the uterine tissue of juvenile mice [257]. In utero exposure to genistein and daidzein, respectively, was also shown to induce hypermethylation of the transposable repetitive elements upstream of the transcription start site [15] and reduces the expression of estrogen receptor- α in the brain of female mice [258], suggesting that ISO may have profound effects on long-term programming of health. To better understand how ISO modulate reproductive health, further information on aberrant DNA methylation and gene expression including high-throughput analysis is required.

Mouse studies investigating the effects of environmental estrogens on programming of reproductive health have typically introduced the intervention in the first 5-days of life because this is the period when endogenous concentrations of sex steroid are low [86-90, 123, 124, 181]. However, mice suckle for the first 21-days of life, which is the time when human infants consume soy protein formula, but reach sexual maturation shortly after the end of suckling. As a result, it difficult to identify the most appropriate duration of exposure to mimic human infants fed soy protein formula. A 10-day protocol was used in this study to more closely mimic the period when human infants are fed soy protein formula while avoiding the time when suckling pups may start to consume mother's diet.

Overall, findings from this study indicate that the induction of numerous abnormalities in the ovaries and uterus of adult female mice is not significantly different between the 5 and 10-day exposure to ISO. In line with this evidence, we recently showed that mice exposed to the same dose of ISO (7 mg/kg body weight/day) used in this study for the first 10 or 21-days of life have comparable disruptions in ovarian folliculogenesis, uterine hyperplasia and atypia at 4 months of age [84]. Taken together, these studies suggest that the

first 5-days of life represent a critical window of developing during which ISO can program reproductive health.

Jefferson et al. previously showed that CD-1 mice exposed to low doses of genistein (0.5 and 5 mg/kg body weight/day) from postnatal day 1 to 5 have an increased number of ovarian corpora lutea, while those exposed to high doses of genistein ($50 \text{ mg} \cdot \text{kg}^{-1}$ of body $\text{wt} \cdot \text{day}^{-1}$) have reduced number of ovarian corpora lutea at 4 months of age [89]. In a separate study, C57BL mice exposed orally to a mixture of genistein (50 mg/kg body weight/day), soy protein formula and corn oil from postnatal day 1 to 5 had increased uterine weight and incidence of multiple oocyte follicles in the ovaries on postnatal day 5, but had normal fertility at 6 months of age [181]. Based on these findings, the authors raised concern that genistein may have adverse effects on reproductive development of human infants fed soy protein formula. However, the effects induced by genistein may be different from those induced by an ISO mixture. This study and another study from our laboratory show that when genistein is administered in combination with daidzein for the first 5, 10 or 21-days of life, female mice have a reduced number of ovarian corpora lutea at 4 months of age [84]. Fewer corpora lutea indicate that ovulation is reduced. Whether these effects are due to daidzein per se or its ability to attenuate the effect of genistein on the hypothalamic-pituitary-gonadal axis has not been elucidated. One study showed that rats treated with 17β -estradiol or genistein for the first 5 days of life have lower lordosis quotient at 2 months of age, while those treated with daidzein have higher lordosis quotient compared to the control group [259]. Lordosis quotient is a measure of sexual behavior and based on these findings it can be concluded that genistein and daidzein have divergent effects on sexual differentiation of the brain and sexual behaviour. Exposure to 17β -estradiol or genistein during neonatal

development may increase responsiveness to estradiol in adulthood, while daidzein exposure may reduce responsiveness. Thus, genistein and daidzein may be inducing biological effects through different mechanisms.

Published studies have shown that exposure to 7 mg of ISO per kg of body weight for the first 5 days of life results in higher body weight at 28 weeks of age [13] but, not at early stages of development [54, 181]. However, this increase in body weight can be observed sooner if pups are exposed to higher doses of ISO in the first 5 days of life [127], or if treatment is extended to first 10 or 21 days of life [84]. In this study, differences in body weight appeared on postnatal day 6 and persisted to 4 months of age when female mice were exposed to ISO from postnatal day 1 to 10. Thus, while duration of ISO exposure affected body weight differently in this study, 5 and 10-day exposure to ISO did not result in marked differences in the number of histological abnormalities in the ovaries or uterus indicating that different tissues respond differently to ISO. The mechanism by which 10-day exposure to ISO increases body weight but has no additional adverse effects on reproductive health remains to be elucidated.

This study is unique in that it compares the 5 and 10-day exposure to better characterize the critical window of development during which ISO program reproductive health in CD-1 mice. It is generally accepted that no animal model exactly represents the human infant, but nonetheless can provide a useful basis for human studies [38, 92, 166]. In conclusion, 5-day exposure represents a sensitive window of development during which exposure to ISO can alter structural development of ovaries and uterus in rodents and may therefore be a factor in precocious human development. These findings raise concern that exposure to ISO during postnatal life may increase the risk of disrupting the structural

development of reproductive organs. Findings from prospective studies of infants fed soy protein formula, cow milk formula and breast milk, some of which are ongoing, are needed to address the paucity of data regarding the safety of ISO on structural and functional development of reproductive organs in adults.

Chapter Nine

OVERALL DISCUSSION

9.0 OVERALL DISCUSSION

9.1 Introduction

This chapter provides an overview of study findings and links the five studies, using the conceptual framework presented in Figure 2-5. Much focus in this section is placed on interpreting study findings to better understand the relationship between early life exposure to ISO and FA on programming of bone and reproductive development. This section also integrates strengths and limitations of the research and discusses the associations uncovered between the CD-1 mouse model and children with precocious puberty. The chapter concludes by identifying the gaps that remain in knowledge and presents potential strategies for future research and knowledge translation.

9.2 Major research findings

The first study (Chapter 4) presented in this thesis showed that oral versus subcutaneous delivery, and single versus multiple oral treatment (with an equivalent total daily dose) does not result in significantly different levels of serum genistein, daidzein, equol and O-DMA in the developing CD-1 mouse model [122]. This information was central to our research because it uncovered novel insight about ISO metabolism in the developing CD-1 mouse model and it helped to more specifically identify practical factors that modulate ISO induced biological effects. The finding that oral administration was as effective as the subcutaneous treatment in elevating total serum ISO concentrations in CD-1 pups revealed that ISO metabolism in the intestine is highly underdeveloped during neonatal life as orally ingested compounds easily passed through to circulation. This observation is consistent with findings showing that intestinal bacteria and phase II metabolism needed to convert aglycones to secondary metabolites (i.e. equol and O-DMA) are underdeveloped during early life [8, 43]. In addition, the finding that route (oral versus subcutaneous) of ISO

exposure does not affect total circulating concentrations of primary and secondary metabolites revealed that subcutaneous exposure to the aglycone form of ISO is a suitable model for oral exposure. This finding is valuable to researchers studying the effects of ISO on CD-1 mice because in the first week of life, oral feeding is very challenging and time consuming, poses a higher risk of death due to perforating tissues/aspiration, and results in a higher variability of serum ISO levels within a treatment group than subcutaneous exposure to the same mixture [88, 181]. Lastly, these findings suggested that cumulative dose and duration of exposure, rather than route of administration and frequency of exposure, might more strongly modulate biological effects of ISO during development. Thus, we designed experiments to investigate how duration of ISO exposure affects bone (study 2, chapter 5) and reproductive (study 5, chapter 8) development of CD-1 mice.

Study 2 (Chapter 5) demonstrated that longer duration of exposure to ISO during development provides greater benefits to bone at adulthood. Female mice exposed to ISO throughout lactation (the first 21 days of life) had a 7% higher BMD, 17% higher Tb.N., 10% lower Tb. Sp. and a 5% higher peak load at the lumbar spine compared to 5-day exposure. More significantly, ISO-treated females had an 18% higher BMD of the femur, 12% higher Tb.N. and 29% lower Tb.Sp. of the femur neck compared to corn-oil treated animals, which in turn resulted in a 30% higher resistance to femur neck fracture. Since fragility fractures are associated with significant morbidity and mortality findings from this study provide support that early life exposure to ISO may provide an optimistic approach for reducing the risk of osteoporosis-related fragility fractures in adulthood. Based on this research, the question of when ISO should be introduced in the developing mouse model to mimic human infants fed soy protein formula arose [38, 205].

Mice suckle for the first 21 days of life and thus, it could be argued that ISO exposure should take place during suckling to mimic the stage of development in which human infants are fed soy protein formula. However, unlike human infants, mice reach sexual maturation 3 weeks post weaning - a much shorter duration between neonatal life and sexual maturity – suggesting that perhaps treatment should be introduced somewhere between 5 and 21-day of life to more closely mimic infants fed soy protein formula. For this reason, we decided to treat CD-1 mice with ISO from postnatal day 1 to 10 in all the subsequent studies. As shown in Figure 9-1, our approach was appropriate because the first 5-days of life represents a critical window of development during which ISO program lumbar spine (a site rich in trabecular tissue that has a high surface-to volume ratio and is very metabolically active) [12, 13, 54]. However, exposure for the first 10-days is needed to program long bones (i.e. femur). The 21-day window of exposure, which requires two extra weeks of treatment, improves both lumbar spine and femur when compared to 5-day exposure, but does not provide added benefits to these skeletal sites when compared to 10-day exposure. Thus, the first 10 days of life are the critical window of development during which ISO improve mouse bone development. We recommend that future studies investigating the effects of ISO on bone development in female and male CD-1 mice administer treatment for the first 10-days of life.

Study 3 and 4 were designed to elucidate the mechanism responsible for the observed improvements in bone outcomes following early life exposure to ISO. As part of the study design, we explored how low, adequate and supplemental levels of FA with or without ISO program bone development of female (Study 3, Chapter 6) and male (Study 4, Chapter 7) CD-1 mice at adulthood. Both genders when exposed to adequate FA+ISO in early life had higher BMD and peak load at the femur and lumbar spine (Figure 9-2, Figure 9-3) than mice

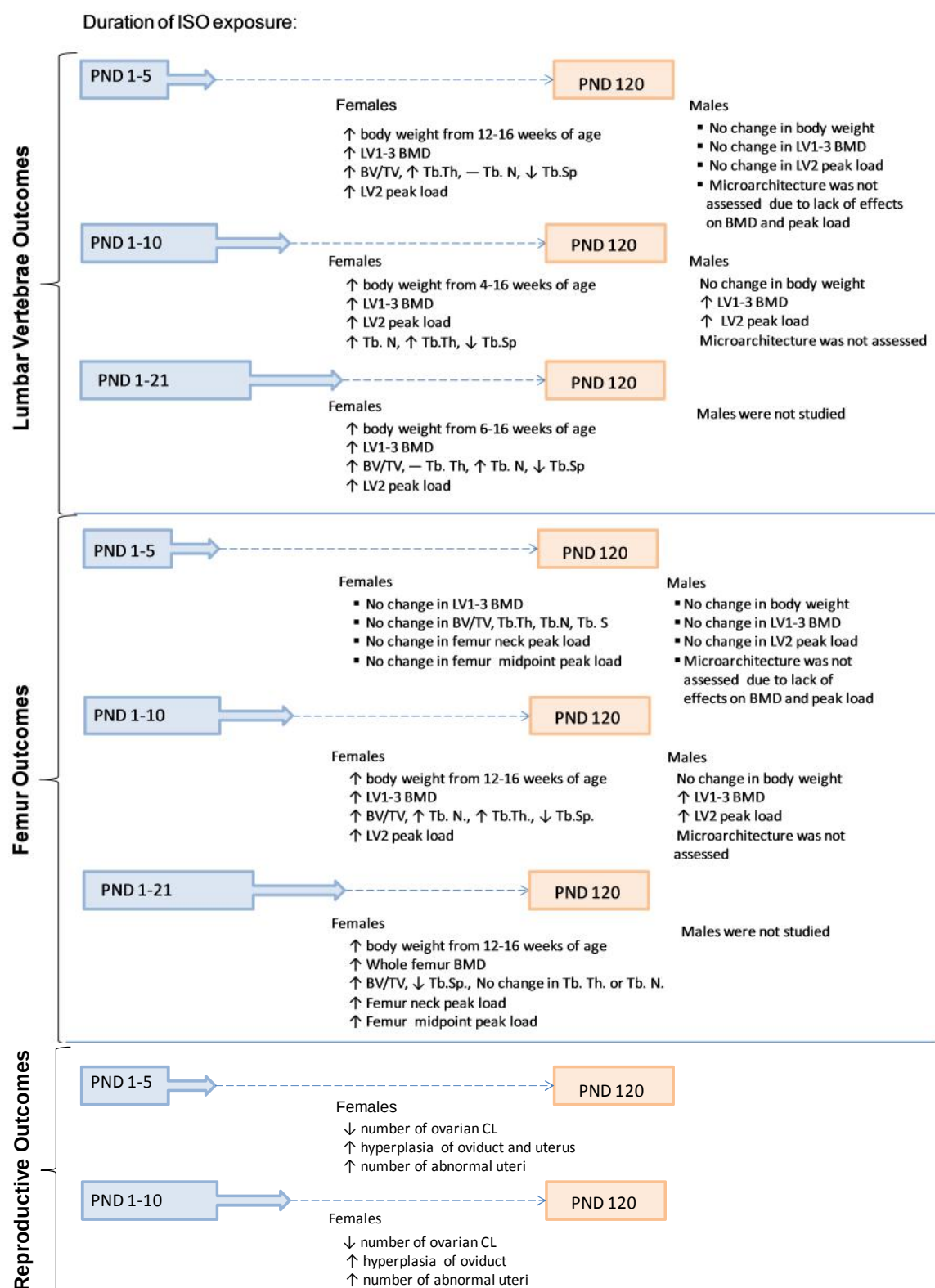


Figure 9-1. Summary of Study 2 through 5 that have characterized the effects of 5, 10 and 21-day exposure to ISO on bone and reproductive outcomes in female and male CD-1 mice at adulthood.

Abbreviations: BMD – bone mineral density; BV/TV – bone volume; CL – corpus lutea; PND – postnatal day; Tb.N – trabecular number; Tb.Sp – trabecular separation; Tb.Th – trabecular thickness

Supp FA	ISO	Supp FA+ISO
<u>Bone Quantity</u> ↑ LV1-3 BMD ↑ Whole Femur BMD	<u>Bone Quantity</u> ↑ LV1-3 BMD ↑ Whole Femur BMD	<u>Bone Quantity</u> ▬ LV1-3 BMD ↑ Whole Femur BMD
<u>Bone Quality</u> ▬ LV2 Peak Load ↑ Femur Neck PL ▬ Femur Midpoint PL ▬ Femur Length	<u>Bone Quality</u> ↑ LV2 Peak Load ↑ Femur Neck PL ▬ Femur Midpoint PL ↓ Femur Length	<u>Bone Quality</u> ▬ LV2 Peak Load ▬ Femur Neck PL ▬ Femur Midpoint PL ↓ Femur Length
<u>Bone Metabolism</u> ▬ OPG ▬ RANKL ▬ OPG/RANKL ▬ Osteocalcin ▬ IGF-I ▬ IGFBP-7	<u>Bone Metabolism</u> ↑ OPG ▬ RANKL ↑ OPG/RANKL ▬ Osteocalcin ↑ IGF-I ↑ IGFBP-7	<u>Bone Metabolism</u> ▬ OPG ▬ RANKL ▬ OPG/RANKL ▬ Osteocalcin ▬ IGF-I ▬ IGFBP-7

Figure 9-2. Summary of effects and mechanisms of supplemental FA, ISO and supplemental FA+ISO on bone development in female CD-1 mice.

Each group is compared to the negative control (exposure to adequate FA alone).

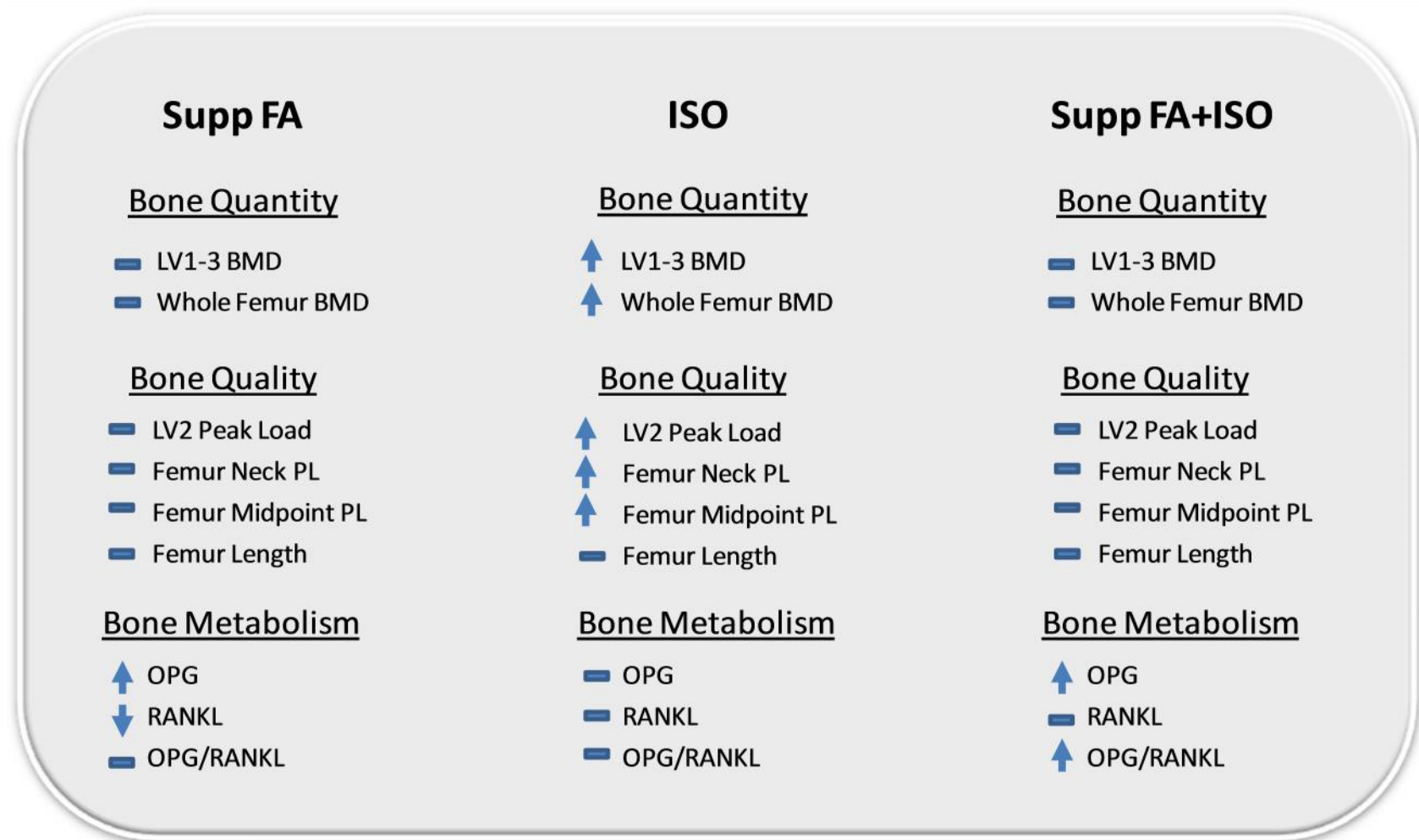


Figure 9-3. Summary of effects and mechanisms of supplemental FA, ISO and supplemental FA+ISO on bone development in male CD-1 mice.

Results from each group are compared to the negative control, which is the group exposed to adequate FA alone.

exposed to adequate FA without ISO. In contrast, female, but not male mice, exposed to supplemental levels of FA had higher BMD at the femur and lumbar spine and greater resistance to fracture at the femur neck than mice exposed to low or adequate levels of FA. Thus, ISO are more potent modulators of bone programming than supplemental FA. Taking that female and male mice were sibling pairs the observed gender difference may be because females in general have lower peak bone mass, weaker bones and a higher incidence of osteoporosis than males. Thus, the effects induced by dietary interventions are often more measurable in females than males.

This thesis is the first to show that ISO-induced benefits to bone depend on the level of FA exposure in both males and females. Exposure to ISO in the presence of low FA provides modest benefits to bone with males exhibiting higher BMD at the femur, and female exhibiting higher BMD and peak load at the lumbar spine. In contrast, both genders when exposed to adequate FA+ISO during development, have higher BMD and peak load at the femur and lumbar spine (Figure 9-2, Figure 9-3) compared to animals exposed to adequate FA alone. This positive effect of adequate FA+ISO on bone development is consistent with published literature as this level of FA (2 mg/kg diet) is found in the AIN93G diet [12, 54]. Thus, we are confident that under adequate FA conditions ISO enhance accumulation of bone mineral at sites rich in trabecular as well as cortical bone. To our surprise exposure to ISO in the presence of supplemental levels of FA did not provide significant benefits to lumbar spine BMD or peak load in female or male mice compared to either treatment alone. However, females did exhibit significantly higher BMD at the femur while males had a two fold increase in serum ratio of OPG to RANKL – suggesting that the combination of supplemental FA and ISO provides modest effects to bone.

Since females were identified to have greater biological responses to supplemental FA

and ISO than males, bone structure and bone-specific gene expression were assessed only in female mice. Both types of analyses provided mechanistic insight. Assessment of bone microarchitecture at multiple skeletal sites by μ CT allowed for identification of how supplemental FA and ISO affect trabecular thickness and connectivity; while, mouse genetics provided the means of identifying the magnitude and type of genes that are affected by early life exposure to supplemental FA and ISO. In general, focal disorganization of the microarchitectural network is an artifact of osteoporosis [260]. Female mice exposed to adequate FA+ISO during development exhibited changes in trabecular connectivity at young adulthood that favour improvements in bone formation. Specifically, neonatal exposure to adequate FA+ISO or supplemental FA led to a rise in Tb.N., while decreasing the overall distance between individual trabeculae at the femur and lumbar spine (Table 6-5). These improvements in bone connectivity are critical in preventing osteoporosis because during aging, loss of trabecular connectivity rather than cortical or trabecular thinning has a more profound adverse effect on bone deterioration. Current drug treatments for osteoporosis can deposit new bone matrix on the existing trabeculae to thicken the overall bone tissue, but they cannot restore the overall number of trabeculae.

The finding that adequate FA+ISO or supplemental FA but not supplemental FA+ISO have positive effects on BMD, bone structure and bone strength in female and male mice provides evidence that these compounds maybe inducing their effects through a common mechanism that has a negative feedback control system. As such, overloading the system may activate a negative feedback that attenuates or prevents the induction of biological effects. It is generally accepted that endocrine factors have continuous feedback systems that work together to signal the glands to regulate the amount of hormones present in circulation. While FA and ISO are not true endocrine factors, they can both stimulate

DNA methylation [91] and indirectly affect the expression of hormones, enzymes and transcription factors. Thus, based on the observed bone outcomes (i.e. BMD, bone structure and strength) we hypothesized that exposure to supplemental FA and ISO in combination during development alters gene expression in such a way that it activates a negative feedback mechanism and in turn, prevents/attenuates the induction of biological effects. In view of this, we discovered that female mice exposed to supplemental FA+ISO during development had higher expression of type 1 parathyroid hormone receptor (PTHr1) and beta catenin (Ctnnb1), which are two factors important for promoting bone resorption and remodeling. Thus, it is very likely that this higher rate of bone resorption is the underlying reason why female mice exposed to supplemental FA+ISO did not have improved bone structure and/or bone strength at adulthood.

Based upon our microarray analyses, we identified that early life exposure to supplemental FA, ISO or the combination alter the expression of 949 genes, which makes up 3% of genes in the mouse genome. Gene ontology revealed that exposure to adequate or supplemental FA+ISO results in lower expression of LOC100046775 gene (a homologue of Histone Macro H2A.1) and higher expression of Top-1mt/2a (topoisomerase) than exposure to adequate FA without ISO (Figure 6-7). LOC100046775 is a protein that inhibits histone acetylation and transcription factor binding [218], while topoisomerase is an enzyme that releases the super coiling and torsional tension of DNA. Thus, by potentially altering the expression of these genes, ISO and FA may activate histone acetylation and assists in releasing the super coiling and torsional tension of DNA. This opening of DNA allows enzymes, proteins and transcriptions factors to more easily regulate DNA methylation and gene transcription. Accordingly, we identified that female mice exposed to adequate

FA+ISO, supplemental FA or supplemental FA+ISO had higher expression of P4hb gene. Taking that P4hb is an enzyme that catalyzing the formation, breakage and rearrangement of disulfide bonds it seems that all 3 treatments can enhance DNA methylation. The number of disulfide bonds created in part depends on the amount of available methyl donors, which is why we originally provided different levels of FA to CD-1 mice. As expected, female mice exposed to supplement FA+ISO had a higher number of differentially expressed genes (560 genes) than females exposed to adequate FA+ISO (260 genes) or supplemental FA alone (259 genes). Importantly, the differentially expressed genes of female mice exposed to supplemental FA with or without ISO were involved in DNA synthesis, stability, integrity or repair, which supports the principle function of folate. On the other hand, females exposed to adequate FA+ISO, had a 5.7-fold lower expression of NPY when compared to females exposed to adequate FA without ISO. NPY is a strong neurotransmitter which when suppressed in bone cells promotes bone formation and osteocalcin production. Interestingly, bone derived osteocalcin is a known regulator of energy metabolism providing support that bone mass accrual and body weight gain may be interrelated. Therefore, findings presented in this thesis are of great conceptual importance as they not only help us to understand how ISO and FA modulate bone development but also provide support that bone is an endocrine organ that regulates body weight.

Aside from bone, there are many estrogen-sensitive tissues that may be programmed by early life exposure to ISO and FA. Until recently scientists have viewed each one of these organs independently but emerging findings including those presented in this thesis highlight the importance of looking at whole organism physiology. As shown in study 3 (chapter 6) and 4 (chapter 7) early life exposure to adequate FA+ISO resulted in higher concentrations of

serum ACTH and IGF-I than exposure to adequate FA alone, indicating that early life exposure to ISO can stimulate both the growth hormone and the stress hormone pathway. Activation of the stress hormone response can induce weight gain. When we began this research there were discrepant findings in the literature as to whether ISO stimulate or inhibit deposition of adipose tissue in animal models. As shown in this thesis and across six of our published studies, neonatal exposure to ISO stimulates weight gain in female but not male mice [13, 54, 83, 85]. Female mice exposed to ISO (7 mg/kg of body weight/day) from birth to 5 days of life had higher body weight than control mice from 28 weeks of age until necropsy (performed at 32 weeks of age) [13], but not at earlier stages of development [54]. In contrast, exposure to higher doses of ISO (50 mg/kg of body weight/day) for the first 5 days of life or longer duration of ISO exposure result in higher body weight at 12 and 6 weeks of age, respectively [85, 129]. This demonstrated that dose and duration of ISO exposure program the time when female mice begin to gain weight. In line with our findings, Newbold et al showed that body weight gain induced by ISO or DES is associated with a higher quantity of adipose tissue and an elevated concentration of circulating triglycerides. Together this suggests that exposure to ISO during early life plays a role in programming the onset of adult obesity [261-263]. From published literature we also know that estrogen is a critical modulator of body composition so it is possible that early life exposure to ISO promotes weight gain through an estrogen mediated mechanism [6].

Study five showed that both 5- and 10-day exposure to ISO have similar adverse effects on structural development of ovaries and uterus in sexually mature female mice. Both 5 and 10-day ISO exposure resulted in a lower number of ovarian corpora lutea and a higher incidence of oviduct hyperplasia, as well as a higher occurrence of hyperplasia, atypia,

polyps and cysts in the uterine tissue structure. These findings are comparable to changes observed in mice with neonatal exposure to potent environmental estrogens (i.e. DES and bisphenol A). Thus, we present evidence that ISO bind to estrogen receptors to induce estrogen like effects in reproductive tissue [126, 128, 250].

The discovery that early life exposure to ISO disrupts serum markers of metabolism (study 3 and 4), induces weight gain (study 2, 3 and 4) and changes the structural development of ovaries and uteri in female CD-1 mice (study 5) is disconcerting. These findings may have significant consequences to adult health. A next step should be to monitor the health of infants fed soy protein formula into adulthood

9.3 ISO-induced effects in CD-1 mice mirror those of humans with precocious puberty: Could there be a link?

Precocious puberty is defined as any sign of secondary sexual characteristics that appears before 8 years of age in females and 9 years of age in males [264]. There are several forms of precocious puberty. Peripheral precocious puberty, which is less common than central precocious puberty, happens without altering the amount of gonadotropin hormone in the brain that triggers the start of puberty [265]. Instead, it develops because of too much estrogen or testosterone in the body. In most cases, a tumour or some metabolic deregulation stimulates endocrine organs to over produce sex steroids and cause early puberty. However, exposure to environmental estrogens during development may also be a trigger [266].

Epidemiological reports indicate that over the past decade the age of puberty onset has stabilized in most European countries [267], while in North America the onset of female puberty, but not the age of menarche, occurs much earlier [268, 269] than it did 20 years ago [270, 271]. Similar trends have also been reported in boys, although not as evident as in girls

[271-273]. This rapid change in both the timing and progression of puberty among North America children suggests that some environment factor(s) present in North America but not Europe may be the underlying cause. A growing body of literature indicates that xenoestrogens present in plastic baby bottles, food storage containers, shampoos, cosmetics pesticides and insecticides contribute to precocious puberty [266, 274]. Over the past decade major public agencies across Europe have been advising the public to avoid the use of soy protein formula, especially for infants younger than 6 months of age, because of its high ISO content and potential safety concerns [26]. As part of this initiative, some European countries have even placed restrictions so that soy protein formula can only be obtained through prescription [275]. In contrast, major public agencies in North America have stated that there is minimal concern that soy protein formula, containing ISO, cause adverse reproductive and/or developmental effects in exposed infants [32]. Due to these policies, in the last decade less than 10% of European infants have been fed soy protein formula compared to 20-25% of infants in North America [23, 276]. Thus, the question arose whether exposure to ISO present in soy protein formula may be a risk factor for precocious puberty. To try to address this question, we compared findings from mice exposed to ISO during development to those of children with precocious puberty. As summarized in Table 9-1 there are many similarities between humans with precocious puberty and CD-1 mice used to mimic ISO exposure of infants fed soy protein formula.

From a biological view, puberty is a process of physical change where a child's body matures so that it can reproduce. It is initiated by gonadotropin hormone in the brain that acts on the pituitary to secrete follicle stimulating hormone and luteinizing hormone, which then trigger sex steroid synthesis from gonads. Sex steroids, including estrogen and testosterone,

Table 9-1. Signs of precocious puberty known in infants and observed in CD-1 mice

Symptoms	Infants	Reference	CD-1 mice	Reference
Body weight and energy metabolism				
Weight gain	?	N/A	Yes	[13, 54, 85] & Study 3
Higher BMI	Yes	[269]	N/A	N/A
Skeletal development and growth				
Reduced bone length	Yes	[277-279]	Yes	Study 3
Higher BMD	?	N/A	Yes	[54, 85] & Study 3
Higher IGF-I	?	N/A	Yes	Study 3 & 4
Reproductive health				
Early breast development	Yes	[279]	?	N/A
Premature menarche	Yes	[279]	?	N/A
Estrogenic ovarian cysts	Yes	[279]	Yes	[84] & Study 5
Ovarian disorders or tumours	Yes	[279]	Yes	[84]

are the most powerful hormones in the body that stimulate growth, function and transformation of brain, bone, muscle, blood, skin, hair, breast, ovaries, testes and uteri. As such, even small changes in sex steroid production, secretion or sensitivity may alter child development and/or risk of disease.

In bone tissue, estrogen stimulates ossification of longitudinal bone growth, which is phenotypically recognized as the time when longitudinal bone growth stops [110]. Because children with precocious puberty are exposed to estrogen at an earlier stage of life than expected their bones stop growing at an earlier age than normal but, have a longer bone maturation period that allows for greater mineral deposition [278]. Consequently, these children are sometimes shorter than expected at adulthood with greater peak bone mass [277]; a phenomenon repeatedly observed in adult CD-1 mice who during neonatal life were treated with ISO (Figure 9-2) [54, 85]. Aside from having altered bone growth, children with precocious puberty often exhibit premature breast development, vaginal bleeding, emotional and social problems and higher risk of breast cancer in adult life. Similarly, findings presented in study 5 demonstrate that early life exposure to ISO induces long-lasting adverse effects on structural development of ovaries and uterus [84]. Female mice exposed to ISO during development had lower number of ovarian corpora lutea, higher incidence of oviduct hyperplasia and higher occurrence of hyperplasia, atypia, polyps and cysts in the uterine tissue at adulthood. In addition, studies have shown that early life exposure to ISO can disrupt estrous cycling and ovulation, as well as reduce fertility and the number of live pups [84]. These findings should be followed up in prospective studies. Moreover, children who experience puberty too early or too late often have a family history of this developmental timing suggesting that it may be passed onto subsequent generation. This begs the question of whether some of the findings presented in Table 9-1 may be transgenerationally inherited.

9.4 Potential for transgenerational inheritance

Findings presented in this thesis (study 3, chapter 6) indicate that perinatal exposure to supplemental FA, ISO or the combination can permanently alter the expression of 949 genes in long bones of F1 female CD-1 mice. The combination of supplemental FA+ISO had the most profound effect with 560 genes being differentially expressed as oppose to only 260 by individual treatments. Of 40,000 genes in the mouse genome, it is believed that only 200 genes are passed on to subsequent generation. However, the specificity of these genes is still under investigation and thus, it remains unclear whether the effects induced by supplemental FA and ISO in bone are transgenerationally inherited.

To date, only two transgenerational studies have examined the effects of developmental [84] and lifespan exposure to ISO on bone health [280]. The first study treated male and female rats from postnatal day 42 to 120 with a diet containing 0, 5, 100 or 500 ppm of genistein and then followed offspring for 4 generations with a subset of mice in each generation being exposed to a different dose and duration of genistein exposure. This study found no effect of treatment on BMD at the femur or lumbar spine across generations. However, interpretation of inter-generational differences in bone size and quality are nearly impossible from this study because it is difficult to tease out cause and effect. Each generation of rats was given genistein for a different duration of time and over a different stage of the lifespan and only one outcome was measured (BMD). In addition, the study was clearly underpowered with not enough mice being analyzed per group. Thus, we believe that this study should not be taken into consideration when assessing whether ISO have transgenerational effects. The second study [172], conducted in our laboratory showed that benefits to bone in F1 generation are transferred to F2 females. Skeletal sites rich in trabecular (lumbar spine) or cortical (femur midpoint) bone of F2 females responded

similarly to ISO treatment in that both sites had higher BMD and could withstand greater forces before fracture. In addition, higher body weight and abnormal sexual maturation observed in F1 generation was transferred to F2, while disruptions in fertility were not. This demonstrates that not all effects induced by early life exposure to ISO are transgenerationally inherited. Further research is needed to more clearly determine which ISO-induced effects are transgenerationally inherited and for how many generations these effects persist.

9.5 Strengths and weaknesses

The research presented in this thesis has many strengths. Firstly, the approach used to conduct this research has provided findings that can be translated from basic science to clinical research. For example, the utilization of the CD-1 mouse model that has a similar response to estrogens and selective estrogen receptor modulators in rodents and humans, allowed for comparison and identification of ISO induced biological effects in bone, uteri, ovaries and adipose tissue. In addition, the confirmation that circulating ISO concentrations and metabolism of CD-1 pups compare to that of human infants fed soy protein formula allowed for more direct comparison between species (study 1, chapter 4). The examination of effects induced by multiple durations of ISO exposure and multiple doses of FA exposure on bone (study 2, chapter 5) and reproductive outcomes (study 5, chapter 8) helped to identify important factors that should be taken into consideration in future prospective clinical studies. Thus, our approach was appropriate and provided information that can be translated from bench to bedside.

Secondly, a comprehensive set of outcomes that are indicative of bone quality and quantity were measured (studies 2, 3 and 4) to determine how supplemental FA and ISO affect bone development. This approach was appropriate because bone removal differs

widely among patients with osteoporosis, and thus, there is a large grey scale in how much any one of the measures used to assess bone quality and quantity can vary before a fracture occurs. According to the World Health Organization, BMD assessed by DXA is the primary measure used in diagnosing osteoporosis and assessing bone mass over time in both treated and untreated patients [182]. Thus, many models of fracture risk have focused predominantly on examining areal bone density through DXA analysis. However, DXA provides 2D information that does not directly reveal structural properties or the force that the bone can withstand before it fractures [281]. Thus, in addition to measuring BMD, serum markers of bone metabolism, bone structure and biomechanical stress testing were analyzed at two skeletal sites (femur and lumbar spine) that differ in the proportion of cortical and trabecular bone. These analyses provide an in depth assessment of bone integrity and in conjunction with the microarray finding provide some mechanistic information about how supplemental FA and ISO improve bone development.

Lastly, the research presented in this thesis explored the effects of early life exposure to ISO on whole-organism physiology to help characterize potential benefits as well as adverse effects. The premise of whole-organism physiology is that no function is determined by one organ alone; so exploring how ISO modulate body weight gain and structural development of ovaries and uteri, in addition to bone health provided a mode of identifying potential interactions between organs. For instance, the discovery that ISO induced positive effects on bone are accompanied by weight gain and structural changes in reproductive organs demonstrates that early life exposure to ISO disrupts the coordinated regulation that exists between bone, energy metabolism and reproduction. However, at the present time we do not understand how ISO do this. Published studies suggest that endocrine factors such as leptin,

NPY, osteocalcin, insulin and FGF-23 may coordinate regulation of bone, energy metabolism and reproduction. Mouse genetics and observation of internal medicine are two tools commonly used to study whole-organism physiology [226]. In this thesis, we used microarray analyses to decipher the genes that are altered by each treatment, and then by knowing the gene function we were able to predict how early life exposure to supplemental FA, ISO or the combination programs bone development. These analyses have significant implications for future clinical studies because bone is one of the last tissues to appear during evolution and most of its genes have conserved function from mice to humans [226]. Moreover, the decision to compare functional, histological and biochemical measures from our studies to those found in children with precocious puberty extended our understanding of how ISO modulate whole-organism physiology. Retrospective and prospective studies are needed to determine whether consumption of soy protein formula, abundant in ISO, during development induces similar effects.

In addition to these strengths, there are few limitations of this research. First, CD-1 pups were treated with a combination of daidzein and genistein because it was impossible to achieve the ISO content of infants fed soy protein formula by orally feeding pups with soy protein formula. However, since whole foods may have different biological effects on programming of human health than pure ISO, it is possible that the observed findings do not translate to infants fed soy protein formula. Secondly, to determine whether route and frequency of exposure affects serum ISO levels we measured total ISO concentrations. However, knowing the concentration of conjugated and free forms of ISO would unquestionably provide more insight about ISO metabolism in the developing CD-1 mouse model and thus, further studies should be done to characterize how oral versus subcutaneous

route of delivery affects the concentration of conjugated versus free ISO in circulation. Thirdly, in this thesis we did not examine body composition of ISO-treated animals because the sensitivity of our DXA did not allow for detection of lean and fat mass in such small animals. Further studies are needed to better understand how early life exposure to ISO in combination with FA programs visceral fat deposition. Moreover, in performing the microarray analyses we identified a subset of genes (PTHr1, Ctnnb1, NPY, etc) that were differentially expressed by early life exposure to adequate FA+ISO, supplemental FA or supplemental FA+ISO, which may help to explain the observed changes in bone outcomes. However, we did not yet perform studies to confirm the expression of these genes via real-time PCR or their protein level. Moreover, we have not done the experiments to show that ISO affect histone acetylation and DNA methylation in bone tissue. Lastly, there is always the limitation that even the most characterized animal model may not fully mimic the human scenario in terms of lifespan and biology. Thus, prospective studies in humans are needed to determine whether consumption of soy protein formula, abundant in ISO, during development induces effects that mimic those observed in CD-1 mice.

Chapter Ten

CONCLUSIONS

10.0 CONCLUSIONS

STUDY 1 – Female and male CD-1 mice

- Oral and subcutaneous route of exposure to ISO (5 mg of genistein + 2 mg of daidzein/kg body weight/day) resulted in similar serum levels of genistein, daidzein, equol and O-DMA in the developing CD-1 mouse model.
- Exposure to 5 mg of genistein plus 2 mg of daidzein per kg of body weight once daily or divided into 4-hour treatments of 0.83 mg of genistein and 0.33 mg of daidzein per kg of body weight in the first 5-days of life resulted in similar serum genistein, daidzein, equol and O-DMA concentrations in the developing CD-1 mouse model.
- Regardless of route or frequency of ISO exposure males had significantly lower concentrations of serum genistein and daidzein than females.

STUDY 2 - Female CD-1 mice

- Exposure to ISO (5 mg of genistein + 2 mg of daidzein/kg body weight/day) from birth throughout suckling (postnatal day 1-21) versus only the first 5 days of life with a conventional diet (AIN93G) resulted in higher BMD, improved trabecular connectivity and higher peak load at the lumbar spine, and unlike 5-day exposure, also had favorable effects on femur outcomes (i.e. higher BMD, improved trabecular connectivity and greater resistance to fracture) in female CD-1 mice.

STUDY 3 - Female CD-1 mice

- Exposure to adequate FA and ISO (2 mg of FA per kg of diet in combination with 5 mg of genistein + 2 mg of daidzein/kg body weight/day) resulted in higher BMD, improved trabecular connectivity and greater resistance to fracture at the femur and lumbar spine in

female CD-1 mice compared to adequate FA without ISO. Similarly, exposure to supplemental FA (8 mg of FA per kg of diet) without ISO resulted in higher BMD at the femur and lumbar spine, improved trabecular connectivity at the lumbar spine and greater resistance to fracture at the femur neck than exposure to adequate FA without ISO. In contrast, exposure to supplemental FA+ISO was limited to higher BMD at the femur neck in female CD-1 mice compared to adequate FA without ISO.

- The effects of FA and ISO on bone development were, in part, mediated by changes in gene expression that are programmed through epigenetic regulation. Thus, the favorable effects of early life exposure to adequate FA and ISO on bone may in part be attributed to lower NPY gene expression that stimulates osteoblast to produce OPG and promote bone formation. In contrast, the lack of effects observed among females treated with supplemental FA+ISO may in part be attributed to higher rate of bone resorption driven by over-expression of PTHr1 and Ctnnb1.

STUDY 4 - Male CD-1 mice

- Early life exposure to adequate FA and ISO (2 mg of FA per kg of diet in combination with 5 mg of genistein + 2 mg of daidzein/kg body weight/day) improved BMD and bone strength of the femur and lumbar spine in male CD-1 mice at young adulthood, while improvements induce by supplemental FA and ISO (8 mg of FA per kg of diet in combination with 5 mg of genistein + 2 mg of daidzein/kg body weight/day) were limited to a higher level of serum OPG.

STUDY 5 - Female CD-1 mice

- Abnormalities in ovaries and uteri of adult female mice were similar regardless of duration of exposure to ISO (5 mg of genistein + 2 mg of daidzein/kg body weight/day), and were comparable to our previous study showing effects with ISO exposure throughout suckling (postnatal day 1-21). Therefore, the first 5-days of life represent the sensitive window of development during which exposure to ISO can alter structural development of ovaries and uteri in CD-1 mice.

Chapter Eleven

FUTURE RESEARCH

10.1 Future research

The findings presented in this thesis have identified a number of questions that will steer the next frontier of animal and clinical research involving plant estrogens. For instance, the discovery that short term ISO exposure during neonatal life results in shorter femur length has raised the question of whether ISO stimulate growth plate closure through an estrogen mediated mechanism. In addition, the discovery that the amount of FA status dictates whether ISO induce benefits to bone has raised a number of questions. For example, does the amount of FA affect how ISO modulate biological responses in tissues (i.e. ovaries, uteri, testes, mammary gland, etc.) other than bone? Why are higher levels of FA needed to stimulate serum OPG secretion in males but not females? Do ISO enhance DNA acetylation and/or alter the expression of DNA methyltransferases? How does exposure to adequate FA+ISO suppress NPY gene expression in bone tissue? Is the up-regulation of PTHr1 responsible for ISO-treated female having shorter bone length? And finally, which bone-specific genes are transgenerationally inherited? To address this last question we are currently running a microarray that will examine the expression of femur genes from F2 females whose mothers were exposed to adequate or supplemental levels of FA with or without ISO during development. Through these future findings we hope to define more clearly the underlying connections between different generations of mice who during development were exposed to ISO and FA. In addition to animal research, both retrospective cohort and prospective studies are needed to determine how soy protein formula affects skeletal and reproductive development in humans. Investigation to adult life is crucial since the ISO effects do not manifest until later in life. The use of DXA and ultrasounds may prove to be useful for identifying changes in bone or abnormalities in reproductive organs such as the occurrence of polycystic ovaries in women.

Chapter Twelve

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11.0 REFERENCES

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