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ORIGINAL ARTICLE

Isoflavone exposure throughout suckling results in improved adult bone health in mice

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

Introduction

'Nutritional programming' is a concept used to describe a permanent change in the structure or function of an organism caused by a food or food component during early development. Programming by early diet may provide a safe and practical approach for optimizing bone health throughout life. Although some aspects of programming by early diet can be investigated directly in humans, mechanistic studies often require appropriate use of animal models to closely mimic the human scenario. The CD-1 mouse model is commonly used to assess biological effects of exposure to isoflavones (ISO) during early life, including effects on bone development.[1–3] We previously reported in CD-1 mice that neonatal exposure to ISO, resulting in similar total serum ISO levels as human infants fed soy protein formula, favorably programs bone health in females at adulthood. The benefits include higher vertebral bone mineral density (BMD) and improved structure (greater trabecular thickness and connectivity) that translate to stronger lumbar vertebrae (LV) at 4 months of age, representing young adulthood.[2,3] Although the mechanism of these programming effects has not been elucidated, ISO are

selective estrogen receptor modulators that can bind to estrogen receptors to elicit estrogen-like responses in bones, ovaries, uteri, prostate, the central nervous system and the cardiovascular system but inhibit estrogen stimulation in the breast and endometrium.[20] As such, exposure to ISO during sensitive stages of development offers the possibility of permanent alterations in bone-specific gene expression[4] or rapid non-genomic action that modulates a diverse array of intracellular signal transduction cascades that affect processes associated with bone metabolism.[5] To date, published studies investigating early life exposure to ISO and bone development have used a 5-day dosing protocol, starting at postnatal day (PND) 1 and ending on PND 5. To more closely represent the duration of exposure (first year of life) that human infants are fed soy protein formula, the protocol was lengthened such that mice are exposed to ISO throughout suckling (the first 21 days of life). The objective of this study was to determine if exposure to ISO from birth throughout suckling enhances the previously observed positive effects of 5-day exposure to ISO on LV and, unlike the 5-day protocol, has favorable effects on femur outcomes at young adulthood.

Methods

Animals and treatment

Six-week-old outbred CD-1 mice (Charles River Laboratories, St Constant, Quebec, Canada) were housed in the Department of

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Comparative Medicine at the University of Toronto under standard environmental conditions (12 h light and 12 h dark cycle; 23°C), were provided water *ad libitum* and fed a semi-purified casein-based diet devoid of ISO (AIN93G; Dyets Inc., Bethlehem, PA, USA).[6] After 2 weeks of acclimatization to the environment and diet, mice were bred harem style. Once females were identified as being pregnant they were housed in individual cages until pups were born. Offspring of six different dams that delivered on the same day were studied. To avoid a littermate effect, cross-fostering was performed at birth and a maximum of one male and one female pup from each dam was assigned to each of the six litters. Cross-fostered litters were subsequently randomized to corn oil or ISO treatment from PND 1 through 21. Control (CON) pups ($n = 24–30$) received corn oil as it is used as the vehicle for ISO.[2,3] The groups receiving ISO ($n = 24–30$) were administered a daily dose containing daidzein and genistein, the two major ISO in soy infant formula. The dose of ISO, 2 mg of daidzein and 5 mg genistein/kg of body weight, resembles the quantity and ratio of each ISO in soy protein formula,[7] and mimics the total circulating ISO levels of human infants fed soy protein formula.[2,8] Treatments were administered via a single daily subcutaneous injection with a total volume of 20 µl/pup/day. On PND 21, gender was determined and females were housed 3–4 per cage (male offspring were not studied beyond PND 21). Body weight was measured once weekly and mice were studied to 4 months of age, which is the time when peak bone mass is established in this mouse strain.[9] Femurs and LV1–LV4 were excised and stored at −80°C until analyses were performed. All experimental procedures respected the policies set out by the Canadian Council on Animal Care and were approved by the University of Toronto Animal Ethics Committee.[10]

Bone mineral content (BMC) and BMD of femurs and LV1–LV3

BMC and BMD of the femur and intact spine (LV1–LV3) was determined by dual energy X-ray absorptiometry (pSabre, Orthometrix, White Plains, NY, USA) and a specialized software program (Host Software Version 3.9.4; Scanner Software Version 1.2.0) that scanned bones in air at a speed of 2 mm/min with a resolution of 0.01 mm × 0.01 mm as previously described.[2,3]

Microarchitecture of the femur and LV4

Microcomputed tomography (GE Healthcare System, Model No. MS0900325-0010) was used to analyze the microarchitecture of trabecular bone as previously described.[2,3] Trabecular bone was evaluated at LV4 and femur neck. To analyze a specific bone volume, a contoured region of interest (ROI) was created using the advanced ROI module (Micro-View Version ABA 2.2). For femur neck analysis, the ROI was defined from the top of the growth plate to the narrowest part of the femur shaft.

Biomechanical strength properties of femurs and LV2

Biomechanical strength properties of the right femur and LV2 were measured using a material testing system (Model 4442, Intron Corp., Canton, MA, USA) and specialized software (Series IX Automated Materials Tester, Version 8.15.00) as previously described.[1–3,11]

Statistical analyses

Statistical analyses were performed using SigmaStat (Version 3.5, Jandel Scientific, San Rafael, CA, USA). Results are expressed as mean ± S.E.M. Student's *t*-test was used to compare the outcomes between the CON- and ISO-treated groups. Pearson coefficient of determination (r^2) was used to examine the relationship between vertebral bone volume/total volume (BV/TV) and stiffness as well as BV/TV and peak load for the CON- and ISO-treated groups. Statistical significance was defined as $P < 0.05$.

Results

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

The ISO intervention resulted in higher ($P < 0.05$) BMD of LV1–3 (Table 1); higher ($P < 0.05$) trabecular number (Tb.N.) and lower ($P < 0.05$) trabecular separation (Tb.Sp.) of LV4 (Table 1); and greater ($P < 0.05$) fracture load of LV2 (Table 1) compared with the CON group. The ISO intervention also resulted in higher ($P < 0.05$) BV/TV of LV4 (Table 1) compared with the CON group. Qualitative assessment demonstrated that the ISO group had improved trabecular network at LV4 compared with the CON group (Fig. 1). There was no significant coefficient of determination for vertebral BV/TV and stiffness, or BV/TV and fracture load among mice exposed to corn oil. In contrast, the coefficient of determination of vertebral BV/TV and stiffness ($r^2 = 0.64$) as well as vertebral BV/TV and fracture load ($r^2 = 0.78$) were significant ($P < 0.05$) for the ISO group (Fig. 2). These findings indicate that the variability in vertebral BV/TV can predict the variability in vertebral stiffness and fracture load by 64% and 78%, respectively.

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

Table 1. *BMD, fracture load and trabecular bone parameters at the spine and femur with 21-day ISO exposure*[a]

Measured outcomes[b]	CON	ISO	*P*-value
Lumbar spine			
LV1–3 BMC (mg)	0.024 ± 0.002	0.029 ± 0.002	NS
LV1–3 BMD (mg/cm^2)	0.066 ± 0.004	0.078 ± 0.003*	0.024
LV2 ~~fracture~~ load (*n*)	62.0 ± 4.85	84.0 ± 6.22*	0.013
LV4 BV/TV (%)	29.7 ± 1.00	37.3 ± 0.70*	<0.001
LV4 BS/BV (mm^2/mm^3)	39.5 ± 2.29	38.9 ± 2.83	NS
LV4 Tb.Th. (mm)	0.051 ± 0.003	0.053 ± 0.004	NS
LV4 Tb.N. (/mm)	5.69 ± 0.309	7.23 ± 0.512	0.031
LV4 Tb.Sp. (mm)	0.123 ± 0.009	0.089 ± 0.006	0.008
Femur			
Whole femur BMC (mg)	32.4 ± 1.30	41.0 ± 0.988*	<0.001
Whole femur BMD (mg/cm^2)	79.7 ± 2.57	97.8 ± 1.87*	<0.001
Femur midpoint ~~fracture~~ load (*n*)	31.0 ± 2.14	37.0 ± 1.87*	0.014
Femur neck fracture load (*n*)	23.1 ± 1.36	31.8 ± 3.87*	0.049
Femur neck BV/TV (%)	52.5 ± 4.30	61.9 ± 4.10*	<0.001
Femur neck BS/BV (mm^2/mm^3)	19.8 ± 3.43	19.8 ± 3.70	NS
Femur neck Tb.Th. (mm)	0.104 ± 0.020	0.104 ± 0.018	NS
Femur neck Tb.N. (/mm)	5.20 ± 0.956	6.14 ± 1.18	NS
Femur neck Tb.Sp. (mm)	0.094 ± 0.020	0.065 ± 0.018*	0.011

BMD, bone mineral density; ISO, isoflavones; CON, control; LV, lumbar vertebrae; BMC, bone mineral content; BV/TV, bone volume/total volume; BS/BV, bone surface area/bone volume; Tb.Th., trabecular thickness; Tb.N., trabecular number; Tb.Sp., trabecular separation.

[a] Values are expressed as mean ± S.E.M. Statistical significance was defined as $P < 0.05$.

[b] Sample size was $n = 10$–14 for BMC, BMD and fracture load; and $n = 6$–7 for outcomes of bone structure.
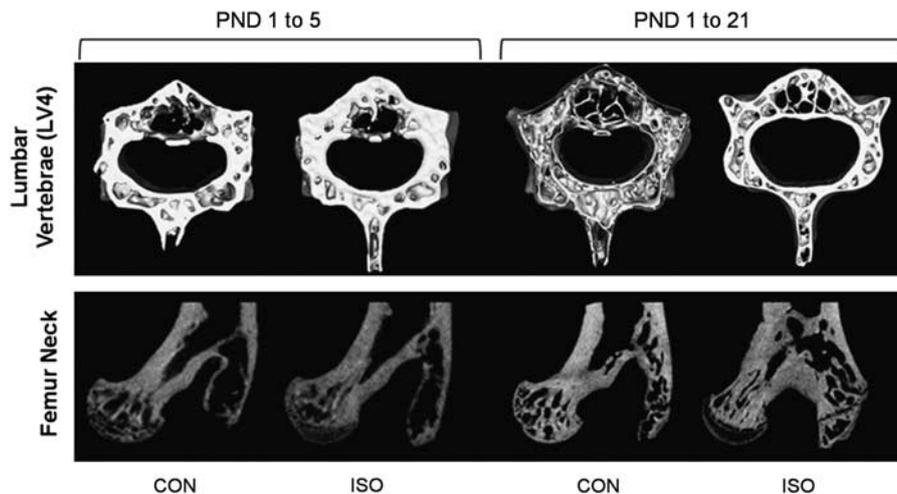

Fig. 1. Representative microcomputed tomography images of the lumbar vertebrae (LV4) and femur neck. The trabecular network is visibly improved at the lumbar spine in ISO-treated females compared with ~~control.~~ The cortical thickness of ISO-treated females is visibly improved at the femur neck compared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

Discussion

Exposure to ISO from birth to 21 days of age resulted in greater effects at the lumbar spine and femur than 5-day exposure to ISO. Although it had been previously shown that the first 5 days of life provides a window of opportunity for programming of bone development by early diet,[1–3] findings from this study suggest that the window of opportunity exists beyond this age. Thus, duration of exposure to ISO is a factor to consider when using this animal model to mimic the human infant scenario.

Our studies have shown that skeletal sites rich in trabecular bone (i.e. lumbar spine ~~and femur neck~~) are more easily
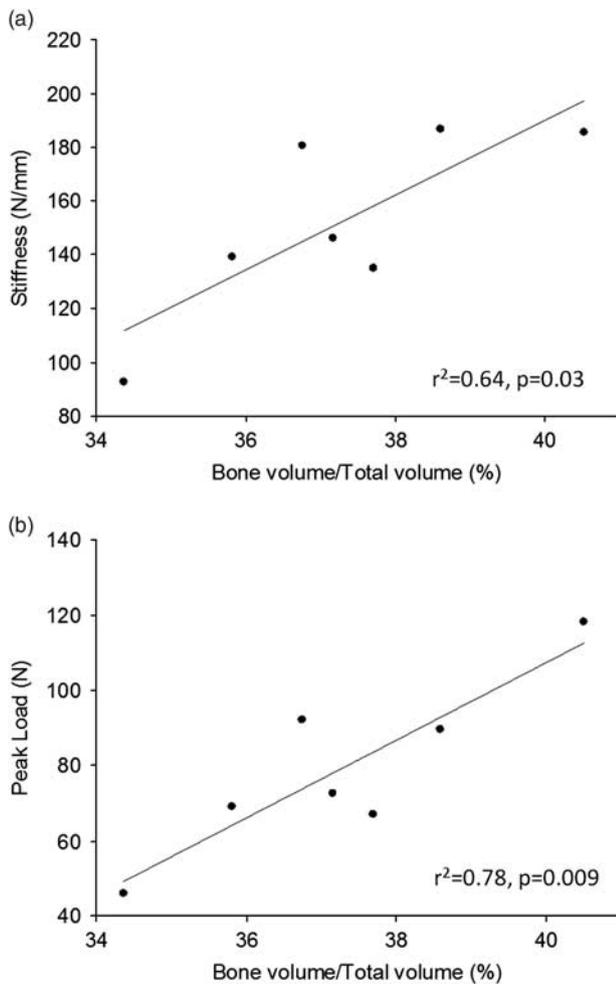
Fig. 2. Relationship of (a) vertebral bone volume/total volume (BV/TV) and stiffness or (b) fracture load of female mice exposed to isoflavones.

influenced by ISO than sites rich in cortical bone (i.e. femur midpoint).[1–3] This may be because trabecular bone has a higher surface-to-volume ratio and is more metabolically active. Although both the 5- and 21-day exposure to ISO improved trabecular connectivity of the lumbar spine in female CD-1 mice by increasing Tb.N. and decreasing Tb.Sp., only the 21-day exposure improved the apparent bone density (BV/TV) at the lumbar spine as well as the femur neck. These improvements in apparent bone density (BV/TV) suggest that longer duration of ISO exposure is needed for trabeculae to be significantly resorbed with bony tissue and merged into cortical bone, which is abundant in the femur. This is important because a small improvement in bone mass and structure during adolescence lowers the risk of fracture in later life.[12] Observational studies indicate that a 5% increase in bone mass at young adulthood can reduce fracture risk by 40% during aging.[13,14]

The apparent bone density (BV/TV) of ISO group was significantly correlated with stiffness ($r^2 = 0.63$) and fracture load ($r^2 = 0.78$) at the lumbar spine. This discovery is similar to findings in humans and identifies that improvements in

bone volume induced by ISO exposure can predict the probability of vertebral fracture by 78% in the mouse model. Chevalier et al.[15] showed that there is a strong correlation between increases in BV/TV and increases in vertebral stiffness and failure load among alendronate and risedronate treated postmenopausal women with osteoporosis. Thus, the relationship of BV/TV and stiffness, and BV/TV and failure load exists in both this mouse model and older humans. Vertebral fractures are the most common manifestation of osteoporosis and account for nearly half of all fractures.[16] Fracture prevention has largely focused on attenuating the rate of age-related bone loss and reducing falls during older age. Findings from this study suggest that early life nutrition is also an important consideration when developing lifestyle approaches to improve bone health throughout the life cycle.

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

The timing of when ISO exposure should be introduced in the developing mouse model to mimic human infants fed soy protein formula has been debated.[18,19] Mice suckle for the first 21 days of life and thus, it could be argued that ISO exposure should take place during suckling to mimic the stage of development in which human infants are fed soy protein formula. However, unlike human infants, mice reach sexual maturation 3 weeks post weaning, a much shorter duration between neonatal life and sexual maturity. To better understand the time of life when peak bone mass and strength is reached in the CD-1 mouse, we previously established that BMC, BMD and fracture load of femur and lumbar spine were similar between 3 and 4 months of age, demonstrating that peak bone mass is attained by 4 months of age in the CD-1 mouse.[9]

It is hypothesized that the programming effects on bone may be mediated through permanent estrogen receptor-induced changes in gene expression[4] or through non-genomic action that modulates a diverse array of intracellular signal transduction cascades.[18,20] Moreover, exposure to ISO during early postnatal life has the potential to exert biological effects that would otherwise be diluted in the presence of higher endogenous sex steroid concentrations that exist at later stages of the life cycle.

Establishing an animal model to study ISO exposure to human infants requires careful consideration of the dose and composition of ISO, the route of ISO administration, as well as the duration and frequency of ISO exposure. Our previous

research has shown that the dose and ratio of ISO (5 mg of genistein and 2 mg of daidzein per kg of body weight) used in this study result in total serum levels of ISO, specifically daidzein and genistein, that are similar to those of human infants fed soy protein-based infant formulas.[2] Moreover, during early postnatal life, both human infants and rodents have a poorly developed microflora that limits their ability to metabolize DAI to equol, the most estrogenic metabolite of ISO. Route of administration has also been studied.[21] Because of small size of mice from birth through the first days of life it is technically challenging to administer ISO orally and thus, administering ISO by subcutaneous injection is more commonly used. Recent findings have shown that oral *v.* subcutaneous delivery, and once daily *v.* multiple oral doses per day does not result in significantly different levels of total serum ISO in neonatal CD-1 mice.[21] This study is the first to evaluate how duration of ISO exposure during postnatal life affects peak bone mass, bone structure and bone strength of female CD-1 mice.

In conclusion, the present study further characterizes the CD-1 mouse model by identifying that longer duration of exposure to ISO has more profound benefits to bone health at multiple skeletal sites than the previously used 5-day exposure. Future studies should consider the duration of ISO exposure when using the CD-1 mouse model to evaluate the effect of early life exposure to ISO on programming of bone development. This mouse model may also be useful for studying effects of other environmental estrogens on bone development. Examples include bisphenol A, diethylstilbestrol (DES), dichlorodiphenyltrichloroethane (DDT) and dioxin. Duration of exposure to such environmental estrogens may be an important consideration when extrapolating findings to understand effects of exposure to human infants.

Acknowledgments

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