Transgenerational Effects of Early Exposure to Soy Isoflavones on Reproductive Health and Bone Development in CD-1 Mice

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A thesis submitted in conformity with the requirements
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ABSTRACT

Previous studies showed that early exposure to soy isoflavones resulted in improved bone mineral density (BMD) and bone quality that resulted in stronger bones in CD-1 mice. This study investigated whether the benefits to bone health are transferred to second generation (F2) females and if there are any adverse effects on reproductive health. First generation (F1) female CD-1 mice received subcutaneous injections of the isoflavones daidzein (DAI) and genistein (GEN) or corn oil from postnatal day (PND) 1 to 10 or 21. F1 and F2 treated-females experienced earlier pubertal onset and lengthened anogenital distance but only F1 had reduced fertility, histological abnormalities in the uterus and ovaries, and altered estrous cycling. F2 had higher BMD and stronger bones at 4 months of age. In conclusion, early life exposure to soy isoflavones compromise reproductive function but confer a transgenerational benefit to bone development in CD-1 mice.

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LIST OF DEFINITIONS

Long-term programming is used to describe the effects that occur when an external stimulus or insult, during a sensitive developmental period or window, results in a permanent or long-term change in the structure or function of the organism. Long-term programming is a theoretical construct for investigating the biological mechanisms through which external stimuli modulate chronic disease risk reduction.

Epigenetics is defined as a change in gene expression that occurs without alterations in the DNA sequence. Epigenetic alterations may induce long term programming effects or can be inherited through changes in germinal cells and be carried to subsequent generations.

LIST OF ABBREVIATIONS

ACTH - adrenocorticotropic hormone

ALP - alkaline phosphatase

BMC - bone mineral content

BMP -2- bone morphogenetic protein 2

BMD - bone mineral density

CON - control

DAI - daidzein

DES - diethylstilbestrol

ER - estrogen receptor

FSH - follicle stimulating hormone

GEN - genistein

GIN - genistin

GLY - glycitein

hGC – human chorionic gonadotropin

IGF -1 - insulin-like growth factor-1

LH - luteinizing hormone

LV - lumbar vertebrae

MC2R - melanocortin receptor 2

OPG - osteoprotegerin

OSC - osteocalcin

PBM - peak bone mass

PND - postnatal day

RANKL - RANK-ligand

RANK - receptor activator for nuclear factor ß

SBIF - soy-based infant formula

SPI – soy protein isolate

TGF α/ß - transforming growth factor α/ß

MOF- multioocyte follicles

Chapter One

INTRODUCTION

1.0 INTRODUCTION

Soy isoflavones are phytoestrogens with potential hormonal activity due to their similar chemical structure to 17-β-estradiol.[1] The increasing availability of soy isoflavones throughout the food supply and through use of supplements has prompted extensive research on biological benefits to humans in chronic disease prevention and health maintenance.[2] While much of this research has focused on adult populations, infants fed soy based infant formulas (SBIF) are exposed to substantial levels of soy isoflavones, even when compared to adult populations that consume a higher quantity of soy-based foods.[3] Infant exposure to soy isoflavones primarily occurs from birth to one year of life, a stage of development that is particularly sensitive to dietary and environmental stimuli.[4] There is considerable interest in studying the potential hormonal effects of soy isoflavones on health outcomes.

Bone tissue may be particularly responsive to soy isoflavones because of their estrogen-like effects. Findings from our lab have shown that neonatal exposure to soy isoflavones, that result in similar serum levels of isoflavones as infants fed SBIF, improves bone health in later life.[2,5] It has been demonstrated that early exposure to isoflavones in a developing CD-1 mouse model results in higher bone mineral density (BMD) and improved bone structure leading to stronger bones. Moreover, greater thickness and connectivity were observed at adulthood. Although both males and females were studied, female mice were most responsive.[5] Importantly, these benefits to bone health at young adulthood protect against the deterioration of bone tissue that accompanies the cessation of endogenous estrogen production after ovariectomy.[5]

While it has been shown that soy isoflavones confer protective effects in bone using the CD-1 mouse model, there is concern regarding the effects of soy isoflavones on reproductive health.[6] Studies using higher doses of isoflavones, particularly GEN, in the developing CD-1

mouse model have demonstrated several harmful consequences – such as altered uterine and ovarian morphology, disrupted estrous cyclicity, subfertility or infertility, pregnancy loss associated with fewer implantation sites and increased resorption as well as development of uterine adenocarcinoma in later life. [7, 8, 9] Such studies, however, used doses of isoflavone genistein (GEN) (50 mg genistein/kg body weight [7, 8, 9] that are 7 fold greater than the dose of isoflavones used in the present study (7 mg daidzein (DAI)+GEN)). With this lower dose of isoflavones, we did not anticipate adverse effects on in reproductive organ development or function.

The mechanism by which soy isoflavones modulate bone and reproductive organ development may be due to epigenetic changes. Thus, it is of interest to examine transgenerational effects of soy isoflavones on bone health and reproductive health, particularly fertility. The objectives of this study were to investigate whether neonatal exposure to soy isoflavones at a dose and ratio comparable to those present in SBIFs, results in higher BMD and stronger bones without adverse effects on reproduction at young adulthood (4 months of age) in females across two generations. Using the developing CD-1 mouse model, F1 and F2 females were exposed to soy isoflavones during the first 10 or 21 days of life and onset of puberty, fertility and reproductive organ development were assessed. In the F2 females, bone mineral content (BMC), BMD and biomechanical bone strength were measured at young adulthood.

Chapter Two

LITERATURE REVIEW

This literature review was modified from the review: Dinsdale, E.C.; Ward, W.E. Early Exposure to Soy Isoflavones and Effects on Reproductive Health: A Review of Human and Animal Studies. *Nutrients* **2010**, *2*, 1156-1187.

2.0 LITERATURE REVIEW

2.1 Isoflavones in Soy-Based Infant Formulas (SBIF)

Soy protein based infant formula (SBIF) has been used throughout the world for over 100 years.[10] SBIFs were initially developed as an alternative to cow's milk based formula for infants with immunoglobulin E-mediated milk allergies, post-infectious diarrhea due to lactose intolerance, galactosemia, or for infants who required a vegan substitute.[10] SBIFs were originally prepared from soy flour which had lower digestibility and lower protein content compared to the soy protein isolate (SPI) which is used currently.[10] SBIFs have been further modified to include methionine, iodine, carnitine, taurine, choline and inositol.[10] According to the Infant Formula Act of 1980, amended in 1986, SBIFs meet all nutritional requirements for term infants.[10] Data from North America suggest that approximately 37.2% to 43.8% of infants are formula fed three to six months postpartum.[11] Recent data suggests the prevalence of feeding SBIF is 20–25% in Canada[12] and the United States[13] and markedly lower (2–3%) in the United Kingdom [14] and Australia.[15]

SBIFs represent a significant source of soy isoflavones with potential hormone-like activities[16] as they contain a diphenolic ring that allows them to bind to the estrogen receptor (ER).[17]Although soy isoflavones are weakly estrogenic, approximately 100 to 1000 fold less potent than endogenous estrogen[18], infants consuming SBIF have extremely high levels of serum isoflavones.[18] One study reported the isoflavone content of the major brands of commercially available SBIFs as well as the serum concentrations of isoflavones (including GEN, DAI and its metabolite, equol) in four month old infants exclusively fed SBIF, cow's milk formula, or human breast milk. The levels of isoflavones in five different SBIFs varied from 32 to 47 mg isoflavones/L of formula.[16, 18] Thus, infants fed these formulas are exposed to 5.7–11.9 mg isoflavones/kg body weight during the first four months of life. Compared to adults consuming a soy rich diet, which could contain approximately 0.71 mg/kg body weight (assuming a body weight of 70 kg) [19] infants fed SBIF are exposed to a 6–11 fold

higher level of isoflavones on a body weight basis than adults. Additionally, circulating isoflavone levels of these infants were 13 000–22 000 times greater than circulating levels of 17-β-estradiol.[16]

2.1.1 Metabolism and Pharmacokinetics of Isoflavones

The absorption, metabolism and pharmacokinetics of soy isoflavones ultimately determine how these compounds influence physiological activity. There are two predominant types of soy isoflavones, DAI and GEN. DAI and GEN have a 6 to 8 fold higher binding affinity for ER-β than ER-α.[20] Thus, isoflavones in soy protein (i.e. 67.1% GEN, 28.7% DAI and 4.2% glycitein, (GLY))[21] induce greater functional changes in tissue containing a higher population of ER-β than ER-α.[22] Additionally, the dimerization of ER complex indicate that ER (α and β) protein helices are crucial in determining whether isoflavones will induce an agonistic or an anti-agonistic effect.[23]

The oral cavity[2] and intestinal microflora[24] modulate the metabolism of lignans and isoflavones. Partially hydrolyzed isoflavones are further broken down by intestinal glycosidase in the jejunum, which cleaves the β-glucose moieties of glycosidic conjugates to release the bioactive aglycones including DAI, GEN and GLY. [25, 26] Bioavailability of isoflavones depends on β-glucosidase activity.[27, 28] Bioactive aglycones enter portal blood and are transported to the liver [28] where they are conjugated to glucuronic or sulfuric acid and combined with cholesterol making them nearly insoluble in water. As a result, conjugated isoflavones are either excreted in the urine or form bile micelles.[28] The bile micelles can be released into circulation, where they are absorbed by surrounding tissues or reabsorbed by the intestine through a process known as enterohepatic cycling.[29] If bile micelles enter the intestine they become deconjugated by intestinal bacteria (i.e. bile salts and lipases) to release bioactive aglycones, DAI, GEN and GLY. In turn, these aglycones can once again can be metabolized by intestinal bacteria or reabsorbed by the intestine

(enterohepatic cycling). Isoflavone metabolites that are not absorbed by surrounding tissues or intestine are excreted as feces.[28]

Pharmacokinetic studies have demonstrated that peak plasma concentrations are attained 5-7 hours post-consumption for DAI, GEN and GLY [30], and 9-10 hours post-consumption for the ß-glycosides because extra time is required for hydrolytic cleavage of the glucose moiety.[29,3] The rate of absorption of the aglycone is faster than that of ß-glycosides because at normal intestinal pH of 6-6.5, the aglycones can rapidly pass through the membrane by non-ionic diffusion. However, aglycones are also more vulnerable to degradation than ß-glycosides which speeds-up their clearance rate and limits their bioavailability.[27] Notable differences are observed between DAI and GEN pharmacokinetics with plasma concentrations of GEN being consistently higher than those of DAI when equimolar concentrations are ingested. This is due to DAI having a greater volume of distribution and a higher clearance rate than GEN.[27] Understanding pharmacokinetics is important when developing an appropriate model to ensure that isoflavone treatment of the CD-1 mice results in serum isoflavone levels that are comparable to human infants consuming SBIF. Although we administer soy isoflavones by subcutaneous injection rather than delivering these compounds orally, we have confirmed that this method is an appropriate model to use. We have shown that CD-1 mouse pups injected with 7 mg isoflavones (2 mg DAI+5 mg GEN/kg body weight) achieve serum isoflavone levels that are similar to infants.[1, 31] Moreover, subcutaneous injection or oral administration of soy isoflavones at the same dose has been shown to result in similar levels of serum isoflavones.[31]

2.2 Studying the Effects of Soy Isoflavones in Infants Fed SBIF

Nine human studies investigating the effects of early life exposure to soy isoflavones on bone health have been conducted and none have measured bone health into adulthood.

Additionally, only one study has reported on reproductive health outcomes of adults fed SBIF.[32]

2.2.1 Male and Female Reproductive Health: Human Studies

To date, few studies have investigated the impact of early life consumption of SBIF on reproductive function in adult life (Table 2.0). Strom *et al.* reported no differences in more than 30 reproductive health outcomes including the age of onset of puberty and reproductive function in males. Prolonged menstruation as well as increased discomfort during menstruation was more frequently reported in the female group. [32] Increased vaginal cell maturation has been reported in female infants at six months of age, and was considered to be an estrogenic effect attributed to the consumption of SBIF in early life.[33] Other outcomes, including vaginal discharge and breast and genital development were not altered.[33] Another study, which focused on infants at two years of life did however demonstrate differences in breast development.[34] Breast tissue was more prevalent in infants fed SBIF at two years of life compared to those fed cow's milk-based formula or breast milk.[34] Currently, The Beginnings Study, a longitudinal prospective study, is in progress at the Arkansas Children's Nutrition Center to compare growth, development, and health of breastfed or formula-fed children.[35] Findings to date have shown that formula-feeding itself, without discriminating between type of formula, results in greater ovarian volume, increased numbers of ovarian cysts per ovary and lower testicular volume.[35] Consideration in the interpretation of these findings is that 32% of infants in the SBIF group did not consume SBIF until 4–8 weeks of age. Because timing of exposure may modulate effects of later health it will be important to further investigate how timing of exposure may influence reproductive outcomes at later stages of development (*i.e.*, beyond four months of age).

Table 2.0 Studies examining the effect of soy isoflavone exposure in early life on human development

Objective	**Sample Size** **Age of Subjects**	**Intervention Duration**	**Reproductive Health Outcomes**	**Findings**
1. Retrospective cohort study to determine the association between soy infant formula consumption and health in adulthood with focus on reproductive health; Self-reported pubertal maturation, menstrual and reproductive history, height and usual weight [32]	n = 248 SBIF n = 563 cow's milk formula Adults aged 20–34	Adults as infants were treated from age 9 days or before to16 weeks of age; Cow's milk formula; SBIF (soy isoflavone content of the formula was unknown)	**Women:** adult height, body mass index, pubertal maturation, number of days between periods, number of days requiring pads or tampons, regularity of menstrual period, menstrual flow, pain with menstrual period, physical symptoms of pain, breast tenderness during menstrual cycle, premenstrual symptoms, breast size, reproductive outcomes, and education level attained as a proxy measure for intelligence **Men:** adult height, usual weight, education level, pubertal maturation and pregnancy outcomes in sexual partners impregnated by the male study subjects, congenital malformations in the offspring of study subjects, hormonal disorders, testicular cancer in men, and homosexual orientation.	**Men and Women:** No statistically significant differences were reported between groups in either men or women for more than 30 outcomes; **Women:** Significantly longer menstrual bleeding and greater discomfort during menstruation.

2.To pilot techniques for assessing infants' responses to the withdrawal from maternal estrogen and gathered data on breast and genital development in infants at different ages in infants who have consumed SBIF, cow's milk formula or exclusively breast milk [33]	n = 72 equally distributed in SBIF, cow's milk formula and exclusive breast fed 37–41 weeks of age	37–41 weeks of age until 6 months of age	Breast adipose tissue; Breast bud and testicular volume; Observed breast and genital development; Vaginal wall cytology; Vaginal discharge	Breast tissue was maximal at birth and disappeared in older children, consistent with waning maternal estrogen; Genital development did not change by age; Vaginal wall cells showed maximal estrogen effect at birth and then reverted as normal; Female infants on SBIF appeared to show reestrogenization at 6 months, by increased maturation in vaginal cells
3. To evaluate the estrogenic effect of soy-based formulas in female infants [34]	n = 50–92 consumed SBIF for greater than 3 months from age 3-24 months n =232-602 Milk group (both breast milk and cow's milk) 3–24 months of age	3–24 months of age	Breast development	No differences in breast bud prevalence during the first year of life; Infants fed SBIF did not demonstrate a decline in the prevalence of breast tissue during the second year of life, unlike other groups.

To determine if differences exist in hormone-sensitive organ size between infants who were fed soy formula (SBIF), milk formula (MF), or breast milk (BM) [36]	n = 40 BM n = 41 MF n = 39 SBIF Age 4 months; SBIF exclusively fed from birth up to 8 weeks of age and continuing until 4 months of age (32% did not switch to SF until 4–8 weeks of age); MF from birth to 4 weeks until 4 months of age; BM from birth until 4 months of age	BM, MF or SBIF for 4 months	Anthropometry; Body composition; Breast buds, uterus, ovary, prostate and testicular volume	In both formula groups males had lower testicular volume, and females had greater ovarian volume, increased numbers of ovarian cysts per ovary; Other measures were not significantly different between the control and SBIF groups

Due to the paucity of human data, animal models are useful to more fully understand effects in humans. Unquestionably there are considerations that need to be made when extrapolating findings from animal models to humans (Table 2.1). Human and animal studies have different challenges and we propose that both types of studies are important to achieve a comprehensive understanding of the biological effects of soy isoflavones on bone development and reproductive health.

Table 2.1 The challenges of designing and conducting studies in humans or using rodent models to study the effects of soy isoflavones on bone and reproductive health

Humans Studies	Rodent Models
• Long-term commitment (time, funding) to follow birth cohort through to adulthood; • Consideration of environmental factors that can impact on bone and reproductive health (*i.e.*, exposure to endocrine disruptors, physical activity, smoking, age, education, diet and disease history); • Limited to measurement of noninvasive outcomes (*i.e.*, organ size, menstrual cycle, fertility, bone mineral density) that may provide limited insight into mechanisms of action.	• Species-related differences in digestion, absorption and metabolism of isoflavones; • Administering purified isoflavone or isoflavones as part of SBIF; • Route of isoflavone administration: Oral feeding *versus* subcutaneous injection; • Frequency of isoflavone administration: Once daily dose or multiple doses per day; • Composition of soy isoflavone mixture to mimic the ratio and combination of isoflavones present in SBIF; • Equating the timing of the life cycle between rodents and humans.

2.3 Challenges of Human Studies

2.3.1 Long-Term Commitment: Time and Funding

Determining the effect of SBIF on human bone growth or reproductive development requires a long-term commitment, both on the part of the investigator(s) as well as the subjects. Ideally, children would be monitored before puberty and until sexual maturity is

reached. This would allow potential differences in bone strength at adulthood or reproductive organ development to be discerned. Furthermore, reproductive capacity should be assessed throughout potential child-bearing years. As with all long-term studies, subject compliance and retention over this long time period will be difficult. Retrospective cohort studies of individuals who consumed SBIF are another strategy and indeed one such study has been reported.[32] A limitation of retrospective studies is recall bias. Unless a birth cohort with detailed records of infant feeding from birth through at least the first months of life exist, it is difficult to accurately determine the type of feeds an infant received. Retrospective cohort studies are still very time-intensive and recruiting sufficient number of participants and controlling for the multitude of factors that may affect bone development and reproductive capacity may be particularly challenging. From a practical standpoint, prospective studies in particular, as discussed above, are costly and require long-term funding. Retrospective studies would also require a substantial level of funding.

2.3.2 Environmental Factors

Accounting and controlling for various environmental differences that subjects either would be, or were exposed to, is difficult to control. Factors such as level of physical activity, dietary patterns, smoking and history of disease known to influence bone health can be evaluated but it is considerably more difficult to determine the level of subject exposure to known environmental estrogens which may impact bone or reproductive development.

2.3.3 Noninvasive Outcomes

Human studies are limited to measurement of fairly noninvasive outcomes. To study bone health in adult humans, pQCT could be used to examine structure at peripheral sites. Reproductive outcomes previously measured include pubertal maturation, sexual orientation, body weight, menstrual characteristics, congenital characteristics of subject offspring.[32] Determination of the prevalence of hormone dependent cancers (*i.e.*, testicular, ovarian,

breast, prostate), measurement of serum hormone levels, reproductive organ size and morphology would provide a more comprehensive understanding of biological effects of SBIF in human infants.

2.4 Challenges of Using Rodent Models

2.4.1 Species-Related Differences

Rats, mice, marmosets and piglets have been used in animal studies and it is known that there are some species related differences in absorption and metabolism of soy isoflavones.[38] There are, however, some important similarities in isoflavone metabolism among species. For example mouse pups, like human infants, do not produce equol which is the more estrogenic metabolite of DAI.[3] Additionally, the influence of estrogen on bone metabolism and reproductive function in CD-1 mice during development is similar to humans.[39] Other animal models are not as well-suited for the proposed studies as the CD-1 mouse. In rats, the growth plate does not fuse and thus PBM cannot be defined.[40] Pigs may be fed SBIF and are a high quality model for infant bone development but they do not reach PBM until 3 years of age and are extremely large, making it impossible to use pigs for the proposed studies from a time and resources perspective.[40] Unlike mice, both pigs and sheep are not good models of postmenopausal bone loss after estrogen withdrawal.[40] Monkeys most closely resemble humans with respect to bone metabolism but are equol producers in early life and there are various ethical issues and considerations when using this model.[40]

2.4.2 Isoflavones in SBIF *versus* Purified Soy Isoflavones

Isolated soy isoflavones are frequently used in animal studies but it is unknown whether they act differently when present in SBIF, a complex mixture of phytochemicals and peptides. Using rodent models it is not possible to deliver sufficient levels of isoflavones by using SBIF as the volume required is too high. This is likely the basis for why most investigators have administered purified isoflavones rather than SBIF for rodents. A few

studies have fed SBIF directly to animals, such as marmosets or pigs and thus when reviewing the animal data it is important to know the form of isoflavones provided.

2.4.3 Route of Administration—Oral versus Subcutaneous Injection

Additional considerations include the route of isoflavone administration in a study. There have been differences reported in the serum isoflavone levels in rodents depending on whether the isoflavone mixture was given orally or delivered by subcutaneous injection.[41] We have previously shown that subcutaneous injection of GEN and DAI in mice results in serum isoflavone levels that are comparable to human infants fed SBIF.[5] This is significant because it suggests that the use of subcutaneous injection is useful even though injected GEN bypasses first pass metabolism in the gut. Oral feeding in rodent neonates is possible but due to the small size of the pup it is difficult to ensure that all of the isoflavone mixture is consumed during an oral feeding (measurement of serum levels are needed to determine the level of isoflavone exposure). Another issue with oral feeding from birth through the first days of life includes the risk of aspiration requiring premature euthanization. One study used a combination of subcutaneous injection and oral feeding and examined the effects of GEN on postnatal development from birth until PND 21 in rat pups. The authors state that it was not technically practical to orally gavage the rat pups from birth and thus subcutaneous injections were used from birth until PND 7 [42] and pups were fed orally thereafter. Oral feeding may be more desirable to more closely mimic the human infant scenario, and whether differences in metabolism result in different biological effects requires further study.

In one study, mice were given varying doses of GEN in order to determine the oral dose that would achieve serum levels close to human infants consuming SBIF. It was determined that 5–20 mg oral GEN/kg body weight did not have a measurable effect on serum GEN levels but an oral dose of 50 mg GEN/kg body weight resulted in serum levels of 2–3 μM[43] and others have shown that subcutaneous injection at this same dose (50 mg/kg

body weight) also results in similar serum levels (1–5 μM).[30] This is similar to the 1–5 μM total serum isoflavone levels observed in human infants.[3] This demonstrates that there may be no difference in serum levels after oral or subcutaneous administration and that the dose of 50 mg isoflavones/kg body weight may achieve serum isoflavone levels close to human infants.[30, 43] We have shown that CD-1 mouse pups injected with 7 mg isoflavones achieve serum isoflavone levels that are comparable to infants.[1] Importantly, there were no differences in serum levels after subcutaneous injection or oral administration of soy isoflavones.[31]

2.4.4 Composition of Isoflavones

GEN, which is the most abundant soy isoflavone found in SBIF [3] has been the focus of investigation and is often administered in isolation. GEN, however, is not the only active isoflavone in SBIF. DAI is also present in SBIF and accounts for approximately 28.7% of total isoflavone content (GEN accounts for approximately 67.1% of total isoflavones).[3] Therefore, the ratio and dose of isoflavones should be comparable to SBIF, and both GEN and DAI should be administered if isolated soy isoflavones are used rather than SBIF.

2.4.5 Equating the Timing of the Life Cycle of Rodents with that of Humans

The timing of when isoflavone exposure should take place using an animal model, in order to mimic the first year of life in humans, is debatable. Mice suckle for the first 21 days of life and thus it makes sense that soy isoflavone exposure should take place during the age of suckling to mimic the stage of development in which human infants may be fed SBIF. In order to establish an appropriate time of life to evaluate bone development in CD-1 mice, we previously determined how bone changes during the first 4 months of life. BMC, BMD and biomechanical strength properties such as peak load of femur and spine were similar between 3 and 4 months of age, demonstrating that peak bone mass is attained by 4 months of age.[44] A difficulty is that mice also start to reach sexual maturation shortly thereafter,

being able to breed by six weeks of age, whereas humans have a much longer duration before sexual maturation takes place.

2.5 Safety of Soy Isoflavones for Reproductive Health

While the benefits of early exposure to soy isoflavones and SBIF for bone development has been documented in the CD-1 mouse model, several studies have identified adverse effects on reproductive health.[5,36,45] These findings have led several countries to control the availability of SBIF because of concern regarding safety of isoflavone exposure in early life and reproductive development. In Europe, SBIF is only available by prescription.[46] In 1996, the United Kingdom Committee on Toxicity of Chemicals in Foods, Consumer Products and the Environment considered the presence of phytoestrogens, such as soy isoflavones in SBIF, and subsequently supported the advice of the U.K. Department of Health, that human breast milk and cow's milk are preferred sources of nutrition for infants and SBIF should only be recommended when there is clinical indications.[47] The Working Group identified the need for further studies, and stated that due to a paucity of data it is not possible to make a final conclusion. The Canadian Paediatric Society, Dietitians of Canada, Health Canada and the American Academy of Pediatrics as well as many other organizations recognize that breastfeeding is the optimal method for feeding infants.[13,48] The use of SBIFs is recommended for only those infants who cannot have dairy-based products because of health, cultural or religious reasons. Nonetheless, significant numbers of infants are fed SBIFs during the first year of life.

In the United States, The National Toxicology Program Center for the Evaluation of Risks to Human Health Reproduction (NTP-CERHR) convened in December of 2009 to evaluate the safety of SBIF. A panel of experts reviewed and evaluated the quality and strength of available scientific data regarding early exposure to SBIF or its isoflavones and how it may impact human development.[6] In 2006, the NTP previously convened to evaluate

soy formula and GEN, but did not complete the evaluation or issue a final statement due to insufficient data.[6] The substantial number of studies that were released from 2006–2009 prompted the NTP-CERHR to revisit this topic. The NTP-CERHR and expert panel has since concluded that there is "minimal" concern due to a paucity of data focusing on early, critical stages of the life cycle [6] Moreover, it was stated that the design of some studies are not ideal for evaluating the safety of SBIF. These aspects have been discussed and are summarized in Table 2. However, the relatively high number of studies in experimental animals, and a few studies in humans, reported some effect of isoflavones on reproductive health, and this raised the level of concern from "negligible" to "minimal". The report also stated that there is insufficient human data to form a definitive conclusion.[6]

Since there are few studies examining the effects of SBIF in humans, it is prudent to conduct animal studies, to determine the effect of soy isoflavones on human health while keeping in mind the limitations/challenges in extrapolating findings from animal studies to human infants. Before discussing the findings from these animal studies it is useful to review the indicators of reproductive health that have been measured and what information they provide regarding effects on reproductive health (Table 2.2). These indicators are grouped according to whether they are indicators of sexual maturation or endocrine disruption. Multiple measures should be used when determining the effect of an estrogenic compound on reproductive development.

Review of studies to date show that interventions with isoflavones have been conducted at different life-stages, and in some studies the intervention occurred over more than one life-stage. This section focuses on studies in which animals were directly exposed to soy isoflavones during suckling.

Table 2.2 Indicators of sexual maturation and endocrine disruption in rodent models

Sexual Maturation	
Preputial Separation	The separation of the foreskin of the penis from the glans, preputial separation (PPS) is an early marker of the progression of puberty.
Vaginal Opening	The initial marker of the rise in circulating estrogen that signifies the onset of puberty and first ovulation followed by the start of estrous cycling.
Endocrine Disruption	
Anogenital Distance (AGD)	The distance between the anus and genital protuberance in newborns of various species including mouse and rat is used as the sole external sex-differentiating marker (longer in males compared to females) and is used to determine whether or not endocrine disruption has occurred. Under-masculinization is said to have occurred if AGD is shortened compared to control animals.
Sex Organ Histology	Changes in morphology of the mammary gland, ovary, uterus, testes are indicators of estrogenic effects that may ultimately be manifested as enhanced or reduced fertility.
Sex Organ Weight	Higher weight of uterus, ovaries, testes, or prostate may indicate estrogenic effects due to higher rates of cell proliferation within the organ.
Serum Hormones	Measurement of sex steroid hormones (*i.e.*, LH, FSH, GnRH, estradiol, progesterone, testosterone) demonstrates estrogenic perturbations in the endocrine system.
Estrogen Receptor Activity	Elevated transcription of ER-ß or ER-α is indicative of higher estrogenic activity.
Estrous Cycle	Length of time spent in each phase of estrous cycle can be used to understand if fertility may be altered, *i.e.*, if an animal is in prolonged diestrus, lower fertility may result.
Lordosis Quotient	A measure of sexual behavior and is calculated by dividing the number of lordoses (inward curving of a portion of the vertebral column) by the number of mounts.

The doses of soy isoflavones and the route of administration used in the studies reviewed are summarized in Table 2.3. Of note is that few studies measured serum levels of soy isoflavones. Without knowing serum levels of isoflavones it is difficult to directly compare findings to human infants.

Table 2.3 Summary of isoflavone doses, route of administration and serum measurements in rodent models studying reproductive health

	Dose	Route of delivery	Serum Isoflavone Levels	Ref.
Female	0.0001–100 mg GEN or DAI/kg bw	SC	NM *	49
	GEN: 0.5, 5, 50 mg/kg bw	SC	NM *	50, 51, 7
	GEN: 12.5, 25, 50 or 100 mg/kg bw	Oral	NM *	52
	GEN: 50 mg/kg bw	SC Oral	NM *	8-9, 53
	GEN: 5, 20, 50, 100 mg/kg bw	Oral	5, 20 and 100 mg GEN/kg body weight: below desired range; 50 mg GEN/kg body weight resulted in desired serum range of: 2–3μM	43
	GIN: 6.25, 12.5, 25 or 37.5 mg/kg bw/day; Oral GEN: 25, 37.5, 75 mg/kg/day	Oral	Serum levels of oral GIN and GEN were measured at 37.5 mg/kg body weight; GEN AUC/dose = 2.4; GIN AUC/dose = 0.34	41
	GEN: 12.5, 20, 25 mg/kg bw	SC	NM *	41
	GEN: 0.2, 2, 4, 40 mg /kg bw (sexes combined)	SC	SC 4 mg GEN/kg body weight: 0.99 μg/equivalents/h/mL; 40 mg/kg body weight: 5.82 μg/equivalents/h/mL	42
		Oral	40 mg GEN/kg body weight: 0.53 μg/equivalents/h/mL	42
	83 mg GEN or DAI/kg bw	SC	NM	54
	GEN: 500 mg /kg bw	SC	NM	55
Male	GEN: 4 mg/kg body weight	SC	NM	56
	1.6–3.5 mg isoflavones/kg bw	Oral	NM	57, 58
	GEN: 0.2, 2, 4, 40 mg/kg bw (sexes combined)	SC	4 mg GEN/kg body weight: 0.634 μg/equivalents/h/mL; 40 mg/kg body weight: 5.82 μg/equivalents/h/mL	42
		Oral	40 mg GEN/kg body weight: 0.53 μg/equivalents/h/mL	42
	GEN: 12.5 25, 50 or 100 mg/kg bw	Oral	NM	52

*NM: not measured. Previously measured serum GEN levels of 1–5 μM after subcutaneous injection of 50 mg GEN/kg body weight are reported. Infants result in serum concentrations of 1–5 μM total isoflavones after SBIF consumption

2.5.1 Female Reproductive Health: Animal Studies

Studies that investigated the long-term consequences of soy isoflavone exposure during suckling have demonstrated differing effects at adulthood (Table 2.4).

2.5.1.1 Reproductive Organ Morphology

Studies have shown that early exposure to soy isoflavone enhances differentiation of the mammary gland, leading to a mammary gland that is less susceptible to chemically-induced mammary cancer.[55] Furthermore, this effect is present at high levels of exposure, (subcutaneous injection of 500 mg GEN/kg body weight) and did not alter fertility and age at puberty onset. GEN treatment resulted in fewer terminal end buds and advanced development and ductal elongation. It is known that terminal end buds are the most susceptible to carcinogens as they are the least mature terminal ductal structures.[59] A reduction in the numbers of terminal end buds can therefore explain the lower incidence of mammary cancer. Part of the terminal end bud differentiates according to each estrous cycle, giving rise to alveolar buds that consist of lobule structures that are more mature and less susceptible to chemical carcinogens.[60] GEN treatment increased the number of lobules indicating a potential protective effect.[60] Previous findings have confirmed that early life exposure to estrogen causes differentiation in mammary tissue, leading to a mammary gland that is less susceptible to cancer.[61] The mechanism by which GEN influences mammary gland development is yet to be elucidated but these findings suggest GEN is exerting an estrogenic effect. Another reproductive organ that is sensitive to isoflavone exposure is the uterus. Neonatal mice (PND 1–5) treated with GEN had greater uterine gland number (subcutaneous injection of 50 mg GEN/kg body weight) and increased uterine weight and epithelial cell height at higher doses (subcutaneous injection of 100 mg GEN/kg body weight).[49]

Table 2.4. Studies in female animal models examining the effects of soy isoflavone exposure during early life

Objective	Sample Size Subjects (age at time of intervention)	Intervention: Route of administration and dosage	Duration of Intervention	Reproductive Health Outcomes	Findings
1. To determine if the orally administered genistin (GIN), the glycosylated form of GEN, causes adverse effects on the developing reproductive tract GIN is most predominant in soy isoflavone formulas, but infants consuming SBIF have high circulating levels of GEN [41]	N = 4–16 mice/group CD-1 mice, PND 1	SC: GEN: 12.5, 20, 25 mg/kg body weight Oral genistin (GIN): 6.25, 12.5, 25 or 37.5 mg/kg body weight Oral GEN (GEN): 25, 37.5, 75 mg/kg/day	PND 1–5	**SC GEN, Oral GEN, Oral GIN** Uterine wet weight gain Induction of estrogen-responsive gene, lactoferrin (LF) **GIN Group only** Vaginal opening Estrous cycling Fertility Morphologic alterations in ovary/reproductive tract	**SC GEN, Oral GEN, Oral GIN** 20–33% more oral GIN was needed to elicit uterine wet weight gain compared to SC GEN but similar response was observed Oral GEN uterine wet weight gain only observed at much higher doses of 75 mg GEN/kg body weight Induction of LF gene **Oral GIN:** Increased incidence of multioocyte follicles in the ovaries Delayed vaginal opening Altered estrous cycling Decreased fertility Delayed parturition

2. To develop a mouse model that more closely mimics the oral GEN exposure and total serum GEN concentrations. To assess reproductive and nonreproductive organs after dosing and during development [43]	Not determined C57BL/6 mice, PND 1	Oral GEN-soy formula emulsion: 5, 20, 50, 100 mg/kg body weight	PND 1–5	Serum GEN concentration Thymic and uterine weights Follicle numbers Immunohistochemistry for progesterone receptor	**5, 20, 100 mg GEN/kg body weight: below desired range of serum GEN** **50 mg GEN/kg body weight** Increased uterine weight Downregulation of progesterone receptor in uterine epithelia Increased incidence of multioocyte follicles Decrease in thymic weight Altered estrous cycling Normal fertility
3. To investigate the potential of GEN to protect against the development of breast cancer and to cause reproductive and developmental toxicity [55]	Not determined Prepubertal female, suckling, Sprague-Dawley rats	SC GEN 500 mg/kg body weight Oral gavage Carcinogen: Dimethylbenz[a]anthracene (DMBA) 80 mg/kg body weight	GEN: 3 days, every second day PND 16, 18, 20 DMBA: PND 50	Mammary gland differentiation and cell proliferation in the presence of carcinogen DMBA; Offspring body weights; Anogential distance; Vaginal opening; Estrus cycle length; Follicular development	**GEN treatment:** 50% reduction in chemically induced mammary tumorgenesis Increased mammary gland differentiation in immature rats leading to mammary gland less susceptible to mammary cancer No significant changes in fertility, number of male and female offspring, body weight, anogenital distance, vaginal opening, testes descent, estrus cycle, or follicular development among groups

4. To measure the estrogenic responses of several phytoestrogens including GEN, DAI and compare them over a dose range and measuring the transcriptional activation of the estrogen receptor (ER) and an *in vivo* immature mouse uterotrophic assay [49]	Not determined CD-1 mice, PND 17	SC GEN and DAI doses 0.00001 to 1000 mg/kg body weight Positive controls: Diethylstilbestrol (DES) 17β-estradiol: 0.01 to 1,000,000 ug/kg body weight Negative control: corn oil	3 consecutive days (PND 17, 18,19)	Uterine wet weight Uterine epithelial height Uterine gland number	**DAI treatment:** Did not demonstrate any increase in uterine epithelial cell height; Increase in uterine gland number; Did not demonstrate an increase in uterine wet weight; **GEN treatment:** Increase in uterine wet weight; Increase in uterine epithelial cell height; Increase in uterine gland number
5. To determine the biochemical effect of GEN as the induction of ectopic expression of ER in granulosa cells, a morphological effect as the induction of multioocyte follicles (MOFs) in the ovary, and a functional effect as the altered ovarian response to superovulation treatment [50]	n =16/group CD-1 mice, PND 1	SC GEN: 1, 10, 100 ug/pup/day (approximately 0.5, 5 or 50 mg/kg body weight)	5 days PND 1–5	ER-ß and ER-α expression and distribution in ovarian tissues The impact of GEN on ER expression, ovulation and the development of multioocyte follicles	ER-β transcript expression predominated in the ovaries in all stages of life and over ER-α and increased with age GEN did not change ER-β expression but ER-α expression increased on days 5 and 12 ER-β was immunolocalized to granulosa cells ER-α was immunolocalized in interstitial and thecal cells GEN caused major increase in ER-α expression in granulosa cells Superovulated mice had an increase in the number of ovulated oocytes at the lowest dose Dose-related increase in multioocyte follicles (MOFs)

6. To determine the long-term carcinogenic potential in mice treated neonatally with GEN or DES with equal estrogenic dose [9]	n = minimum 8/group CD-1 mice, PND 1	SC GEN: 50 mg/kg body weight DES: 0.001 mg/kg body weight Negative control: corn oil	5 days PND 1–5	Incidence of uterine adenocarcinoma Uterine weight Corpora lutea absence Abnormalities in the oviduct Ovarian tumor	Higher incidence of uterine adenocarcinoma at 18 months with GEN and DES; Higher uterine weight gain with GEN and DES; Higher absence of corpora lutea with GEN and DES
7. To study the formation of multioocyte follicles (MOFs) and potential disruption of the development of the ovary by GEN on ovarian differentiation[7]	n = 24–48/group CD-1 mice, PND 1	SC GEN 50 mg/kg body weight (~100 µg/pup/day)	PND 1–5	Ovarian differentiation	**GEN treatment:** Fewer single oocytes Higher percentage of oocytes not enclosed in single follicles Oocytes nest breakdown was prolonged Fewer oocytes undergoing apoptosis on neonatal day 3

8. To determine the the processes involved in altered mammary gland growth and development after neonatal GEN treatment[53]	n = 3–8/group CD-1 mice, PND 1	SC GEN 0.5, 5 or 50 mg/kg body weight	PND 1–5	Development of the mammary gland	**4-week**: No morphological differences were observed in development **5-week**: Gen50 group had stunted development(less branching) decreased numbers of terminal end buds **6-week:** Gen50 had decreased number of terminal end buds, Gen 0.5 treated mice had advanced development with increased ductal elongation Increased levels of progesterone receptor protein and estrogen receptor-β mRNA in Gen0.5-treated mice compared with controls ER-α expression decreased after all doses of Gen treatment Gen50 treated mice were unable to deliver live pups
9. To elucidate the mechanism by which gensitein leads to infertility[8]	Not determined CD-1 mice, PND 1	SC GEN 50 mg/kg body weight Control: corn oil	PND 1–5	Oocyte developmental competence Timing of embryo loss	**GEN treatment:** Females were not capable of supporting normal implantation of control embryos Oocytes were competent but the oviductal environment and the uterus have abnormalities that result in reproductive failure Complete infertility observed

10. To study the effects of neonatal GEN exposure on attainment of puberty and fertility[51]	Not determined CD-1 mice 2,4,6 months of age	SC GEN: 0.5, 5 or 50 mg/kg body weight	PND 1–5	Vaginal opening Fertility Implantation and pregnancy Ovarian function (number of corpus luteum and ovarian capacity) Estrous cyclicity Serum hormone levels (estradiol and progesterone) before puberty	GEN treated mice had prolonged estrous cycles that had a dose and age-related increase Pregnancy loss was attributed to fewer implantation sites and increased resorption Low dose GEN treated mice had increased numbers of corpora lutea compared to controls High dose GEN treated mice had fewer corpora lutea Similar levels of serum estrogen, progesterone and testosterone were observed before and during pregnancy Mice treated with Gen-50 did not deliver live pups
11. To determine the effects of oral exposure to GEN in order to assess human risk following oral ingestion of GEN [42]	Not determined Alderley Park rat PND 1	**PND 1–6** SC GEN: 0.2 or 2 mg/kg body weight **PND 7–21** Oral gavage GEN: 4 or 40 mg/kg body weight Control: corn oil	PND 1–21	Serum LH, FSH, estradiol, progesterone Vaginal opening Estrous cycling Sex organ weights GnRH	**40 mg GEN/kg body weight**: Increased uterus weights at PND 22 Advanced mean day of vaginal opening Induced permanent estrus Decreased progesterone in mature females **4 mg GEN/kg body weight:** No effects

12. To evaluate whether early exposure of neonates to GEN has any effect on the development of sexual organs and/or reproductive performance[52]	n = 10–24/group Sprague-Dawley rats PND 1	Oral gavage GEN: 12.5, 25, 50 or 100 mg/kg body weight Control: corn oil	PND 1–5	Fertility Vaginal Opening Estrous cycling Histopathological changes in the reproductive organs	Fertility was disrupted at 100 mg GEN/kg body weight Age at vaginal opening was not altered Estrous cycle: GEN-treated had cycle had variation in the amount of time spent in each phase and this was not dose responsive, cycle length was normal Histopathological changes in the uterus and ovary at 100 mg GEN/kg body weight
13. To examine the effect of phytoestrogens on female sexual behavior and ovarian cyclicity[54]	n = 9–10/group Wistar rats PND 1	SC GEN 1 mg/day DAI 1 mg/day Control: sesame oil	PND 1–5	Estrous cycle Vaginal Opening Ovary histology Lordosis quotient (feminine sexual reflexes)	**GEN treatment:** Prolonged estrous cycle Smaller ovaries and no corpora lutea compared to control or DZ group Low lordosis quoteint **DAI treatment:** Corpora lutea seen but ovaries were smaller compared to controls High lordosis quotient

These results also suggest that GEN is mimicking the effect of estrogen on uterus, supporting the hypothesis that GEN acts like estrogen in the reproductive system.[62] Interestingly, DAI did not cause such alterations in the uterus, suggesting that DAI may not have a measurable estrogenic effect on the mouse uterus. In addition, a higher incidence of uterine adenocarcinoma, corpora lutea absence and oviduct abnormalities have been reported in mice following treatment with GEN at subcutaneous injection of 50 mg GEN/kg body weight.[8]

2.5.1.2 Sexual Maturation and Endocrine Function

Estrogenic substances are known to alter endocrine function, especially when exposure happens during critical periods of development.[4] It was previously hypothesized that early exposure to compounds with estrogen-like activity may accelerate the age of puberty onset.[63] Earlier age at time of vaginal opening has been reported at doses of 40 mg GEN/kg body weight and this was administered from PND 1–21 combining both subcutaneous injection and oral gavage.[42] Interestingly however, at higher doses of GEN (subcutaneous injection of 100 mg GEN/kg body weight for PND 1-5) no change in timing of puberty was observed but this may be explained by a shorter duration of treatment.[52] Lower progesterone and differences in the amount of time spent in each phase of the estrous cycle without changes in estrous cycle length have also been observed after the 21 day treatment period.[42] Lordosis quotient may also be affected by GEN and DAI exposure; however they have contrasting effects.[54] One study reported a low lordosis quotient in the GEN treated group, whereas a higher lordosis quotient was observed in the DAI treated group.[54]These results suggest that GEN and DAI may affect sexual differentiation of the brain, ultimately leading to differences in sexual behavior.

2.5.1.3 Fertility

Impaired fertility in females has been documented after soy isoflavone exposure in the neonatal mouse model.[51,7,8] Neonatal GEN treatment resulted in pregnancy loss and was characterized by fewer implantation sites and increased resorption.[51] In the same study, increased numbers of corpora lutea after low dose GEN treatment (subcutaneous injection of 0.5 and 5 mg GEN/kg body weight) and reduced numbers of corpora lutea after higher doses (subcutaneous injection of 50 mg GEN/kg body weight) were observed. A prolonged estrous cycle without changes in serum estrogen, progesterone and testosterone before and during pregnancy was also reported at varying doses but showing a higher incidence of extended estrous at the highest subcutaneous dose of 50 mg GEN/kg body weight. [51] One study reported no corpora lutea after GEN treatment, smaller corpora lutea after DAI treatment and both treatments resulted in smaller ovaries and these were at higher subcutaneous doses of approximately 83 mg GEN and DAI/kg body weight.[54] Other abnormalities observed at subcutaneous doses of 50 mg GEN/kg body weight include the presence of multioocyte follicles (MOFs).[7] Furthermore, MOFs were accompanied by prolonged nest breakdown and fewer oocytes undergoing apoptosis.[7] The implications of these findings are noteworthy since *in vitro* data suggest oocytes derived from MOFs have reduced fertilization capacity compared to single oocytes follicles.[64] More recently, however, Jefferson *et al.* has demonstrated that mice treated with the same subcutaneous dose of GEN had competent oocytes but these mice could not support normal implantation of control embryos and were unable to deliver live pups.[8] Using the same dose and route of administration, ER-α but not ER-β transcription was upregulated in mouse ovaries after exposure to GEN.[50] This is an important finding as the mechanism by which estrogen exerts its effect on the female reproductive tract is predominantly mediated through ER-α.[65] Early exposure to GEN compromises ovarian development and reproductive function in rodent models at serum levels that resemble those of human infants consuming SBIF.

2.5.2 Male Reproductive Health: Animal Studies

2.5.2.1 Reproductive Organ Differentiation and Morphology

Only two studies demonstrated a measurable effect of soy isoflavones on male reproductive development when exposure was limited to the suckling period (Table 2.5). In terms of organ morphology, seminiferous tubule lumen formation and a high sertoli cell nuclear volume that did not match the lumen volume per testis has been documented.[56] This measure is used to determine the capacity for pubertal spermatogenesis and thus indicates that spermatogenesis may be abnormal.[56] Interestingly, these effects were observed at relatively lower doses of GEN (subcutaneous injection of 4 mg GEN/kg body weight). In another report that administered GEN by oral gavage, no consistent morphological changes in the testes, epididymides, ventral prostate, and seminal vesicles were observed at oral doses of GEN up to 100 mg GEN/kg body weight.[52]

2.5.2.2 Male Sexual Maturation, Endocrine Function and Fertility

Most notable is the study that used twin marmoset monkeys [57] as it prompted many European countries to minimize the use of SBIF.[36] Importantly, unlike other studies, the marmoset monkeys were directly fed SBIF. In this study one twin was fed SBIF and the other was fed with cow's milk formula beginning from day four or five of life. Of the twin pair, the marmoset fed SBIF, had a reduction in serum testosterone of 53–70% compared to its twin fed cow's milk formula at 35–45 days of age.[57] Additionally, males fed cow's milk formula had serum testosterone levels that are typical of the "neonatal testosterone rise" observed in human male neonates whereas the SBIF group had consistently reduced testosterone levels.

Table 2.5 Studies in male animals examining the effects of soy isoflavone exposure during early life

Objective	Sample Size Subjects (age at time of intervention)	Intervention: Route of administration and dosage	Duration of Intervention	Reproductive Health Outcomes	Findings
1. To establish if there are any biological consequences of consuming soy formula milk and to study the effects observed during and at the end of the feeding period which encompasses the period of the neonatal rise in testosterone in a non-human primate, the marmoset [57]	n = 15/group (included 13 pairs of twins) Marmoset monkeys 4–5 days old	Hand fed using 1 mL syringe (3–4 times on weekdays, 1–2 times on weekends) Cow's milk formula Soy milk formula Formulas were prepared as per instructions and offered to the marmoset until feeding stopped Approximately 1.6–3.5 mg soy isoflavones/kg body weight	5–6 weeks	Histology: testes, epididymis, pituitary gland Sertoli and germ cell number per testes Leydig cell number Plasma testosterone	Soy formula fed males had mean testosterone levels were consistently lower than milk formula fed males No significant changes in numbers of sertoli cells or germ cells Leydig cell number increased by 74% Paired comparison in soy milk formula and cow's milk formula co-twins showed a 53–70% lower serum testosterone levels at day 35–45

2. To establish if there are any consequences of consuming soy formula milk and to study the effects observed on fertility and testicular structure in a non-human primate, the marmoset [58]	n = 7/group (14 total) Marmoset co-twin monkeys 4–5 days old	Hand fed using 1 mL syringe (3–4 times on weekdays, 1–2 times on weekends) Cow's milk formula Soy milk formula Formulas were prepared as per instructions and offered to the marmoset until feeding stopped Approximately 1.6–3.5 mg soy isoflavones /kg body weight	5–6 weeks	Onset and progression of puberty based on testosterone levels Fertility Testicluar morphology	Normal progression of puberty Normal fertility Sertoli and leydig cell numbers/testes were significantly increased
3. To investigate whether neonatal exposure of estrogenic compounds altered pubertal spermatogenesis and whether the changes observed resulted in long-term changes in testis size, mating or fertility [56]	Not determined Wistar rats, PND 2	SC GEN 4 mg/kg body weight Control: corn oil	PND 2–18	Mating and fertility Sertoli cell and germ cell nuclear volume per testis Germ cell apoptotic index Seminiferous tubule lumen formation Plasma FSH	Few experienced impaired mating and fertility and low sample size was considered Slowed lumen formation Increased germ cell apoptotic rate High sertoli cell nuclear volume that did not match the lumen volume per testis Suppressed plasma FSH at PND 18

4. To evaluate whether early exposure of neonates to GEN has any effect on the development of sexual organs and/or reproductive performance[52]	Not determined Sprague-Dawley rats PND 1	Oral gavage GEN: 12.5, 25, 50 or 100 mg/kg body weight Control: corn oil	PND 1–5	Preputial separation Fertility Sperm count Serum testosterone Histopathological changes of reproductive organs	Preputial separation, was not effected Male fertility was not effected Sperm counts and serum testosterone was not effected No histopathological changes in the gonads
5. To determine the effects of oral exposure to GEN on neonatal rats to assess human risk following oral ingestion of GEN [42]	Not determined Alderley Park rats, PND 1	PND 1–6: SC GEN: 0.2 or 2 mg GEN/kg body weight PND 7–21: Oral gavage GEN: 4 mg/kg body weight 40 mg/kg body weight Control: corn oil	PND 1–21	Serum FSH, LH, testosterone Preputial separation Testes descent	No consistent effects observed in males at either dose

At the end of the formula feeding an increased number of leydig cells were reported and may indicate compensation or adjustment for leydig failure. Of note is the fact that monkeys in this study were exposed to 1.6–3.5 mg isoflavones/kg body weight, which is less than half the level of exposure compared to a human infant consuming SBIF.[3] In a later study however, using the same subject group and feeding protocol normal fertility and progression of puberty was demonstrated.[58] Moreover, isoflavone metabolism in marmosets compared to human infants may be markedly different. A study in cynalogous monkeys demonstrated a markedly higher conversion of DAI to equol, a more estrogenic isoflavone metabolite, than in human infants.[63] It is speculated that marmosets would also have a high rate of conversion from DAI to equol.

There was no difference in testes weight in the preliminary results of a study examining SBIF consumption and reproductive health in neonatal pigs, which metabolize isoflavones in a similar way to human infants.[36] It should be noted however, that the sample size was relatively small, n = 4/group and these measurements were taken at postnatal day 21, prior to sexual maturity. Furthermore, normal testes weight does not provide confirmation for normal testicular development and multiple measures, as discussed in Table 2, should be used to determine if disruption in sexual maturation or endocrine function has occurred.

Based on the data gathered from rodent studies, there appears to be no effect of soy isoflavones on sexual maturity in males. Preputial separation, fertility, sperm count and testosterone levels were unaffected by soy isoflavone treatment at oral doses of 100 mg GEN/kg body weight. Depressed plasma FSH has also been reported after GEN treatment (subcutaneous injection of 4 mg/kg body weight) [56] yet these results contrasted with those reported by others who observed no changes in FSH, LH, or testosterone after using a comparable dose of GEN when it was administered orally.[42]

2.6 Studying the Effects of Isoflavones on Bone Metabolism

Various studies have been conducted to investigate potential benefits to bone health after early exposure to soy isoflavones. Outcomes of bone development that have been measured in rodent models are shown in Table 2.6

Table 2.6 Outcomes of bone development in rodent models *

Method	Tissues of Measurement	Outcome
Dual X-ray Absorptiometry (DEXA)	Femur, lumbar vertebrae	Determines bone mineral content and bone mineral density
Biomechanical Strength Testing	Femur neck and lumbar vertebrae that are rich in trabecular bone. Femur midpoint that is rich in cortical bone.	Determine the maximum force that the bone can withstand before fracturing, measured as the peak load. This is a surrogate measure of fracture risk.
Micro-Computed Tomography (µCT)	Femur neck and lumbar vertebrae, rich in trabecular bone, femur midpoint, rich in cortical bone	To determine the 3D structural quality of the bone such measures include trabecular thickness, trabecular separation and trabecular number
Biochemical Markers of Bone Metabolism	OPG, OSC, RANK, RANKL	Higher levels of osteoprotegerin (OPG) and osteocalcin indicate higher osteoblast activity and bone formation. Higher RANKL indicates osteoclastogenesis and higher bone resorption

*OPG - osteoprotegerin, OSC - osteocalcin, RANK - receptor activator for nuclear factor ß, RANKL - RANK-ligand

2.6.1 Bone Growth and Peak Bone Mass

Bone tissue undergoes constant changes in structure, size and shape throughout the life cycle.[66] During puberty, sex steroid production increases and there is an up-regulation of bone formation, ultimately resulting in a 45% increase of bone mass deposited onto the growing skeleton.[67,68] The acquisition of bone mineral continues until early adulthood when peak bone mass (PBM) is reached.[68] PBM, is the maximal amount of bone tissue present at the end of skeletal maturation and is a key determinant of risk of osteoporotic fractures.[66, 69] Therefore,

optimizing PBM in early life may be one strategy to attenuate the decline of bone tissue in later life. The concentration of sex steroids plateaus in early adulthood when PBM has been established. Subsequently, the skeleton undergoes remodeling, whereby minerals and the collagen matrix are resorbed at the same rate or more rapidly than new bone tissue is produced.[68] Bone mass thus remains mostly constant with an average annual loss of approximately 0.5 to 1%.[70] During adulthood, cessation of endogenous sex steroid production upregulates the rate of bone resorption, resulting in a change in annual loss of 3 to 5% of female bone tissue compared to only a 1 to 3% in males.[70]

If a higher PBM is achieved during adolescence there will be more bone to lose during aging and a lower risk of fracture in later life.[69] Small changes in PBM can have a profound effect on fracture risk. A 5% increase in PBM for instance, can result in a 40% decrease in fracture risk.[71] Although prenatal, neonatal and adolescent stages of life account for most of an individuals' bone mass accrual, external stimuli or insult during early development has the more profound effect on long-term programming of bone metabolism.[72,73] Accordingly, effective strategies to reduce the risk of the developing osteoporosis during adulthood could target the neonatal stages of life. A number of factors modulate PBM (Figure 2.0).

2.6.2 Human Studies

Presently, there is paucity of clinical data in human infants examining the effects of SBIF on bone development and none have monitored bone health into adulthood. A total of nine human studies have been conducted and these findings imply that SBIF does not significantly change body length, head circumference, body weight, BMC or biochemical markers of bone formation at 1 year of age (Table 2.7). Interestingly, several studies have demonstrated that SBIF may not be beneficial to bone development in human infants up to one year of life.

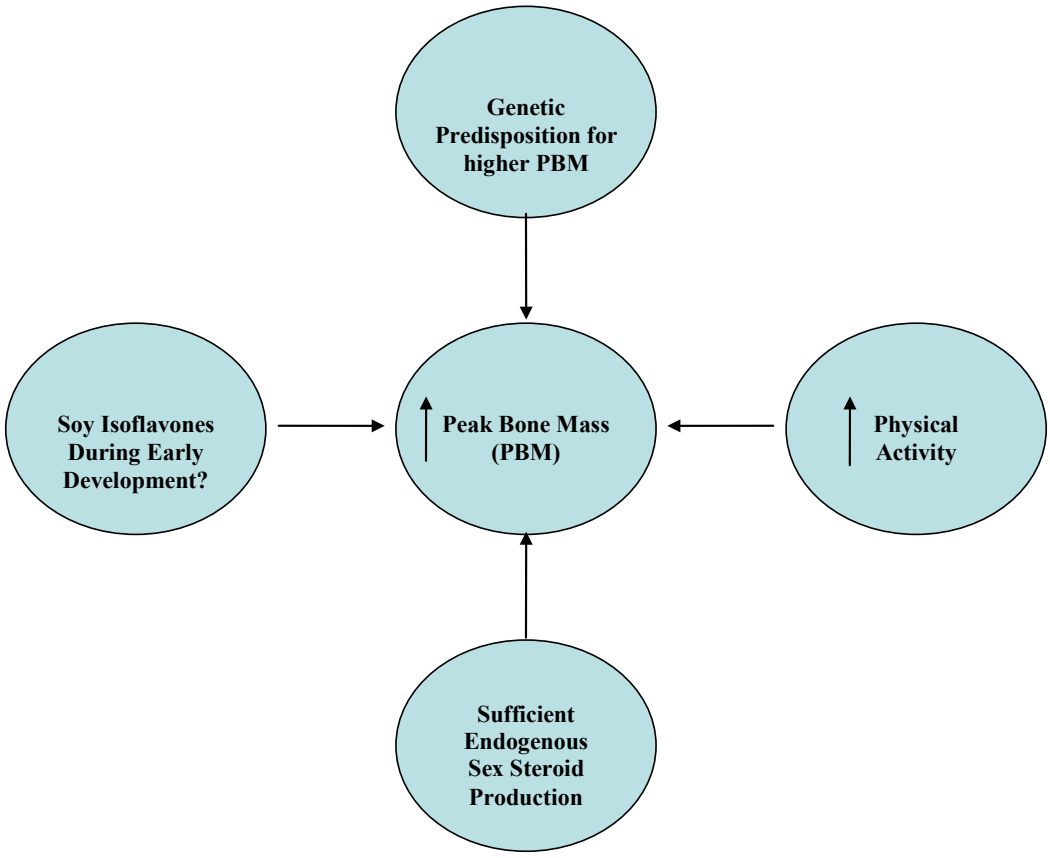


Figure 2.0 Factors that contribute to the acquisition of peak bone mass (PBM): nutrition, genetics, physical activity and sex steroid production

One study demonstrated that infants fed SBIF had lower BMC compared to infants fed cow's milk formula at 3,6,9 and 12 months of age.[74] This study however, did not include a group assigned to human breast milk and instead may simply mean that cow's milk formula may be more beneficial compared to SBIF.[74]Lower bone mineralization and maturation at 3 and 4 months of age have been reported by two studies of infants fed SBIF when compared to breast fed infants but at 6 months of age these differences disappeared.[75] Of note is the fact that none of these studies examined infants after 12 months of age. It is possible that protective effects of early exposure to soy isoflavones may not be evident until later childhood or adult life. Thus, studies in which infants are followed to older ages are warranted. Because such studies require many years to complete, using animal models can provide useful insight into potential benefits to human infants. Such studies can also provide a basis for studies in humans.

2.6.3 Animal Studies

2.6.3.1 Animal Studies during the Neonatal Period

Five animal studies have investigated the effects of soy isoflavones on bone development during the neonatal period (Table 2.8).[5, 45, 83-45, 84] Two studies have investigated the effects of neonatal exposure to soy isoflavones on skeletal development in a piglet model and three in the CD-1 mouse model.[84-85]

2.6.3.2 Early Isoflavone Exposure Using a Piglet Model

Findings from these two studies using the piglet model are inconsistent. One study demonstrated that oral consumption of soy protein isolates from PND 9 to 43 had no significant effect on piglet growth but rather a decrease in bone calcium.[86]

Table 2.7 Summary of human studies: effects of soy isoflavones on bone mass, growth and biochemical markers of bone metabolism

Objective	Sample Size Subjects (age at time of intervention)	Intervention	Duration of Intervention	Findings
1.To evaluate some hormonal and metabolic effects of long-term (more than 6 months) SBIF feeding [76]	HM (n=18) SBIF (n=48) 7 months	SBIF	7-96 months	Bone age was within the normal range Serum ALP osteocalcin and PTH were within the normal values
2. To test the hypotheses that bone mineral content (BMC) is similar in infants fed SBIF and human milk (HM) and higher in infants fed cow milk-based formula (CBF) [77]	HM (n=10) CMF (n=10) SBIF (n=42) Days 2-7 of life	SBIF Isomil (44 mg/L of isoflavones) Prosobee (45 mg/L of isoflavones)	8, 16, 26, and 52 weeks of age	BMC was not significantly different among treatment groups at 8, 16, 26 and 52 weeks

3. To study the hypothesis that ingestion of a modified SBIF with an improved mineral suspension system may result in bone mineral content similar to that observed in infants fed human milk or cow milk-based formulas[78]	SBIF (n=20) CMF (n=19) HM (n=17) 0-8 weeks	SBIF*	8, 16, and 24 to 26 weeks'	ALP, Ca, P, PTH and Mg were not different at 8, 16, 24 or 26 weeks Infants fed SBIF had significantly ↑ BMC at 16, 24 and 26 weeks than infants fed HM Bone width was higher among SBIF fed infants at 16 weeks
4. To determine the bone mineral content (BMC) of the midportion of the humerus term infants fed human milk (9 infants), cow milk-based formula (11 infants), or SBIF (11 infants) over the first year of life[79]	SBIF (n=11) CMF (n=11) HM (n=9) <3 Weeks	SBIF*	2, 4, 6, 9, and 12 months	BMC of the middle region of the humerus ↑ during the first year of life amongst all treatment groups
5. The purpose of this study was to evaluate mechanisms of mineral homeostasis and mineralization in term infants with recommended vitamin D intakes. Infants fed human milk (nine), cow milk-based formula (11), or SBIF (11) were studied at 2 weeks and at 2, 4, 6, 9, and 12 months of age[80]	SBIF (n=11) CMF (n=11) HM (n=9) Within the first 2 weeks of life	SBIF*	2 weeks and at 2, 4, 6, 9, and 12 months of age	Ca, P, PTH, BMC and bone growth were not different

6. To determine the effects of SBIFs on mineral metabolism in term infants[81]	SBIF (n=40) HM (n=10) Within the first 2 weeks of life	SBIF*	2 weeks, 2 months, and 4 months of age	Intervention did not alter weight, length, head circumference, 25-hydroxyvitamin D; Ca, P, Mg, Cu, or ALP at 2 and 4 months of age Infants fed HM had ↑ Zn, BMC and BMD at 2 and 4 months of age infants fed SBIF
7. The purpose of this study was to evaluate the adequacy of a soy protein-based formula versus a cow milk protein-based formula for body growth and skeletal mineralization in the first year of life [82]	SBIF (n=18) CMF (n=17) Within the first 24 hours of life	SBIF – Isomil (44 mg/L of isoflavones)	First 12 months of life	Infants fed SBIF had ↓ BMC at 3, 6, 9 and 12 months of age Treatment had no effect on weight, length and head circumference
8. To compare growth of infants between six and twenty-six weeks of age on breast milk, cow's milk formula, or soy formula[75]	SBIF CMF HM (n=12-26) < 6 weeks	SBIF*	First 12 months of life	No change in body length and skin fold thickness SBIF infants demonstrated a slower mineralization and maturation of bone at 3 months of age, but not at 6 months of age

CMF - cow's milk formula; HM - human milk OC - osteocalcin; ALP - Alkaline phosphatase; Ca – calcium; Mg – magnesium; P – phosphorous; PTH – parathyroid hormone

* - type of SBIF was not specified; but it is known that commercial soy based infant formulas contain between 40 and 50 mg/L of isoflavones.

It is important to note that piglets do not reach PBM until 3 years of age such that the duration of the study may have been too short to determine an influence of soy isoflavones on bone development. A more recent study examining the impact of early life SBIF consumption on bone development has demonstrated higher BMC and BMD at PND 35. This beneficial effect is noteworthy since this study used SBIF rather than soy protein isolates, which more closely resembles the experience of human infants fed SBIF. Additionally, compared to breast fed piglets, piglets fed SBIF had improved serum markers of bone formation (i.e. ALP, OSC, OPG, RANKL, CTX and BMP-2).[45]

2.6.3.3 Early Isoflavone Exposure Using a CD-1 Mouse Model

While the piglet model allows for direct feeding of SBIF, it is both time and resource intensive due to the 3 year time period required to ensure that measurements are taken after PBM has been reached. Thus, the CD-1 mouse model has been used widely in studies examining endocrine function and bone development. The influence of sex steroids on bone metabolism and reproductive function in CD-1 mice during development is similar to humans.[39,87-88] Furthermore, we have previously characterized the age at which CD-1 mice reach PBM as age 4 months.[44] Regarding reproductive health, it is known that early exposure to environmental estrogens (i.e. DES) in the CD-1 mouse model elicits physiological effects on offspring that are comparable to women who were exposed to DES during pregnancy.[89]

Moreover, both human infants and neonatal mice produce little equol since, intestinal bacteria needed for conversion of aglycones to secondary metabolites (i.e. equol) is underdeveloped during early stage of life.[21, 90] The CD-1 mouse model, is useful in predicting possible benefits and adverse effects as caused by early life exposure to soy isoflavones in human infants.

Our lab has conducted 3 studies examining the effect of soy isoflavones using the CD-1 mouse model using a dose of isoflavones that result in serum isoflavone levels comparable to infants fed SBIF. In one study, female and male mice treated with GEN from PND 1-5 had stronger lumbar vertebra (LV3) at young adulthood compared to control mice. Male and female mice who were treated with GEN had a higher BMD at LV1-LV4 and had a greater peak load meaning that they were more resistant to compression fracture.[85] Although this study demonstrated that male as well as female mice benefited from early GEN exposure, two studies showed that early exposure to soy isoflavones may have a greater benefit to females than males.[5] Females treated with soy isoflavones from PND 1-5 had greater BMD, structure and strength at the lumbar vertebra compared to controls but there were no discernable effects in males. The combination of DAI+GEN did not have greater effects on bone than either treatment alone. Additionally, it was shown that early exposure to soy isoflavones offered protection after the cessation of endogenous sex steroid protection induced by ovariectomy. Females treated with DAI, GEN or DAI+ GEN had greater BMD and improved trabecular connectivity at the femur neck and lumbar spine 4 months post-ovariectomy. [91] This effect however was not observed in males after orchidectomy.

2.7 Potential Mechanisms by Which Isoflavones Modulate Development and Transgenerational Effects

The mechanism by which soy isoflavones may be able to induce long-term programming effects on bone metabolism and reproductive health include stimulating intracellular estrogen-mediated gene expression by binding to ERs[2], causing direct or indirect effects through signaling or metabolic pathways [2, 92] and altering gene expression.[93]

Table 2.8 Summary of animal studies: effects of soy isoflavones on bone mass, growth and biochemical markers of bone metabolism

Objective	Sample Size Subjects (age at time of intervention)	Intervention: Route of administration and dosage	Duration of Intervention	Findings
1. To determine whether these improvements in bone quantity and quality at 4 mo of age provide protection against the deterioration of bone tissue that occurs after a decline in endogenous sex steroid production[83]	Mouse (CD-1) PND 1 to PND 5	Sex organs removed at 16 weeks, mice studied to 32 weeks	DAI alone (2 mg/kg bw/d) GEN alone (5 mg/kg bw/d) DAI + GEN (7 mg/kg bw/d) Subcutaneous	Females treated with DAI, GEN or DAI+ GEN had ↑ BMD and improved trabecular connectivity at the femur neck and lumbar spine that resulted in stronger bones at 4 months post-ovariectomy Exposure to DAI and GEN attenuated deterioration of bone in ovariectomized females but not orchidectomized males
2. To determine whether early exposure to a combination of the soy isoflavones DAI and GEN that naturally exists SBIF results in greater benefits to bone at adulthood than either treatment alone.[5]	Mouse (CD-1) (n=8-16/group) PND 1 to PND 5	16 weeks	DAI alone (2 mg/kg bw/d) GEN alone (5 mg/kg bw/d) DAI + GEN (7 mg/kg bw/d) Subcutaneous	Females treated with DAI and GEN had improve BMD, structure and strength at the lumbar vertebra There were no differences after exposure to DAI+GEN combination compared to single isoflavone exposure Isoflavone exposure did not have a benefit nor an adverse effect on bone in males

3. To determine whether neonatal exposure of mice to GEN resulted in higher bone mineral density (BMD) and greater resistance to fracture at adulthood[85]	Mouse (CD-1) PND 1 to PND 5	16 weeks	GEN (4 mg/kg bw/d) Subcutaneous	Females: BMD was↑ at the femur and lumbar vertebrae (LV1-4) among DES and GEN groups compared to CON; these improvements resulted in ↑ LV3 peak load among GEN and DES groups Males: lumbar vertebrae BMD and peak load were ↑ among GEN group compared to CON and/or DES
4. To evaluate the effects of commercially available SBIF relative to breast milk and cow milk formula on neonatal piglet bone development [45]	Piglet (n=3-5/group) PND 2 to PND21/35	3 or 5 weeks	SBIF Oral	Piglets fed SBIF: ↑ BMC and BMD ↑ osteoblastogenesis in ex vivo bone marrow cell culture ↑ serum osteocalin and bone specific ALP ↓serum CTX ↑tibial BMP2 and ALP mRNA expression ↑tibial expression of extracellular signal-regulated kinases ↓tibial RANKL expression 35 day old male piglets had ↑osteoblast number, ↓ osteoclast number, ↑BV/TV, ↑ trabecular bone formation rate and ↑ mineral apposition rate in the proximal tibia
5. To determine the effects of milk protein-based infant formulas and SBIFs on the blood and tissue cholesterol concentration, bone calcium, and body composition in weanling pigs[84]	Piglet PND 9-12 to PND 41-43	~ 6 weeks	SBIF Oral	Bone calcium, was ↓ in pigs fed SBIF than breast milk SBIF had no effect on growth and development

It has been postulated that isoflavones may modulate the hypothalamus-pituitary-ovary axis by up or down-regulating ER-β or ER-α in the hypothalamus and pituitary.[94] Additionally, it has recently been postulated that soy isoflavones may modulate DNA methylation patterns and induce epigenetic changes.[95]

2.7.1 Estrogen-Mediated Mechanism

In both reproductive and bone tissue, estrogen-like interactions involving soy isoflavones and ER-β and ER-α have been documented.[96] The effect of soy isoflavone GEN on estrogen receptors in the uterus has been compared to 17β-estradiol in ovariectomized adult female rats.[96] It was found that the dietary GEN exposure resulted in a greater uterine weight gain following external ER-α stimulation, similar to 17β estradiol. Moreover, it was concluded that lifelong exposure to GEN significantly enhances the uterine responsiveness to ER-α mediated estrogenic stimuli in female rats.[96]

Studies have also demonstrated that by directly binding to ERs, estrogen can modulate nuclear gene transcription which in turn can regulate bone cell production. Estrogen can bind to ERs in both osteoblasts and osteoclasts to increase the production of transforming growth factor-ß (TGF-ß), an inhibitor of bone resorption.[97] Thus, it is hypothesized that dietary estrogens such as isoflavones can induce similar effects. To date, studies have shown that GEN can stimulate the production of TGF-ß from human osteoclasts[98] and can upregulate the production of OPG (a bone resorption inhibitor) through human osteoblasts.[99,100,101] In addition, isoflavones have been shown to upregulate markers of bone formation such as IGF-1, OSC and ALP, which together promote proliferation and differentiation of osteoblastic cells.[102-104]

2.7.2 Epigenetic Transgenerational Actions

Epigenetics is defined as a change in gene function that occurs without alterations in the DNA sequence but does result in transgenerational inheritance of a given trait.[105] To date, it is known that environmental estrogens have the capacity to induce transgenerational effects.[106] DES, a potent environmental estrogen that was administered to women to prevent miscarriages, is associated with a higher incidence of adverse health effects in the offspring of daughters who were exposed in utero.[107] There was a higher prevalence of miscarriages, reproductive abnormalities and potential hearing loss amongst second generation females[108] and increased risk of hypospadias in males.[109] The mechanisms by which these genetic modifications occur require future investigation. DNA methylation is the most well characterized epigenetic modification and involves the regulation of gene transcription, chromosomal positioning, activation of X-chromosome, and gene imprinting.[110] DNA methylation occurs when there is attachment or substitution of a methyl group for a hydrogen atom on a cytosine residue[110]. Although soy isoflavones cannot directly donate or accept a methyl group, it has been proposed that soy isoflavones may enhance histone acetylation through interaction with ERs, and that this interaction may open up the methylation region.[111] If this happens, methyl group donors such as folate, choline, and SAM may stimulate DNA methylation.[95] Evidence to support this theory has been previously documented.[95] One study determined that exposure to GEN resulted in hypermethylation of six CpG sites in the agouti mouse genome and resulted in coat colour and body weight changes. Another study demonstrated that prenatal exposure of mice to GEN (270 mg/kg feed) resulted in an increase in granulopoiesis, erythropoiesis, and mild macrocytosis at the adult age of 12 wk. Furthermore, GEN exposure was associated with hypermethylation of certain repetitive elements, which coincided with a significant down-regulation of estrogen-responsive genes and genes involved in hematopoiesis in bone marrow cells of GEN-exposed

mice.[112] Interestingly, estrogen receptor (ER)-α, which has at least eight promoters that contain CpG islands and are tightly regulated by DNA methylation[113] are present in both bone and reproductive organ tissue.[114] As a result, it is possible that isoflavones may be able to modulate bone and reproductive health by regulating DNA methylation and inducing transgenerational effects.

Chapter Three

OBJECTIVES AND HYPOTHESES

3.0 OBJECTIVES AND HYPOTHESES

3.1 Objectives

- **Study 1:** To determine if neonatal exposure to soy isoflavones during either half (PND 1-10) or the complete suckling period (PND 1-21) causes adverse effects on reproductive health in female F1 exposed to soy isoflavones and female F2 offspring whose mothers were exposed to soy isoflavones
- **Study 2:** To determine if neonatal exposure to soy isoflavones mimicking the quantity and ratio of isoflavones consumed by human infants fed SBIF during either half or the complete suckling period results in greater bone mineral and stronger bones at adulthood in female F2 offspring whose mothers were exposed to low dose soy isoflavones

3.2 Hypotheses

- **Study 1:** Because adverse effects to reproductive health have been previously reported after neonatal exposure to high doses of isoflavone (5-500 mg GEN/kg body weight), it is hypothesized that there will be no adverse effects to reproductive health in either F1 females exposed to soy isoflavones or F2 females whose mothers were exposed to low levels soy isoflavone (5 mg GEN +2 mg DAI/kg body weight).
- **Study 2:** Because soy isoflavones are known to alter DNA methylation patterns, female F2 offspring whose mothers were exposed to soy isoflavones during early stages of development will have higher BMD and stronger bones at young adulthood.

Chapter Four

STUDY 1: TRANSGENERATIONAL EFFECTS OF EARLY EXPOSURE TO ISOFLAVONES ON BODY WEIGHT AND REPRODUCTIVE HEALTH IN FEMALE CD-1 MICE

4.0 STUDY: 1 TRANSGENERATIONAL EFFECTS OF EARLY EXPOSURE TO ISOFLAVONES ON BODY WEIGHT AND REPRODUCTIVE HEALTH IN FEMALE CD-1 MICE

4.1 Abstract

Soy based infant formula (SBIF) can be a significant source of soy isoflavones during early life. Because soy isoflavones have the capacity to mimic endogenous estrogen and thereby exert hormone-like effects, there is concern regarding reproductive health. The objectives were to determine if neonatal exposure to soy isoflavones altered reproductive health in females and if so, whether such effects are transferred to subsequent generations. Mice were bred and F1 offspring were cross-fostered at birth and randomized to one of four treatments: 7 mg soy isoflavones/kg body weight or corn oil from postnatal day (PND) 1-10 or from PND 1-21 (n=8-13 females/group). Mice were subsequently bred to control males on PND 56 to obtain F2 (n=10-15 females/group). F1 mice that received isoflavones had greater body weight during 6 through 16 weeks and markedly reduced fertility with a 55-60% success rate. Reduced fertility was associated with abnormal estrus cycles, a lower number of corpus luteum in ovaries and increased incidence of hyperplasia and atypia in the uteri. In F2, body weight was higher during 6 through 16 weeks of age and fertility was normal. In summary, early exposure to soy isoflavones, at serum isoflavone levels similar to human infants fed SBIF, reduced fertility in F1 but not F2 but higher body weight observed in F1 was also observed in F2.

4.2 Introduction

There is much debate regarding the safety of soy based infant formula (SBIF) on reproductive health and development. In 2009, a report from the National Toxicology Program Center for the Evaluation of Risks to Human Health Reproduction (NTP-CERHR) in the United States concluded that that there is insufficient human data to form a definitive conclusion regarding the safety of SBIF.[6] Much of the concern originates from the fact that soy isoflavones have potential estrogen-like activities due to structural similarities to estrogen that allow isoflavones to bind to the ER and thereby exert a biological effect.[17]

There are few human studies. The sole prospective study that has examined the long-term biological effect of consuming SBIF at adulthood used phone interviews to question subjects about a number of outcomes including those pertaining to fertility, sexual preference, age at puberty, and menstrual characteristics.[32] Prolonged menstruation and increased discomfort during menstruation was evident in women who consumed SBIF as infants.[32] More recently, a study in 6-month old female infants fed SBIF were shown to have greater vaginal cell maturation compared to infants consuming cow's milk or breast milk.[33] Another study reported persistent breast tissue at 2 years of life in female infants who consumed SBIF.[34] Findings from these studies suggest an estrogenic effect that may be mediated by isoflavones present in SBIF. Studies using animal models, particularly mouse models in which purified isoflavones – not SBIF based infant formula - are administered during the early postnatal life are prevalent and have demonstrated a variety of estrogenic effects including enhanced mammary gland differentiation[55], greater uterine wet weight gain[9], higher uterine epithelial cell height[49] and accelerated onset of puberty.[42] Studies from our research group have also demonstrated benefits to bone health[83, 5] using a mouse model. Modulation of endocrine development by estrogenic

substances[4] may have life-long beneficial or adverse effects to health that are manifested at adulthood, and possibly transferred to subsequent generations.[5, 83, 115]

Extrapolating the findings from animal studies to the human infant fed SBIF is complex. These complexities include differences in the form of isoflavones (SBIF versus purified compounds), route of administration (oral versus subcutaneous injection), frequency of administration (every few hours versus once daily) and differences in metabolism of isoflavones.[116] However, due to the paucity of studies in humans, data from animal studies have an important role in identifying potential biological effects and mechanisms that may otherwise be difficult or impossible to study in human infants. Indeed, the NIH Workshop on Designing, Implementing and Reporting Clinical Studies of Soy interventions acknowledges that preclinical model systems can provide useful information - a specific example includes use of rodent models that demonstrated the protective effect of early isoflavone exposure against breast cancer.[117]

Our previous findings using the CD-1 mouse model demonstrated that early exposure to soy isoflavones, in which serum isoflavone levels are similar to human infants fed SBIF, results in greater bone mineral density, improved bone structure and stronger bones at young adulthood[5] and that these benefits to bone health at young adulthood protect against the deterioration of bone tissue that accompanies the cessation of endogenous estrogen production after ovariectomy.[83] Studies using higher doses of isoflavones, particularly GEN, in the developing CD-1 mouse model have demonstrated adverse effects on reproductive health – these effects include altered uterine and ovarian morphology, disrupted estrous cyclicity, subfertility or infertility pregnancy loss associated with fewer implantation sites and increased resorption as well as development of uterine adenocarcinoma in later life. [7-9, 41, 50-51] Although these doses are comparatively higher to those used in our studies investigating bone

development, the serum isoflavone levels are reported to be similar to an infant consuming SBIF.[30]

The objective of this study was twofold: to determine whether or not a combination of DAI and GEN at a dose that has been previously shown to have beneficial effects on bone development, results in adverse effects on reproductive health; and whether any adverse effects are transgenerational since environmental estrogens can mediate epigenetic modifications.[106-107] Studies that have investigated transgenerational effects have exposed mice across several life stages, ie. in utero through adulthood but not exclusively during suckling.[118] We hypothesized that no changes to reproductive health would be observed because of the relatively low dose of isoflavone that is used in our model system.

4.3 Materials and Methods

4.3.1 Animals and Treatment

All experimental procedures followed the policies set out by the Canadian Council on Animal Care[119] and were approved by the Animal Ethics Committee at the University of Toronto. Six week-old CD-1 mice (F0) (n=5 males, n=13 females) were obtained from Charles River Laboratories Canada (St. Constant, QC,) and fed a control diet (AIN93G, Dyets Inc. Bethlehem, PA) that contained no known estrogenic compounds.[120] Mice (F0) were bred harem-style after a two week adaptation period to standard environmental conditions (12-h-light cycle; 23°C). Pregnant females were housed individually and continued to be fed fresh control diet and water, ad libitum, every 2-3 days. All litters had 8-12 pups (F1) and pups were cross-fostered (n=8-18 females/group). Litters were subsequently randomized to 1 of 4 groups: 10 days of corn oil (CON-10), 21 days of corn oil (CON-21), 10 days of DAI+GEN (DG-10; 2 mg DAI·kg body weight^{-1}·d^{-1} + 5 mg GEN·kg body weight^{-1}·d^{-1}); or 21 days of DAI+GEN (DG-21; 2 mg

DAI·kg body weight^{-1}·d^{-1} +5 mg GEN·kg body weight^{-1}·d^{-1}). The 10d protocol was selected to represent early postnatal life and the 21d protocol was chosen to represent the entire period in which humans could be fed SBIF. DG was solubilized in 1 mL dimethyl sulfoxide and then suspended in corn oil.[5] Treatments were administered each morning from PND 1 until PND 10 or PND 21 via subcutaneous injection with a total volume of 20 µL·pup^{-1}·d^{-1}. The solution was designed such that the ratio and dose of DG would resemble the quantity and ratio of soy isoflavones found in SBIF[3] and result in serum DG levels that are similar to human infants fed SBIF[5]. Purified GEN (Catalogue #G6649) and DAI (Catalogue #D7802) were purchased from Sigma-Aldrich (Mississauga, ON). Mice were weaned at PND 21. Only female mice were studied after PND 21 because our previous findings have shown that female mice have a greater response to soy isoflavone exposure.[83] Body weight of F1 and F2 was measured at 3, 4, 6, 8 and 16 weeks of life. At 8 weeks of age, F1 females were bred with control (untreated) males to obtain F2 (n=10-14 F2 females/group). When F2 females reached 8 weeks of age they were bred with control (untreated) males to obtain F3. F1 and F2 mice were killed at 4 months of age and F3 at PND 21 using carbon dioxide followed by cervical dislocation.

4.3.2 Assessment of Sexual Development and Puberty Onset in F1 and F2

At weaning (PND 21) and at PND 65, anogenital distance (AGD) was measured as a marker of sexual development using digital calipers that measure to two decimal places in mm. Anogenital distance was measured as the distance from the proximal end of anus to the midpoint of the genital papilla.[121] Age at puberty onset was determined as the age at which vaginal opening occurred. Vaginal opening was monitored daily, each morning, from PND 21 and was considered to have opened when the epithelium was pierced.

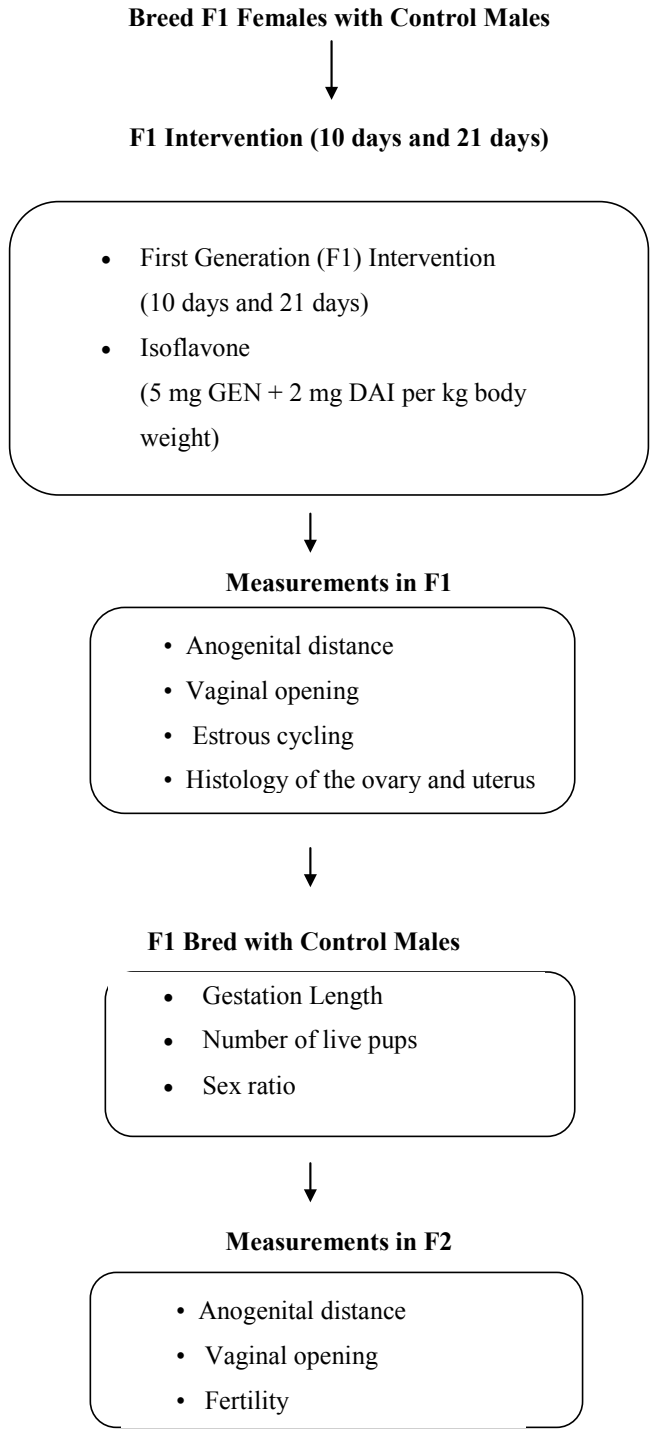


Figure 4.0 Study 1 design

4.3.3 Fertility Assessment in F1 and F2 at Adulthood

Fertility was assessed at 8 weeks of age by placing an 8 week old control male with each female. Each mating pair remained together until either a copulation plug was detected or when 14 days was completed if no copulation plug was detected. Mice were subsequently housed singly. Monitoring for copulation plugs was done twice daily. To detect the presence of a copulation plug, a metal probe was carefully inserted into the vagina of the female and the cage was emptied to verify whether it had fallen out and into the cage. Gestation day one (GD1) was considered to be the day the copulation plug was detected. For those females who did not become pregnant, a second breeding trial was conducted using the same protocol with males that were known to be fertile. At the end of the first 14-day breeding trial, non-pregnant female mice were singly housed for 4 weeks to confirm that the first breeding trial was not successful. The second breeding trial was then conducted.

4.3.4 Characteristics of litters born to F1 and F2

At PND 1, pups (F2 and F3) were counted and weighed in order to determine the litter size and total litter weight, respectively. AGD was used to determine sex at PND 21. The pups were sexed in order to determine the ratio of male to female pups in each litter.

4.3.5 Estrous Cycling in F1

Estrous cycling was performed by a blinded observer in F1 mice after the fertility trials were complete. Estrous cycling was conducted each morning from 8-9 am for 14 days. Each mouse was housed singly to prevent cyclic synchronicity that may result from cohabitation in mammals.[122] Microbrush disposable applicators (2.0 mm) were dipped into saline solution and the vaginal canal was gently scraped by turning the swab in a clockwise motion. The swab was

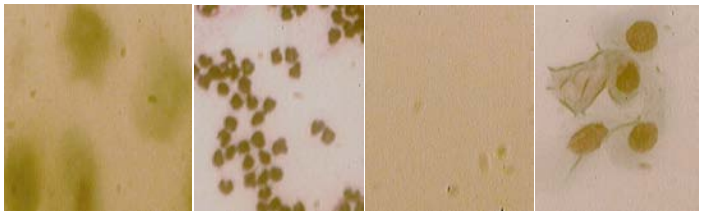
then rolled onto a Fisherbrand Superfrost Plus precleaned slide coated with polylysine. Slides were air dried for 15-30 minutes and stained according to the Diff-Quik ™ Stain Set (including Diff-Quik Solution I ™, Diff Quik Solution II ™ and Diff-Quik Fixative ™). Slides were analyzed using a Nikon light microscope at a magnification of 40. Each phase of the estrus cycle was identified as follows:

Estrus: >50% cornified epithelial cells

Metestrus: First appearance of leukocytes

Dioestrus: Blank with some debris or epithelial cells, leukocytes, no cornified cells present

Proestrus: >50% round epithelial cells



Estrus **Metestrus** **Dioestrus** **Proestrus**

Figure 4.1 Phases of the estrous cycle

4.3.6 Histological Assessment of Reproduction Tissues from F1.

At necropsy, reproductive tissues including ovaries, uterus and cervix were removed and fixed in 10% buffered formalin. 6-8 samples per group were embedded in paraffin and sectioned at 5 µM. Tissue sections were stained with hematoxylin and eosin (H&E), and evaluated by light microscopy. Both uterine horns were cut from 1 mm from the uterine body. The cervix was excised 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.

4.3.7 Statistical Analyses

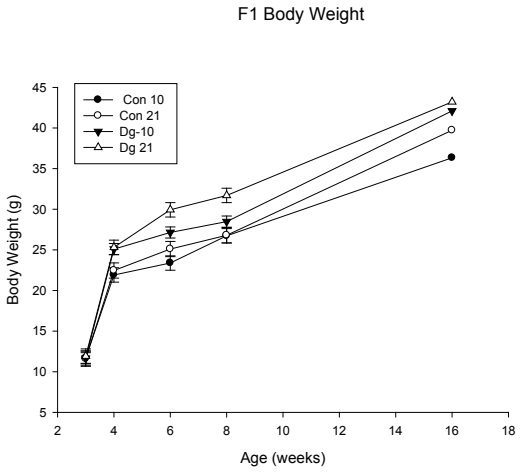
Statistical analysis was performed using Sigma-Stat (Version 3.5, Jandel Scientific, Chicago, Illinois). Results are expressed as mean ± SEM except for the gender ratio of litters and fertility and histological data that are presented as a percentage. Repeated measures two-way ANOVA, with time and treatment as factors, was performed to determine the differences in body weight over time. One-way ANOVA was performed to determine differences in litter characteristics (weight, sex ratio), vaginal opening and anogenital distance, and the number of follicles and corpus luteum (CL) among groups. All differences in fertility and other reproductive health outcomes were evaluated by Chi-Square (Fisher exact) test. Student Newman Keuls test was used for comparison of multiple means when statistical differences were detected. Statistical significance was defined as $p \leq 0.05$.

4.4 Results

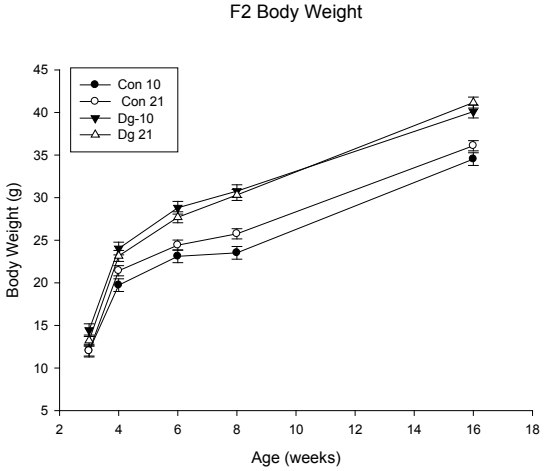
4.4.1 Body Weight

F1: There was an overall effect of treatment on body weight from 6 through 16 weeks of age. The DG-21 group had higher ($p \leq 0.05$) body weight than all other groups and the DG-10 group had higher ($p \leq 0.05$) body weight than both control groups (CON-10, CON-21) at 6 weeks. DG-21 and DG-10 had higher body weight at 8 and 16 weeks than both control groups (Figure 4.2 A).

F2: At 6 through 16 weeks of age, the DG-10 and DG-21 groups were heavier ($p \leq 0.05$) than both control groups (Figure 4.2 B).



A



B

Figure 4.2 Body weight of F1 and F2 female mice

Body weights of (A) F1 female mice that received subcutaneous injections of vehicle (CON) or (DG) from PND 1 to 10 or 21 from wk 3-16; and (B) F2 female mice from wk 3-16 whose mothers were exposed to subcutaneous injections of vehicle (CON) or (DG) from PND 1 to 10 or 21 . Values are means ± SEM, (F1, n =8-18/group; F2, n=10-14). The closed circle represents the control treated from PND 1-10, the open circle represents the control group treated for PND

1-21, the closed triangle represents the mice treated with DG from PND1-10 and the open triangle represents the mice treated with DG from PND 1 to PND 21. (A) At 6 weeks of age the DG-21 group had higher ($p \leq 0.05$) body weight than all other groups. At 8 through 16 weeks of age the DG-10 and DG-21 groups had higher ($p \leq 0.05$) body weight than both control groups (CON-10, CON-21); (B) At 6 through 16 weeks of age, the DG-10 and DG-21 groups had higher ($p \leq 0.05$) body weight than both control groups (CON-10, CON-21)

4.4.2 Anogenital Distance and Vaginal Opening in F1 and F2

F1: Vaginal opening was significantly earlier ($p \leq 0.05$) in the DG-21 group compared to the CON-10 group and there were no significant differences among other groups (Table 4.0). Anogenital distance (AGD) was not significantly different among groups at PND 21. At PND 65, AGD was longer ($p \leq 0.05$) in the isoflavone groups (DG-10 and DG-21) compared to controls (CON-10 and CON-21). There was no statistically significant difference in AGD at PND 65 between the DG-10 and DG-21 groups.

F2: Vaginal opening was significantly earlier ($p \leq 0.05$) in the DG-10 group and compared to the CON-10 group (Table 4.0). At PND 21 the DG-10 group had a longer ($p \leq 0.05$) AGD than the CON-10 and CON-21 groups and was not significantly different from the DG-21 group. The DG-21 group had an AGD that was significantly longer than the CON-10 group. There were no significant differences between the controls. At PND 65 both the DG-10 and DG-21 groups had longer ($p \leq 0.05$) AGD than CON-10.

4.4.3 Fertility in F1 and F2

F1: The fertility in both isoflavone groups (DG-10 and DG-21) was significantly reduced ($P \leq 0.05$) compared to the controls during the first breeding trial (Table 4.1). In the second breeding trial there were no significant differences in fertility but the two remaining control mice did have litters whereas none of the DG-21 mice and less than half of the DG-10 mice had litters. There were no significant differences in the number of copulation plugs, the indicator of normal mating behavior, detected among groups.

F2: There were no significant differences in fertility between the control and treatment groups (Table 4.1).

Table 4.0 Age at vaginal opening and AGD at PND 21 and 65 for F1 and F2 female mice†

	CON-10	CON-21	DG-10	DG-21
F1				
Age at Vaginal Opening (d)	30±1.31^{ab}	31±1.50^{a}	29±1.59^{ab}	29±1.58^{b}
AGD at PND 21 (mm)	5.49±0.19	5.65±0.33	5.19±0.49	5.25 ± 0.33
AGD at PND 65 (mm)	6.85±0.62^{b}	6.81±0.64^{b}	7.66±0.76^{a}	7.8±0.73^{a}
F2				
Age at Vaginal Opening (d)	31±3.17^{a}	29±1.15^{b}	26 ±2.36^{b}	27±3.40^{ab}
AGD at PND 21 (mm)	4.20±0.86^{c}	4.68±0.57^{bc}	5.57±0.54^{a}	5.17±1.08^{ab}
AGD at PND 65 (mm)	5.18±0.58^{b}	6.00 ± 0.85^{ab}	6.54±0.62^{a}	6.91±0.72^{a}

† Data are presented as mean ± standard error of mean, different superscripts in a row denote significant differences among groups, $p \leq 0.05$.

Table 4.1 Fertility of F1 and F2 female mice bred to control males†

	CON-10	CON-21	DG-10	DG-21
F1				
Breeding Trial 1				
No. plug positive	11/13	8/8	18/18	10/10
No. pregnant (%)	10/11 (91%)[a]	8/8(100%)[a]	10/18 (55%)[b]	6/10 (60%)[b]
Breeding Trial 2*				
No. plug positive	2/3	N/A	8/8	3/4
No. pregnant (%)	2/2(100%)		3/8(37%)	0/4(0%)
F2				
Breeding Trial 1				
No. plug positive	12/12	13/14	10/10	12/12
No. pregnant (%)	12/12 (100%)	13/13 (100%)	9/10 (90%)	12/12 (100%)

† Data are presented as the fraction of total mice that mated (plug positive) or had a litter (pregnant). Different superscripts in a row denote significant differences among groups, $P \leq 0.05$.
*Second breeding trial was conducted for only mice that did not get pregnant during the first breeding trial. Numbers in brackets represent the percent of mice, out of the total number of mice that were plug positive, that became pregnant.

4.4.4 Characteristics of Litters from F1 and F2

F1: There were no significant differences in the length of gestation, litter weight at birth, number of live pups, or the ratio of female to male pups in each litter (Table 4.2).

F2: There were no significant differences in the length of gestation, litter weight at birth, number of live pups, or the ratio of female to male pups in each litter (Table 4.2).

4.4.5 Estrous Cycling for F1

Both the DG-10 and DG-21 groups had a shorter ($p \leq 0.05$) estrous phase compared to the controls (Table 4.3). Metestrus was significantly longer ($p \leq 0.05$) in both treatment groups compared to the control groups. There were no significant differences in the length of dioestrus among groups. Proestrus was significantly shorter ($p \leq 0.05$) in treated groups (DG-10, DG-21) compared to the control groups. The total number of complete cycles in the treatment groups was less ($p \leq 0.05$) than the control groups.

4.4.6 F1 Histology

4.4.6.1 Ovary and Oviduct

There was no abnormal change observed in the ovaries in the control groups (Figure 4.3A, 4.3 B). There was no significant difference in the number of follicles, from secondary to preovulatory follicles, among groups (Table 4.4). The number of corpus luteum (CL) was lower ($p \leq 0.05$; Table 4.4) in the treated groups compared to controls, and 50% of mice in the treated groups did not have CL ($p \leq 0.05$; Table 4.4) and these ovaries were filled with interstitial cells in the stroma (Figure 4.3 C, 4.3D). Two mice from DG-10 (33%) and one from DG-21 (17%) showed abnormal cyst-like structure, where the wall of the cyst was lined with simple cuboidal

Table 4.2 Characteristics of litters from F1 and F2 female mice bred to control males †

	CON-10	CON-21	DG-10	DG-21
F1				
Gestation Length (d)	19±1	20±0.5	20±1	20±1
Litter Weight (PND 1)	19.90±3.6	19.90±4.0	20.77±5.6	19.94±7.6
Number of Live Pups	12±1	10±3	9±3	10±3
Sex Ratio (female:male)	0.5±0.2	0.5±0.1	0.5±0.2	0.4±0.2
F2				
Gestation Length (d)	20±0.5	20±0.4	20±0.4	20±0.5
Litter Weight (PND 1)	24.14±4.2	21.45±4.9	23.74±3.8	23.17±3.6
Number of Live Pups	12 ±2	12±2	13±2	11±3
Sex Ratio (female:male)	0.5±0.2	0.4±0.1	0.4±0.2	0.5±0.1

† Data include litter characteristics from breeding trial 1 and 2 and are presented as mean ± standard error of mean

Table 4.3. Estrous cycling in F1 female mice †

	CON-10	CON-21	DG-10	DG-21
Number of Days Spent in Phase:				
Estrous	4.1±1.7[a]	3.9±1.6[a]	1.4±2.6[b]	2.1±3.2[ab]
Metestrus	6.3±3.7[b]	7.4±1.5[b]	11.2±3.5[a]	10.7±4.1[a]
Dioestrus	0.9±1.4	1.4±1.5	1.0±2.3	0.6±1.5
Proestrus	2.1±1.4[a]	1.8±1.0 [a]	0.3±0.5[b]	0.6±1.0[b]
Total Number of Cycles	1.8±1.2[a]	1.5±0.8[a]	0.3±0.5[b]	0.3±0.7[b]

† Mice were monitored daily over a 14 day period. Data are presented as mean ± standard error of mean. Different superscripts in a row denote significant differences among groups, $p \leq 0.05$.

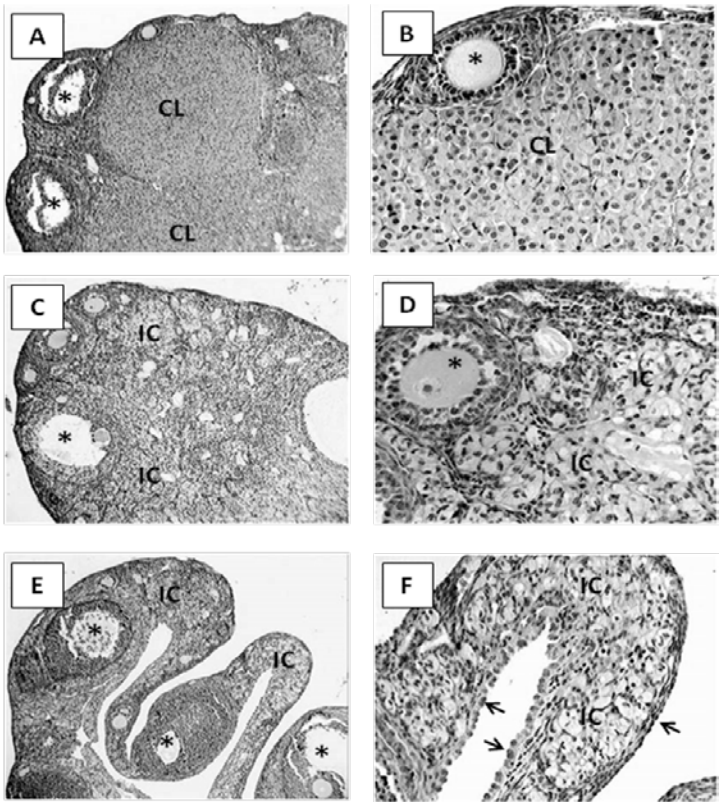


Figure 4.3 Histological structures of F1 ovaries

(A) Representative ovary from mouse control group (CON-10); CL, corpus luteum; *, follicle; H&E, 100X. (B) High magnification of Figure 4.3 A showing corpus luteal (CL) cells; *, follicle; H&E, 400X. (C) Ovary from mouse treated with DG for 21 days (DG-21), showing interstitial cells (IC); *, follicle; H&E, 100X. (D) High magnification of Figure 4.3C showing interstitial cells; *, follicle; H&E, 400X. (E) Ovary from mouse treated mouse treated with DG for 21 days (DG-21), showing cystic changes in the ovary with folding of the wall that consists of follicles (*) and interstitial cells (IC); H&E, 100X. (F) High magnification of Figure 4.3E, showing that the wall of the cystic structure is lined with simple cuboidal epithelium on both sides (arrows); IC, interstitial cells; H&E, 400X.

Table 4.4. Histological abnormalities of ovaries, oviducts, uteri and cervix of F1 female mice
†

	CON-10	CON-21	DG-10	DG-21
Ovary				
Follicles (number/ovary)	8.35±0.79	8.09±1.36	7.06±0.92	9.07±0.55
Corpus Luteum (CL) (number/ovary)	8.50±0.85[a]	10.47±1.28[a]	1.15±0.46[b]	3.31±1.75[b]
No CL (%)	0/8 (0)	0/6 (0)	3/6 (50)	3/6 (50)
Cystic change (%)	0/6 (0)	0/6 (0)	2/6 (33)	1/6 (17)
Oviduct				
Abnormal (%)	0/8 (0)	0/6 (0)	0/6 (0)	0/6 (0)
Uterus				
Hyperplasia (%)	0/8 (0)[a]	0/6 (0)[a]	6/7 (86)[b]	5/6 (83)[b]
Polyps (%)	0/8 (0)	0/6 (0)	2/7 (29)	2/6 (33)

† Data are presented as mean ± standard error of mean or the fraction 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.

or columnar epithelium, and some follicles and interstitial cells appeared between two layers of the wall (Figure 4.3E, 4.3F). This change may be the result of abnormal morphogenesis in the early development of the ovary. The oviducts did not show evident change among groups.

4.4.6.2 Uterus

No evident histological changes were observed in the control groups (Figure 4.4A and 4.4B). However, there was a higher incidence of endometrial hyperplasia among treated groups (Table 4.4). The endometrial epithelium of hyperplasia consisted of simple, pseudostratified or stratified columnar cells (Figure 4.4C to 4.4F). The endometrial polyps (Figure 4.4C), abnormal projections of endometrium into lumen, were found in more ($p \leq 0.05$) mice from treatment group compared to controls (2 out of 7 treated mice had epithelium lined with irregular pseuodostratified or stratified cuboidal and columnar cells) (Figure 4.4D and 4.4F). The gland complex, consisting of multiple 'daughter glands', or glandular crowding with multiple glands interconnecting, was present in treated mice ($p \leq 0.05$) (Figure 4.4E and 4.4F) and not observed in controls. Moreover, hyperplasia was observed only in the treated mice ($P \leq 0.05$) (Figure 4E and 4F) and not in controls (Table 14). Adenomyosis, the endometrial glands within myometrium, were also present in treated groups but not control groups (Table 14, Figure 4D), though not severe. Due to enlargement of endometrium, the wall of myometrium was much thinner in the treated mice compared to the control mice (Figure 4C and 4E).

4.4.6.3 Cervix

The microglandular hyperplasia in the mucosa were observed in treated mice at an incidence of 43% (3/7) and 33% (2/6) for DG-10 and DG 21 groups, respectively, but not in the control groups (Table 4.4 Figure 4.4G and 4.4H).

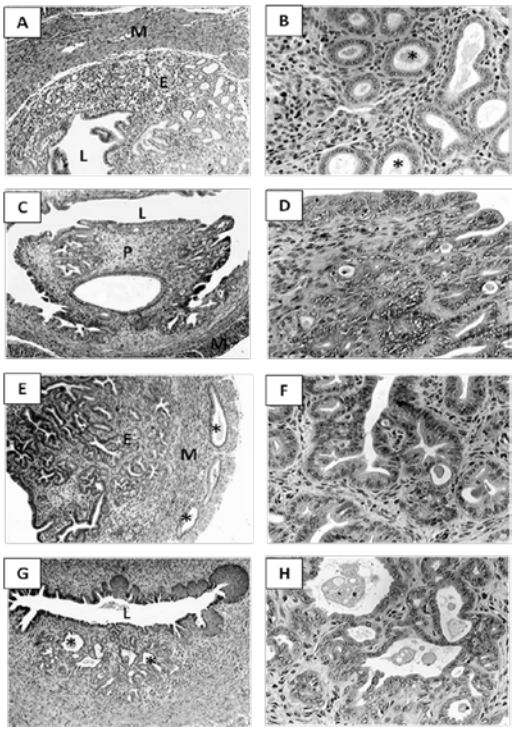


Figure 4.4 Histological structures of the uterus and cervix of F1 mice

(A) Representative uterus from mouse of control group (CON-10); L, lumen; E, endometrium; M, myometrium; H&E, 100X. (B) High magnification of Figure 4.4A showing that the endometrial glands (*) with regular shape are lined with simple cuboidal epithelium; H&E, 400X. (C) Uterus of mouse treated with DG for 21 days (DG-21), showing uterine polyp (P) in lumen (L); M, myometrium; H&E, 100X. (D) High magnification of Figure 4.4C, showing dysplastic glandular epithelium; H&E, 400X. (E) Uterus of mouse treated with DG for 10 days (DG10) showing endometrial hyperplasia (E) with glandular crowding, glandular complex and cytological atypia. Note glands (*) seen in the myometrium (M); H&E, 100X. (F) High magnification of Figure 4.4E showing conglomerate glands; H&E, 400X. (G) Cervix of mouse

treated with DG for 10 days (DG-10) showing microglandular hyperplasia in the mucosa; *, glands; L, lumem; H&E, 100X. (H) High magnification of Figure 4.4G; H&E, 400X.

4.5 Discussion

Soy isoflavone exposure during the first 10 or 21 days of life affected body weight, fertility, reproductive organ development and sexual maturation in female CD-1 mice at young adulthood in the F1. F1 fertility was markedly reduced and was likely due to the lower number of days these mice spent in the estrus phase and the overall lower number of estrus cycles experienced. Moreover, histological abnormalities in reproductive organs likely compromised fertility. In contrast, fertility among F2 mice was not compromised. F1 females exposed to soy isoflavones for the entire suckling period experienced earlier onset of puberty and were heavier at adulthood.

Higher body weight in adulthood due to soy isoflavone exposure in early life has been previously documented[5, 123] and our present data showing higher body weights in F1 mice from 6 to 16 weeks of age that were exposed to isoflavones from PND 1 through 10 or 21 supports such findings. ER knockout mice gain weight at sexual maturity[124] and it has been suggested that early exposure to soy isoflavones may be acting antagonistically on ER receptors in adipose tissue to cause the weight gain. The F2 group, whose mothers were treated with soy isoflavones during the first 10 or 21 days of life, had significantly higher body weight at weeks 6-16, indicating that a transgenerational effect on body weight occurred. Although mice exposed to isoflavones experienced a higher adult body weight, the weight was considered to be within the normal body weight range reported by the commercial supplier.[125] Nevertheless, body weight at adulthood, even among F2 females, was influenced by exposure to isoflavones and postnatal growth is critical in determining body weight in later life[126]. With childhood and adult obesity

currently a major public health concern in Canada[126]and the U.S [127], the mechanisms of weight gain in this study as well as the implications to body weight in human infants require further investigation.

Fertility of F1 but not F2 exposed to soy isoflavones was severely compromised compared to the control groups. Amongst F1 control mice, fertility was nearly 100% compared to the treated F1 mice that had success rates of approximately 55-60%. Importantly, copulation plugs were detected 100% of the time for the treated mice and suggest that normal mating behaviors existed. To confirm the reduced fertility observed in this group, a second breeding trial, with known fertile males, was conducted for F1 mice that did not deliver pups. During the second breeding trial, mice from the 10 day treatment group continued to experience a low fertility rate (37%) and mice treated for the longer time period experienced no pregnancies compared to 100% fertility for the control mice. Thus, the results of the second breeding trial confirmed the pattern of low fertility among both groups treated with isoflavones.

The lower fertility may be related to the disruptions in estrus cycling that were observed. Treated mice demonstrated both a lower number of total estrous cycles and a lower number of days spent in the estrous phase compared to controls. Notably, mice exposed to either subcutaneous GEN or altered genistin both experienced disruptions in estrous cyclicity[43, 52] demonstrating that the route of exposure is not as significant as simply the presence of soy isoflavones during early development. Prolonged estrous cycle and permanent estrus have been observed after administration of much higher doses of 40-100 mg GEN/day.[42, 52, 54] However, at lower exposures of GEN throughout the life cycle, shortened estrus and prolonged dioestrus has been observed.[128] This indicates that early exposure to soy isoflavones may cause different changes to the estrus cycle depending on the dose that is used. An explanation for the changes in estrous cyclicity observed in the present study is that soy isoflavones are disrupting the

hypothalamic pituitary-gonadal axis. GEN alters pituitary responsiveness to gonadotropin releasing hormone (GnRH) and this can interfere with normal estrous cyclicity.[129]

Structural abnormalities of ovaries from mice exposed to soy isoflavones during early life have been documented.[50] In the present study, fewer corpus luteum in the ovaries of F1 treated females were present compared to the controls. Because the presence of a corpus luteum indicates that ovulation has taken place, the finding suggests that ovulation was disrupted.[130] GEN treated mice have been found to be anovulatory.[8] It has been demonstrated that ovulation can be stimulated after treatment with pregnant mare serum gonadotropin (PMSG) followed by human chorionic gonadotropin (hCG). It was also found that the numbers of eggs ovulated from the control and GEN treated mice did not differ and egg morphology was normal. Furthermore, eggs from GEN treated mice may be fertilized normally suggesting that egg quality may not be a factor concerning infertility. Although structural changes to the ovaries were observed, previous findings suggest that the reduced fertility was likely due to hormonal disturbance rather than egg development, though it may not be occurring normally as a result of changes in hormonal status.

Although MOFs were not observed in the present study they have been reported after GEN exposure.[7,50] Neonatal mice treated with 0.5, 5 and 50 mg GEN/kg/d had higher numbers of MOFs in the ovary at 2 months of age.[7] Importantly, MOFs were also observed after neonatal mice received oral doses of GIN (12.5, 25 and 37.5 mg/kg) at PND 19.[41] Thus, although the routes of administration differed, both methods yielded similar results. The fact that MOFs were not observed in the F1 females at 4 months of age may be related to timing at which the tissue was collected. Because MOFs are formed during very early life when the oocyte clusters do not separate, it is possible that later in life these structures are able to differentiate and disappear.

Abnormal uterine structure, including endometrial hyperplasia, may also explain why F1 mice failed to become pregnant. The altered structure of the uterus could create a hostile environment for a developing embryo and prevent normal implantation. Previous findings at higher doses of GEN (50 mg GEN/kg body weight) have shown that exposure to GEN in early life reduced the number of implantation sites and increased the incidence of embryo resorption[106]. One explanation for this is that neonatal exposure to estrogen alters uterine responsiveness to steroid hormones, preventing normal implantation to occur.

There were no significant differences in fertility among F2 groups, indicating that this adverse effect was not transferred to the subsequent generation. In both F1 and F2, there were no significant differences regarding litter characteristics. Gestation length, litter weight, number of live pups and sex ratio of the pups were normal. Differences in sexual development and maturation were also observed in both F1 and F2 females. F1 mice experienced earlier timing of vaginal opening, used to determine the onset of puberty, and this appeared to be dependent on the duration of exposure as only the longer exposure resulted in earlier onset of puberty. This finding is consistent with previous studies that demonstrated earlier age at vaginal opening at higher doses of 40 mg GEN/kg body weight that were administered during suckling PND 1-21.[42] The AGD of F1 females were significantly longer in both treatment groups at adulthood compared to control groups. Among F2 females, both groups of treated mice had significantly longer AGD compared to the 10d control group at the end of suckling and during adulthood. While the earlier age at vaginal opening and longer AGD in F2 suggests a transgenerational effect is taking place, the biological significance is unclear as fertility of F2 was not compromised. AGD is used to determine the sex of the pup and can be a measure for endocrine disruption[131] and longer AGD and earlier onset of puberty been observed in female and male mice exposed to estrogen.[131]

In conclusion, early soy isoflavone exposure to F1 at doses that result in comparable serum isoflavone levels in human infants[16] modulates weight gain and has some adverse effects on reproductive outcomes, including reduced fertility, reproductive organ structure and estrous cycling, in the developing CD-1 mouse model. While modulation of body weight gain was transferred to F2, fertility was normal in this generation. The mechanism by which body weight gain was programmed requires further investigation. As the first study to report findings on body weight and reproductive health in F2 mice whose mothers were exposed to soy isoflavones during early life, as well as the first study to investigate DG combination, the present study contributes to the body of literature using animal models to understand potential effects in human infants. As acknowledged in the Introduction, extrapolation of findings from animal models to the human scenario are complex but can provide guidance for more fully understanding the implications for infants consuming SBIF.

Chapter Five

STUDY 2: TRANSGENERATIONAL PROGRAMMING OF BONE DEVELOPMENT IN FEMALE CD-1 MICE BY EARLY EXPOSURE TO SOY ISOFLAVONES

5.0 STUDY 2: TRANSGENERATIONAL PROGRAMMING OF BONE DEVELOPMENT IN FEMALE CD-1 MICE BY EARLY EXPOSURE TO SOY ISOFLAVONES

5.1 Abstract

We previously reported that neonatal exposure of female mice to soy isoflavones, at levels similar to those in soy-based infant formula, can favorably program bone development such that greater bone mineral, improved bone structure and greater bone strength are observed at young adulthood and these effects are sustained after ovariectomy. The primary objective of the present study was to determine if these benefits to bone health are transferred to the second generation (F2). A secondary objective was to determine if lengthening the duration of isoflavone exposure to F1 resulted in greater benefits to bone development in F2. Female CD-1 mice (F1, n = 8-13 pups/group) were randomized to subcutaneous injections of soy isoflavones, (5 mg GEN +2 mg DAI ·kg body weight^{-1}·d^{-1}) or corn oil (CON) from postnatal day (PND) 1-10 or 1-21, weaned at PND 21 and fed control diet (AIN93G) until 2 months of age. These same females (n = 8-13 pups/group) were then bred to control males to obtain F2 females. F2 females (n=14-22/group) were fed control diet until necropsy at 4 months of age, representing young adulthood, and measured outcomes included bone mineral content (BMC), bone mineral density (BMD) and biomechanical bone strength at the femur and lumbar vertebrae. F2 females whose mothers were exposed to DAI+GEN for either 10 d or 21 d had greater ($p<0.05$) BMC and BMD at the lumbar vertebrae and femur. Femur neck BMD and peak load was also greater ($p<0.05$) amongst F2 females whose mothers were treated with isoflavones compared to controls. In conclusion, favourable benefits to BMC, BMD and bone strength in F1 are transferred to F2 females.

5.2 Introduction

Soy isoflavones have received much attention for their potential modulatory effects to bone health.[2] Consumed frequently as part of soy-based foods such as tempeh, tofu or miso, soy isoflavones are commonly found in the diet of many Asian populations.[132-133] Epidemiological studies have demonstrated that there are a lower incidence of hip fractures in Asian countries and it has been postulated that this may be due to the wide consumption of soy foods in the diet.[134-135] Human infants fed soy-based infant formula are a unique population that is exposed to high levels of soy isoflavones. Soy-based infant formula (SBIF) contains approximately 32 to 47 mg of isoflavones (i.e. 67.1% GEN and 28.7% DAI) per liter of formula[21]. This translates to approximately 8 mg soy isoflavones·kg body weight^{-1}·d^{-1} which is 6 to 11 fold greater than levels consumed in a typical Asian diet.[21, 28] Although neonatal exposure to soy isoflavones using rodent models have demonstrated long-term protective effects on bone [5], there is a paucity of data from human studies. While several studies have measured bone outcomes in human infants who have consumed SBIF, none of these studies have followed infants into adulthood.

Studies have demonstrated that piglets fed SBIF for 3 or 5 weeks have higher levels of serum markers of bone formation (i.e. alkaline phosphatase and osteocalcin) and decreased serum levels of markers of bone resorption (RANKL). Increased osteoblastogenesis in bone marrow cell cultures and improvements in bone structure were also observed.[45] Previous findings from our lab using the CD-1 mouse model demonstrated that early exposure to soy isoflavones, in which serum isoflavone levels are similar to human infants fed SBIF, results in greater bone mineral density (BMD), improved bone structure and stronger bones in female mice at young adulthood[5]. Moreover, these benefits to bone health protected against the decline of bone tissue associated with the cessation of endogenous estrogen production after ovariectomy.[83] We have also previously observed that adverse effects on reproductive health

using this same mouse model were transferred to F2. Moreover, higher weight gain in F1 was also observed in F2. Whether or not the beneficial effects to bone outcomes in F1 female mice are transferred to subsequent generations is unknown but other environmental estrogens have demonstrated transgenerational effects.[136, 137, 138] Pregnant rats treated with the estrogenic pesticide methoxychlor or the anti-androgenic fungicide vinclozolin have subfertile offspring and the male offspring pass this trait through the male germ line for at least four generations.[138]

The primary objective of the present study was to determine if these benefits to bone health are transferred to the second generation (F2). A secondary objective was to determine if lengthening the duration of isoflavone exposure to F1 resulted in greater benefits to bone development in F2. Specifically, BMD, BMC and biomechanical strength properties at the femur and lumbar vertebrae of female F2 mice (F2) whose mothers were exposed to soy isoflavones in early life were measured.

5.3 Materials and Methods

5.3.1 Animals and Treatment

All experimental procedures followed the policies set out by the Canadian Council on Animal Care[119] and were approved by the Animal Ethics Committee at the University of Toronto. Six week-old CD-1 mice (F0) (n=5 males, n=13 females) were obtained from Charles River Laboratories Canada (St. Constant, QC,) and fed a control diet (AIN93G, Dyets Inc. Bethlehem, PA) that contained no known estrogenic compounds.[120] To obtain F1, mice (F0) were bred harem-style after a two week adaptation period to standard environmental conditions (12-h-light, 12-h-dark cycle; 23°C). Pregnant females were housed individually and continued to be fed fresh control diet and water, ad libitum, every 2-3 days. All litters had 8-12 pups (F1) and pups were cross-fostered. F1 pups were subsequently randomized to 1 of 4 groups: 10 days

of corn oil (CON-10), 21 days of corn oil (CON-21), 10 days of DAI+GEN (DG-10; 2 mg DAI·kg body weight^{-1}·d^{-1} + 5 mg GEN·kg body weight^{-1}·d^{-1}) or 21 days of DAI+GEN (DG-21; 2 mg DAI·kg body weight^{-1}·d^{-1} +5 mg GEN·kg body weight^{-1}·d^{-1}). CON pups received corn oil since it is used as a vehicle for DAI+GEN that was solubilized in 1 mL dimethyl sulfoxide and suspended in corn oil.[5] Treatments were administered each morning from PND 1 until PND 10 or PND 21 via subcutaneous injection. A total volume of 20 μL·pup^{-1}·d^{-1} was administered. The solution was designed such that the ratio and dose of DAI and GEN would resemble the quantity and ratio of soy isoflavones found in soy-based infant formula[3] and result in serum GEN and DAI levels that are similar to human infants fed SBIF [5] Purified GEN (Catalogue #G6649) and DAI (Catalogue #D7802) were purchased from Sigma-Aldrich (Mississauga, ON). Mice were weaned at PND 21. Because our previous findings have shown that female mice have a greater response to soy isoflavone exposure, only female mice were studied after PND 21. Body weight of F1 and F2 was measured once weekly until they reached 8 weeks of age. At 8 weeks of age, F1 females were bred with control (untreated) males to obtain F2 (n=13-22/group). At 4 months of age F2 mice were killed using carbon dioxide followed by cervical dislocation. Femurs, lumbar vertebrae (LV1-3) and sex organs were collected. Femurs and LV 1-3 were cleaned of soft tissue and stored at -80°C until analyses were performed.

5.3.2 BMC, BMD of Lumbar Vertebrae (LV1-LV3) and Femurs

BMC and BMD of the left femurs and LV1-LV3 were measured using dual energy x-ray absorptiometry (DEXA) (pSabre, Orthometrix, White Plains, NY), in conjunction with a software program (Host Software Version: 3.9.4; Scanner Software Version: 1.2.0). Individual femurs or LV1-LV3 segments were placed in a fixed position in the x-ray field and scanned in the air. The DEXA parameters used included a speed of 2 mm/min and resolution of 0.01 mm x

0.01 mm. The CV for femur and LV1–LV3 BMC were 1.4 and 4.0%, respectively. The CV for femur and LV1–LV3 BMD were 1.8 and 4.4%, respectively.[83]

5.3.3 Biomechanical Strength Testing of Lumbar Vertebra 2 (LV2) and Femurs

LV2 and the right femur biomechanical strength were determined using three-point bending and compression testing. Because the proportion of cortical and trabecular bone varies depending on the femur region, the femur midpoint which contains mostly cortical bone and the femur neck, which contains mostly trabecular bone, were selected for testing. A material testing system and specialized software (Model 4442, Instron Corp., Canton, MA; Series IX Automated Materials Tester, Version 8.15.00) was employed. The femur neck (hip) and lumbar spine are common sites of fracture in humans so measuring the strength of these regions is relevant to the human situation.[83]

5.3.4 Compression Testing of Lumbar Vertebra 2 (LV2)

LV2 were hydrated in 0.9% saline solution for 2 hrs. The compression test was conducted by placing a single LV2 flat on a stainless steel plate and applying a vertical force at a rate of 2 mm/min. Peak load was determined as the first peak of the load-deformation curve[83].

5.3.5 Femur Three-Point Bending

Three-point bending was performed at the femur mid-point to determine the peak load at a skeletal site rich in cortical bone of the femur. Right femurs were removed from the -80°C freezer and hydrated for 2 hours in 0.9% saline solution. Three-point bending was conducted by placing the femur midpoint directly under the crosshead with either end of the femur on two supports of the bending jig set 6 mm apart. The crosshead, which was rounded to reduce shear force, was lowered at a constant speed of 2 mm/min until fracture occurred.[83]

5.3.6 Femur Neck Fracture Test

The femur neck compression test was conducted by placing the right femur vertically in a customized jig such that the force is applied by the crosshead until fracture occurs. The force is applied at a constant speed of 2 mm/min on the proximal femur head.[83]

5.3.7 Statistical Analyses

Statistical analyses were performed using SigmaStat (Version 3.5). Results are expressed as mean ± SEM. Two-way ANOVA was performed with duration of treatment and treatment as factors. Student-Newman Keul's test was used for comparison of multiple means when statistical differences were observed. Statistical significance was determined as $p \leq 0.05$.

5.4 Results

5.4.1 Body Weight at PND 21 and 4 Months of Age

At PND 21, body weight was greater ($p<0.05$) in the DG 21 group compared to all other groups (data not shown). At 4 months of age the DG 21 group had a higher ($p<0.05$) body weight than all other groups (data not shown).

5.4.2 BMC and BMD of Intact Lumbar Vertebrae (LV1-LV3) and Biomechanical Strength of LV2

There was an overall effect of treatment ($p<0.05$) and duration on BMC and a treatment effect ($p<0.05$) on BMD and peak load of LV2 (Table 15). DG 10 and DG 21 groups had a higher ($p<0.05$) peak load of LV2 compared to either CON group (Table 5.0).

Table 5.0 BMC and BMD of LV 1-3 and peak load of LV2.

	Treatment	Mean±SEM	Duration of Treatment	Treatment	Time x Duration
LV 1-3					
BMC (mg)	CON 10	24.5±1.25			
	CON 21	28.1±1.21			
	DG 10	28.9±1.21			
	DG 21	30.4±1.33	0.042	0.010	NS
BMD (mg/cm^2)	CON 10	$65.5±2.25^c$			
	CON 21	$72.3±2.25^b$			
	DG 10	$73.1±2.25^a$			
	DG 21	$73.9±2.25^a$	NS	0.05	NS
LV2					
Peak Load (N)	CON 10	$53.1±5.00^a$			
	CON 21	$60.5±5.00^a$			
	DG 10	$71.4±5.00^b$			
	DG 21	$71.2±6.00^b$	NS	0.008	NS

Values are expressed as mean ± SEM. Different superscripts denote a significant difference among treatments
n=13-22

5.4.3 Whole Femur BMC, BMD and Peak Load at Femur Midpoint

A treatment effect was observed for whole femur BMC, BMD and peak load at the femur midpoint (Table 5.1). DG 10 and DG 21 groups had greater whole femur BMC ($p<0.001$) and BMD ($p=0.018$) compared to controls. Higher peak load ($p=0.009$) at the femur midpoint was observed for both DG 10 and DG 21 groups compared to controls.

5.4.4 Femur Neck BMC, BMD and Peak Load

DG 10 and DG 21 groups had greater ($p=0.019$) femur neck BMD compared to controls (Table 5.2). Additionally, the femur neck of DG 10 and DG 21 groups was more resistant ($p=0.022$) to compression fracture (Table 5.2).

5.5 Discussion

This study is the first to show that exposure in F1 female mice to soy isoflavones, for the first 10 or 21 days of life increased BMD and strength in F2 at all skeletal sites measured. There were no significant differences in bone outcomes between the 10 and 21 day exposure. That the benefits to bone development previously observed in F1 are also present in F2 female mice demonstrates a transgenerational effect.

Table 5.1. Whole femur BMC and BMD and peak load of femur midpoint

	Treatment	Mean±SEM	Duration of Treatment	Treatment	Time x Duration
Whole Femur					
BMC (mg)	**CON 10**	32.0±0.10[a]			
	CON 21	34.4±0.10[a]			
	DG 10	37.1±0.10[b]			
	DG 21	36.4±1.02[b]	NS	<0.01	NS
BMD (mg/cm^2)	**CON 10**	77.8±1.74[a]			
	CON 21	82.8±1.84[a]			
	DG 10	84.4±1.74[b]			
	DG 21	84.9±1.84[b]	NS	0.018	NS
Femur Midpoint					
Peak Load (N)	**CON 10**	30.7±1.44			
	CON 21	35.9±1.37			
	DG 10	36.1±1.30			
	DG 21	38.2±1.53	0.012	0.009	NS

Values are expressed as mean ± SEM. Different superscripts denote a significant difference among treatment n=13-22/group

Table 5.2 BMC and BMD of femur neck and peak load of femur neck

	Treatment	Mean±SEM	Duration of Treatment	Treatment	Time x Duration
Femur Neck					
BMC (mg)	**CON 10**	87.7±2.47			
	CON 21	100.0±2.47			
	DG 10	102.0±2.40			
	DG 21	157.0±2.53	NS	NS	NS
BMD (mg/cm^2)	**CON 10**	83.1±2.40[a]			
	CON 21	89.5±2.40[a]			
	DG 10	92.3±2.33[b]			
	DG 21	91.8±2.46[b]	NS	0.019	NS
Peak Load (N)	**CON 10**	16.8±1.12[a]			
	CON 21	16.2±1.09[a]			
	DG 10	20.5±1.16[b]			
	DG 21	18.0±1.3[b]	NS	0.022	NS

Values are expressed as mean ± SEM. Different superscripts denote a significant difference among treatments n=13-22/group

Because DG21 was heavier than controls at weaning and adulthood, there was the potential for differences in BMC to be present because of differences in body size. The fact that

BMD, which accounts for differences in bone size, also differed among DG10 and DG21 compared to controls indicates that bone size did not alter bone mineral. Although animal studies have demonstrated increased BMD, BMC and strength after early life exposure to soy isoflavones [45,5] human studies have not followed infants fed SBIF beyond 1 year of age. Lower BMC has been reported at 3 and 4 months in infants fed SBIF when compared to breast fed infants.[81] At 6 months of age however these differences disappeared.[81] It is quite possible that protective effects of early exposure to soy isoflavones may not be evident until later childhood or adult life.

In addition to our previous findings which demonstrated transgenerational body weight gain, acceleration of sexual maturation and development, we now report transgenerational benefits to bone health. This finding is in agreement with other environmental estrogens having known transgenerational effects. While it is unknown how soy isoflavones are eliciting transgenerational effects in bone, mechanisms may include epigenetic changes to DNA. Epigenetics is defined as the study of heritable changes in gene expression that occur without a change in the DNA sequence.[139] Epigenetic changes are inherited when there is alteration of the DNA molecule in the sperm or egg cell that then contributes to the embryo.[140] Dietary changes such as calorie or protein restriction[141] or folate and selenium supplementation[142] of different nutrients or compounds has shown to alter DNA methylation patterns. Estrogen receptor (ER)-α, has at least eight promoters that are tightly regulated by DNA methylation [113] and have been located in bone tissue.[114] Specifically, methylation of CpG islands in the ER-α gene promoter F, a common promoter in osteoblastic or bone forming cells, causes a decrease in ER-α gene expression which can lower effects of estrogen on bone turnover. DNA methylation may also alter the estrogen receptor (ER-α: ER-β) ratio thereby modulating bone metabolism.[113] Osteoblasts contain both ER-α and ER-β and ER-α transactivation modulates the expression of

various cytokines, such as decoy receptor osteoprotegrin (OPG), involved in bone formation.[143] Notably, both 17-β estradiol and GEN have been associated with enhanced OPG production through ER-α and suppression of RANKL gene expression which is associated with an inhibition of osteoclastogenesis and a decrease in bone resorption.[144,145,146,100]

Another regulator of osteoblasts is adrenocorticotropin (ACTH) which is processed and secreted from the pituitary to stimulate cortisol production in the adrenal gland.[147] ACTH binds to a melanocortin receptor (MC2R) expressed in osteoblastic cells. MC2R expression is strongest at sites of active bone deposition thus higher ACTH levels may indicate osteoblastic activity or differentiation.[147] GEN has been shown to have an inhibitory effect on adrenocorticotropin-stimulated cortisol production in cultured fetal and postnatal adrenal cortical cells [148]. In vivo, GEN has been shown to increase the adrenal weight of weanling rats and higher adrenal weight is most often related to the higher serum levels of ACTH.[149] Although the connection between ACTH regulation and soy isoflavones is unknown it is an area for investigation.

In conclusion, the findings from our study suggest that early exposure to soy isoflavones that result in serum levels similar to those of human infants fed SBIF, improves bone health and increases body weight using a CD-1 mouse model.[16] Notably, both 10 d and 21 d exposure to soy isoflavones during the neonatal period have significant and similar biological effects on bone health. Further investigation is required to elucidate the mechanisms by which soy isoflavones are mediating transgenerational effects on body weight and bone outcomes.

Chapter Six

GENERAL DISCUSSION AND CONCLUSIONS

6.0 GENERAL DISCUSSION AND CONCLUSIONS

This research has shown that early exposure to soy isoflavones has distinct and profound effects on both reproductive health and bone development using the well-established CD-1 mouse model. That these effects are transferred to the subsequent generation emphasizes the ability of soy isoflavones to program distinct aspects of development. Altered sexual maturation, estrous cycling, reproductive organ development and reduced fertility were observed in F1 and although there were no changes to fertility in F2, abnormal sexual maturation was also observed (Figure 6.0). Higher adult body weight was recorded in F1 exposed to isoflavones as well as their F2 pups. Assessment of bone health in F2 revealed that there was an increase in BMD, BMC and biomechanical strength at femur and lumbar vertebrae skeletal sites (Figure 6.0).

6.1 Overall Impact of Soy Isoflavones on Health

This report sought to understand the adverse effects on reproductive health and the beneficial effects to bone health as a result of early exposure to soy isoflavones, across two generations. Studies 1 and 2 have demonstrated adverse and beneficial aspects of early life consumption of soy isoflavones. Although beneficial effects indicate the potential for early life exposure to soy isoflavones to protect against deterioration of bone tissue in later life, the changes to reproductive health in both generations are disconcerting and represent significant consequences. It is important to determine whether or not soy isoflavone exposure should be recommended or restricted in early life. Thus, it is important to consider the effects of soy isoflavones in different organ systems.

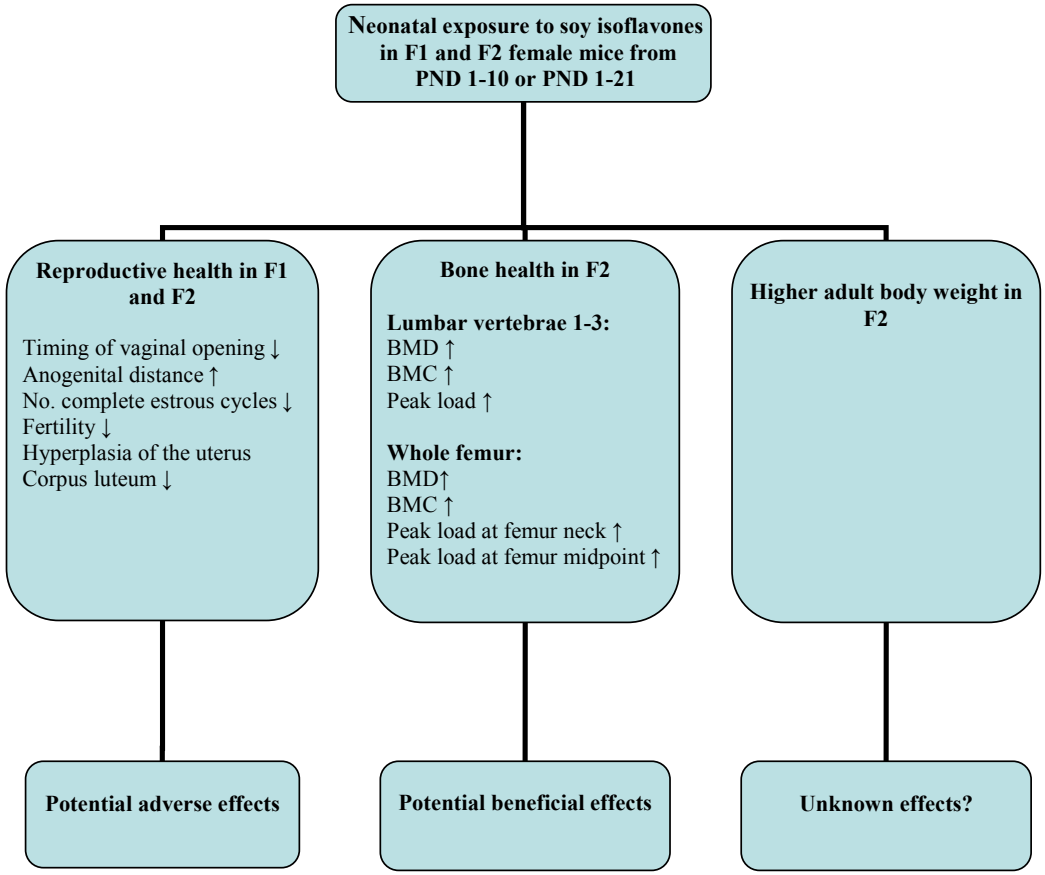


Figure 6.0. Summary of study findings classified as adverse, beneficial or unknown effects

Study 1 was designed to elucidate adverse effects on reproductive health in female CD-1 mice exposed to soy isoflavones at a dose comparable to infants fed SBIF and discern any transgenerational effects on reproductive health. A novel finding was that adverse effects were determined at low doses of the combination of soy isoflavones unlike previous studies which have mainly used higher doses of the single soy isoflavone GEN. Early onset of puberty, reduced fertility and abnormalities of the uterus and ovaries has been previously reported after 50 mg GEN/kg body weight but not the dose that was used in Study 1, 7 mg GEN+DAI which included only 5 mg GEN/kg body weight. Because such low doses were used, adverse effects on reproductive health were not anticipated.

Changes in sexual maturation and markers of endocrine function were observed in F1 treatment groups. Earlier onset of puberty, as determined by timing of vaginal opening was demonstrated in female mice in both F1 and F2. This contrasts with one study which demonstrated delayed vaginal opening after F1 exposure of 6.25 mg/kg oral genistin[41] and other studies that demonstrated no changes to timing of vaginal opening after exposure to much higher doses including subcutaneous 500 mg GEN/kg body weight [55] or 100 mg GEN/kg body weight.[52] Still, 40 mg GEN/kg body weight has demonstrated advanced day of vaginal opening[42] consistent with our findings. Administration of other estrogenic compounds such as DES, bisiphenol-A, zearalenone or zeranol have also shown early onset of puberty when exposure takes place in early life.[150] Thus, our findings suggest that specifically lower doses of soy isoflavones may accelerate pubertal onset transgenerationally.

Altered estrous cycling and fewer numbers of complete estrous cycles were observed in F1. These changes may explain the reduced fertility associated with this group. Altered estrous cycling has been previously reported after exposure to both oral and subcutaneous GEN[41]

although these studies demonstrated induced estrus rather than prolonged metestrus. Metestrus is characterized by the presence of leukocytes and may indicate that low grade inflammation in the vaginal canal may be occurring. Multigenerational exposure to GEN has been shown to result in prolonged periods of dioestrus, also characterized by the presence of leukocytes, when exposure took place during neonatal life and adulthood.

Fertility was significantly reduced among female mice that were exposed to DAI and GEN for either 10d or 21d with a success rate of approximately 50% compared to the controls. Importantly, fertility was normal in F2, demonstrating that, adverse effects are not transgenerational. Studies examining fertility after GEN exposure have shown different effects depending on the compound and dose. Low-dose oral genistin exposure of 6.25 mg genistin/kg body weight[41] and subcutaneous injection of 50 mg GEN/kg body weight has demonstrated reduced fertility in female CD-1 mice[8] but much higher doses of GEN, such as 500 mg GEN/kg body weight has shown no changes.[55] It is therefore possible that lower doses of soy isoflavones, such as those used in our study, may be associated with more harmful consequences to fertility. Although the second generation did not experience changes to fertility, consistent with the multigenerational study using GEN-dosed feed throughout the life cycle, a trend toward reduced litter sizes was reported.[128]

Although the mechanism by which soy isoflavones are impairing fertility is unknown, histological assessment of the ovaries and uterus indicate that structural changes in these organs are occurring. Lower numbers of corpus luteum and endometrial hyperplasia were observed. Similar structural changes to the ovaries and uterus after exposure to GEN, including lower numbers of corpus lutea and MOFs have been reported.[7, 50] Studies using DAI have not demonstrated such changes to the ovary but have shown increases in epithelial cell height[49], which is consistent with the changes we observed in the uterus. We are the first to examine

structural development of the ovaries and uterus using a combination of soy isoflavones. Future studies should include measuring serum hormones, estrogen, testosterone, lutenizing hormone and follicle stimulating hormone to determine if hormonal disruption may explain the reduced fertility in this group. The hyperplasia in the endometrium requires further investigation and measurements should include: ER status, cell signaling, markers of cell proliferation and apoptosis. Additionally, mice exposed to isoflavones during neonatal life should be followed beyond 4 months of age to discern any potentially carcinogenic effects.

Higher body weight gain was observed in both the first generation exposed to soy isoflavones and in the second generation compared to the controls. Importantly, these findings indicate that soy isoflavones may elicit transgenerational effects on body weight. Findings from our lab have previously demonstrated higher body weight at adulthood as a result of exposure to soy isoflavones but this is the first study to report transgenerational effects. Although the adult body weight in both F1 and F2 was higher compared to the controls, the body weights were within the normal body weight range for female CD-1 mice. Thus, the higher body weight observed may not necessarily indicate adverse effects as a result of soy isoflavone exposure. Because we cannot discern if the difference in body weight is due to higher lean mass, higher fat mass or both, further investigation is required. As well, studies related to how soy isoflavones may influence appetite and metabolism to determine potential mechanisms for these changes are needed.

Study 2 was designed to determine whether or not the benefits to bone due to early exposure to soy isoflavones, previously demonstrated, are transferred to the subsequent generation. Two sites were selected, the lumbar vertebrae and femur (neck and midpoint), since they contain varying amounts of trabecular bone which is more metabolically active than cortical bone. Consistent with findings in F1, we observed higher BMC and BMD at the femur

and lumbar vertebrae in F2. Biomechanical strength testing confirmed the higher BMC and BMD resulted in bone that is resistant to fracture at 4 months of age. It is known that estrogen binding to ER-α regulates osteoblastogenesis and osteoblast number and apoptosis through DNA-binding.[151] Because we know that soy isoflavones have the capacity to bind to ER-α and can regulate DNA methylation it is not surprising that these transgenerational effects are observed.

Studies in human infants have not examined bone health beyond one year of life. Ongoing studies in human infants are examining effects of SBIF bone health at later life stages. This is the first study to report bone outcomes to the second generation after exposure to soy isoflavones.

6.2 Future Directions

These studies were conducted in an animal model and it is unclear how these findings may relate to humans. As stated in table 2.1 there are various limitations to this study including species-related differences between rodents and humans, administering purified isoflavones, the subcutaneous route of isoflavone administration and frequency of isoflavone administration. Human studies are necessary to truly determine how soy isoflavones influence human development. Animal studies, however, can provide useful mechanistic information that may not otherwise be gathered from human subjects. The future directions of both human and animal studies are discussed below. The biological effects of soy isoflavone exposure as a result of SBIF consumption are controversial and inconclusive. Only 1 retrospective study has reported effects of feeding SBIF on health outcomes at adulthood and few studies have examined infant reproductive or bone health after exposure to SBIF. While studies using a variety of animal models report positive and negative effects of soy isoflavone exposure during development, it is

unclear whether these data can be extrapolated to human infants. Animal models do however provide the biological plausibility needed in order to justify conducting a study in humans. Both studies in humans and using appropriate animal models are needed. Table 6.0 identifies aspects of bone and reproductive development that would be useful to measure in either humans or using animal models. Together, the findings from such studies will provide a more comprehensive understanding of the biological effects of isoflavones in SBIF on reproductive health.

Table 6.0. Future directions for human studies or using animal models

Outcomes to Measure in Human Studies	Outcomes to Measure Using Animal Models
Prospective Cohort • BMC, BMD • Bone biomarkers of resorption and formation • Incidence of fracture • Sexual maturity • Reproductive organ morphology, development and function • Serum hormone levels • Fertility • Testicular, prostate, ovarian, uterine cancer **Retrospective Cohort** • BMC, BMD • Bone biomarkers of resorption and formation • Incidence of fracture • Serum hormone levels • Fertility • Reproductive organ morphology and function • Testicular, prostate, ovarian, uterine cancer • Offspring characteristics: (birth weight, sex ratio)	**Mechanisms:** • Altered hormone receptor expression and/or activity • Changes in gene expression • Organ and bone histopathology • Bone biomarkers • Serum hormones at various life stages • Transgenerational effects

6.2.1 Future Directions for Human Studies

Both retrospective cohort and prospective studies are needed to determine how SBIF may be affecting human bone and reproductive health. Prospective studies that monitor bone development outcomes of infants who are currently consuming SBIF, at various life stages or abnormalities in reproductive organ development[35], are needed. Investigation to adult life is

crucial since the impact of consuming SBIF may not be evident until adulthood. Although one retrospective study has been previously conducted [32] there is a need to increase the number of health outcomes measured. Performing ultrasounds may be useful for identifying changes in bone or abnormalities in reproductive organs such as the occurrence of polycystic ovaries in women. Serum hormones in post-pubescent females should also be taken at specific time points in the menstrual cycle to obtain a quantifiable characterization of the menstrual cycle. Additionally, measuring BMC and BMD in adults who consumed SBIF as infants is necessary to determine potential beneficial effects. Fertility should be closely examined in this population as well since abnormalities in the reproductive system of both males and females do not necessarily compromise fertility. Because animal studies have shown a higher incidence of cancer in animals consuming high levels of soy isoflavones[7], cancer screening should be considered for adults who have consumed SBIF in infancy. As well, offspring of those who have consumed SBIF should be monitored for differences in growth and development. Many individuals in North America have consumed SBIF as infants, and so the potential to more extensively assess bone and reproductive health is possible.

6.2.2 Future Directions for Animal Studies

Because bone development and sexual maturation is similar across species [152], animal models can provide a practical design for studying development. Numerous endocrine-mediated events involved in this progression in the rat for example, are comparable to other mammalian species such as humans.[64] In both humans [153] and rodents [154], pubertal onset is associated with similar physiological changes such as the attainment of a body mass, chronic inflammatory states, thyroid disease, and growth hormone deficiency. The control of gonadotropin releasing hormone (GnRH), the release of gonadotropins from the pituitary, and the steroid positive and

negative feedback controls are fairly consistent across mammalian species.[155] Due to these species related similarities, the animal model may provide mechanistic support for investigations in humans. By determining how soy isoflavones modulate gene expression and hormone receptor activity, transgenerational effects may be more fully understood. There are various outcomes that cannot be measured in humans such as organ weight and histology of such organs. Other environmental estrogens, such as diethlystillbesterol are known to cause harmful transgenerational effects [51, 107] and there has been concern that soy isoflavones may act in a similar way. Animal models allow for controlled and time efficient transgenerational data to be collected.

A mouse model could be refined to more closely characterize the human scenario. Ideally, the animal would be exposed orally, in order to ensure that first pass metabolism in the gut is occurring. As well, exposure should take place more than once per day, if technically possible, in order to mimic the multiple feedings that an infant would receive. Exposure should only take place during early postnatal development starting at the first day of life and during suckling, although the duration during suckling requires further study. Transgenerational studies, in order to characterize the offspring, are easily performed by breeding the treated animals to known controls and should also be conducted. Characterizing the ideal animal model for studying the effects of isoflavones in SBIF on human infants is an ongoing area of investigation.

6.3 CONCLUSIONS

- Neonatal exposure to soy isoflavones during either half (PND 1-10) or the complete suckling period (PND1-21) adversely affected female reproductive health in F1 by accelerating puberty onset, altering sexual development and altering estrous cyclicity, reducing fertility and causing abnormal ovarian and uterine structural changes.F2 offspring whose mothers were exposed to isoflavones also experienced earlier puberty and sexual development but no difference in fertility. F1 and F2 mice had higher adult body weight.

- Early exposure to soy isoflavones elicits transgenerational benefits to bone health. Female mice (F2) whose mothers were exposed to soy isoflavones had higher BMC, BMD and peak load at the femur and lumbar vertebrae.

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Chapter Seven

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7.0 REFERENCES

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