

Influence of Lifelong Soy Isoflavones Consumption on Bone Mass in the Rat

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Soy isoflavones (IFs) have shown a bone-sparing effect through epidemiological studies in the Asian population. However, there is no evidence as to whether such protection would result from a lifelong exposure. We investigated the impact of an early exposure to IFs on bone status. Sixty female Wistar rats were fed either a standard diet ($n = 30$) or the same food enriched with IFs (0.87 mg/g of diet) ($n = 30$). After 1 month, they were allowed to mate, and were kept on the same regimen during the whole gestation and lactation periods. At weaning, female pups were each assigned to one of four nutritional groups; within each experimental group, animals were split into two groups, fed either the standard or the IF-rich diet. At 2, 3, 6, 12, 18, and 24 months after birth, 10 animals in each group were sacrificed. Femurs were collected for mechanical testing and bone mineral density (BMD) measurement. The rats perinatally or lifelong exposed to the IF-rich diet exhibited higher body weight and fat mass at 24 months of age. Peak bone mass was achieved between 6 and 12 months and did not differ between groups. In animals perinatally exposed to IF, BMD continued to increase. Thus, at 24 months, femoral total BMD ($P < 0.05$), metaphyseal BMD ($P < 0.01$), and failure load ($P < 0.05$) were higher in the offspring born from mothers provided IF during pregnancy. Postnatal exposure alone did not improve bone parameters. This experiment provides evidence that perinatal exposure to phytoestrogens leads to a higher BMD later in life. It is suggested that these changes may have occurred as a consequence of programming effects, as has been shown for the endocrine and immune systems. *Exp Biol Med* 233:229–237, 2008

Key words: isoflavones exposure; rat; bone mass

Introduction

The bone mass of an individual depends on the peak attained during skeletal growth as well as the subsequent rate of bone loss. Thus, preventive strategies against osteoporosis may aim at either increasing the peak bone mass or reducing the rate of skeletal attrition during aging.

It is likely that environmental influences during early life will interact with the genome and establish the functional level of a variety of metabolic processes involved in skeletal growth. Research into human nutrition has led to an awareness of the health benefits that dietary modification can offer. Indeed, diet provides sufficient nutrients to meet the metabolic requirements of an individual, but also a complex array of naturally occurring bioactive molecules, such as phytoestrogens, that may confer significant long-term health benefits. In this light, there is a general agreement that soy-rich diet can be beneficial to adults (1). Indeed, clinical studies have provided evidence of bone-sparing effects associated with phytoestrogens consumption in postmenopausal women (2–7). However, there is no clear evidence of such a protection over a longer period of exposure. It is possible that the positive association between a diet rich in soy foods and bone mineral density (BMD), as demonstrated in Asian people (8–10), would result from a lifelong exposure. It is thus of the greatest interest to investigate the consequences of an early exposure.

Actually, some effects might be expected due to the exposure of the fetus to isoflavones (IF) from soy products consumed by the mother during pregnancy (11). Indeed, the maternal-fetal intrauterine transfer of IFs in animals fed an IF-rich diet elicits high serum IF levels in new-born rat pups that are maintained throughout the suckling period by passage of IFs into maternal milk (12). Transfer of IFs into breast milk has been shown in feeding studies in which lactating women were challenged with soyfoods. According to measurements by Franke *et al.* (13), with adjustment for body weight, isoflavonoid exposure is four to six times higher in infants fed soy-based formula than in adults eating

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Table 1. Composition of the Soy Protein–Free Powdered Semipurified Diet

Ingredient ^a	Concentration (g/kg)
Casein	200
Sucrose	220
Cornstarch	440
Cellulose fiber	50
Peanut oil	25
Rapeseed oil	25
Vitamin mixture ^b	10
Mineral mixture ^c	25
DL-methionine	3
Choline bitartrate	2

^a Casein (Union des caséineries, Surgères, France), sucrose (Euro-sucré, Paris, France), cornstarch (Cerestar, Saint-Maur, France), cellulose (Durieux, Marne la Vallée, France), oil (Bailly, Aulnay sous Bois, France), vitamin mixture (Roche, Neuilly sur Seine, France), mineral mixture (Prolabo, Fontenay sous Bois, France), and DL-methionine (Jerafrance, Jeufosse, France).

^b Expressed in mg/kg of mixture: retinyl palmitate (250 IU/mg), 2000; cholecalciferol (400 IU/mg), 312; DL- α -tocopherol acetate (0.25 IU/mg), 20,000; menadione, 100; thiamine HCL, 1000; riboflavin, 1000; nicotinic acid, 4500; D-calcium pantothenate, 3000; pyridoxine HCL, 1000; inositol, 5000; D-biotin, 20; folic acid, 200; cyanocobalamin, 1.35; ascorbic acid, 10,000; p-aminobenzoic acid, 5000; choline chlorhyascorbic acid, 10,000; p-aminobenzoic acid, 5000; choline chlorhydrate, 75,000; and sucrose, finely powdered, 871.9 g.

^c Expressed in g/kg of mixture: CaHPO₄ · 2H₂O, 308; K₂HPO₄, 194; CaCO₃, 146; MgSO₄ · 7H₂O, 109; NaCl, 168; MgO, 24.3; FeSO₄ · 7H₂O, 20.9; ZnSO₄ · H₂O, 12.1; MnSO₄ · H₂O, 12.1; CuSO₄ · 5H₂O, 0.0005; CoCO₃, 0.0005; Na₂SeO₃, 0.0005.

a diet rich in soy foods (approximately 30 g/day). Besides, soy-based infant formulas containing significant amounts of phytoestrogens have been in use for more than 30 years in developing countries, where there is a continued search for a suitable substitute for human milk (14). On a body weight basis, the daily exposure of infants to IFs in soy infant formulas is 6- to 11-fold higher than the dose that has hormonal effects in adults consuming soy foods (11).

The safety of soy-based infant formulas (SBF) is being debated (15–17). The basis of this controversy is that soy products generally contain IFs, which can affect the development of the reproductive system (18) and non-reproductive tissues (19). Regarding bone health, Bainbridge *et al.* (20) have compared the results of two studies (21, 22) that measured the bone mineral content (BMC) of infants fed either soy-based formulas or human milk– or cow milk–based formula at various ages. Both studies showed that, in the first year of life, the BMC of infants fed soy formula or human milk appeared to be similar, especially after 6 months of age. However, the BMC of those raised under such a diet was lower than that of infants fed cow milk–based formula. In a report by Kohler *et al.* (23), an initial decrease in bone mineralization at 3 months was described in SBF-fed infants compared with those fed mothers milk or cow milk–based formula. Further research is thus needed to determine the impact on bone health of phytoestrogens exposure via soy infant formula and soy-

based dietary supplements early in life and throughout life. With respect to bone status and metabolism, no long-term experiment targeting the effect of continued exposure to IFs through the perinatal and subsequent periods is available on BMD or biomechanical bone strength properties in humans or rats during aging.

Thus, we aimed to investigate the consequences of early and long-term exposures to dietary phytoestrogens on bone health. The present experiment, conducted in rats, was designed to test the specific effects on bone status of a perinatally and/or a lifelong exposure to soy IFs during aging.

Materials and Methods

Animals and Diets. The study was conducted in accordance with the regional ethics committee on animal experiments. Sixty female Wistar rats (F0 generation, 3 months old) were purchased from Institut National de la Recherche Agronomique (INRA; Clermont-Ferrand/Theix, France). The animals were randomly divided into two groups ($n = 30/\text{group}$) and housed in plastic cages (8 animals/cage) at 25°C with relative humidity of 55% and under a 12:12-hr light:dark cycle.

The F0 generation was fed, *ad libitum* (dry food from INRA, Jouy en Josas, France), either a standard diet devoid of any soy proteins (which were replaced by casein) or the same food enriched with IFs (Novasoy Isoflavones compound 152–400; Archer Daniels Midland Co., Decatur, IL) (Table 1). The powdered soy IF concentrate contained 348 mg/g as total IF (159 mg genistein, 156 mg daidzein, 33 mg glycitin 33; i.e., 0.87 mg of IFs/g of diet). As a consequence, the IFs intake was about 40 mg/kg body wt/day. The animals had free access to water during the entire experiment, and their body weight and food intake were measured weekly.

After an adaptation period of 30 days to their experimental diet, the females were allowed to mate and were kept on the same regimen during the whole gestation and lactation period. Pups were weaned on Day 21. At weaning, female offspring (the F1 generation) were assigned to one of four nutritional groups (Fig. 1): one half of the pups from mothers fed the control diet were given the IF diet (C-IF), and the remaining pups continued to receive the control diet (C-C); one half of the pups from the IF-treated mothers were given the control diet (IF-C), and the remaining pups continued to receive the IF diet (IF-IF).

At 2, 3, 6, 12, 18, and 24 months after birth, and at 2 months after parturition for the F0 generation, 10 animals in each group were intraperitoneally anaesthetized with chloral hydrate. A power calculation was performed based on a previous study targeting the effect of perinatal exposure to phytoestrogens on BMD in the rat (24). To reach the power of 0.97, 8–11 animals were needed in each group. At 48 hrs before death, the body composition was estimated by dual-energy x-ray absorptiometry (DEXA) (25). On Day –1, the

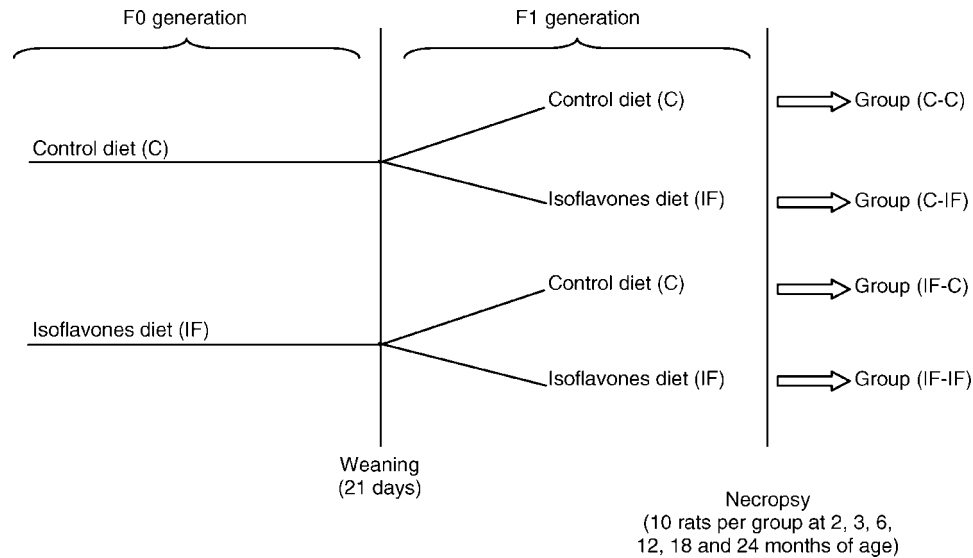


Figure 1. Experimental design.

24-hr urine samples were collected to assess deoxypyridinoline (DPD), a marker of bone resorption (26). The day of death, blood samples were collected by cardiac puncture into ice-cooled heparinized plastic tubes containing 200 peptidase inhibitory units of aprotinin (Iniprol; Choay, Paris, France) per milliliter blood, and centrifuged immediately (3500 g for 5 mins at 4°C). Plasma samples were then frozen at -20°C until measurement of osteocalcin (OC), a marker of osteoblastic activity (27). Uterine horns were removed and immediately weighed. Left and right femurs were cleaned from adjacent tissues and collected for mechanical testing and BMD measurement, respectively.

Physical Analysis. Whole Body Composition. Rats were anesthetized by intraperitoneal injection of chloral hydrate (80 g/L in saline solution; 0.4 ml/100 g body wt). They were scanned by DEXA with a QDR-4500 A x-ray bone densitometer (Hologic, Massy, France). Scans were analyzed. Body fat mass, body lean mass, and whole body BMC were measured.

Femoral BMD. Femoral BMD was assessed by DEXA with the Hologic QDR-4500 A. The total right femur BMD (T-BMD), as well as the BMD of two subregions, was determined. One corresponded to the distal femur metaphyseal zone (M-BMD) and the other to the diaphyseal zone (D-BMD). For M-BMD and D-BMD measurements, scans were cut and analyzed as follow: the first cut of the femur was made at the upper third, and the next cut was made at the lower third. D-BMD, which is rich in cortical bone, corresponded to the density of the second third of the femur. M-BMD, which mainly contains cancellous bone, was calculated as the mean of the femoral proximal metaphysis density and of the femoral distal metaphysis density. Results are given in grams per square centimeter (28). The intra- and interassay precisions

measured on 10 femoral assays were 0.22% and 0.24%, respectively.

Femoral Mechanical Testing. After collection, the length of the left femur and the mean diameter of the femoral diaphysis were measured with a precision caliper (Mitutoyo, Shropshire, UK). Because of the irregular shape of the femoral diaphysis, the femoral diameter used in the calculation was the mean of the greatest and the smallest femoral diaphysis diameters. Femoral failure load was determined by a three-point bending test (29) with a Universal Testing Machine (Instron 4501; Instron, Canton, MA). An upper crosshead roller (6-mm diameter) was applied in front of the middle of the bone and advanced at 0.5 mm/min until rupture was automatically determined by the apparatus. Load (in newtons) at rupture was recorded.

Analysis. Marker of Osteoblastic Activity. OC in plasma was measured by radioimmunoassay (RIA), using rat [¹²⁵I]labeled OC, goat anti-rat OC antibody, and donkey anti-goat second antibody (Biochemical Technologies kit; Stoughton, MA). The sensitivity was 0.01 nM. The intra- and interassay precisions were 6.8% and 8.9%, respectively, in the range of 1.70–14.00 nM.

Marker of Bone Resorption. DPD in urine was determined by competitive radioimmunoassay using rat monoclonal anti-DPD antibody coated to the inner surface of a polystyrene tube and [¹²⁵I]labeled DPD (Pyrilinks-D RIA kit; Metra Biosystems INCO, Mountain View, CA); the sensitivity was 2 nM. The intra- and interassay precisions were 3.9%–5.3% and 6.7%–7.8%, respectively, in the range of 42–163 nM. Results were expressed as nanomoles DPD per millimole of creatinine (30). The urinary creatinine assay, based on a modified Jaffés's method in which picric acid forms a colored solution in the presence of creatinine, was used to adjust DPD values for variation in urine volume (31).

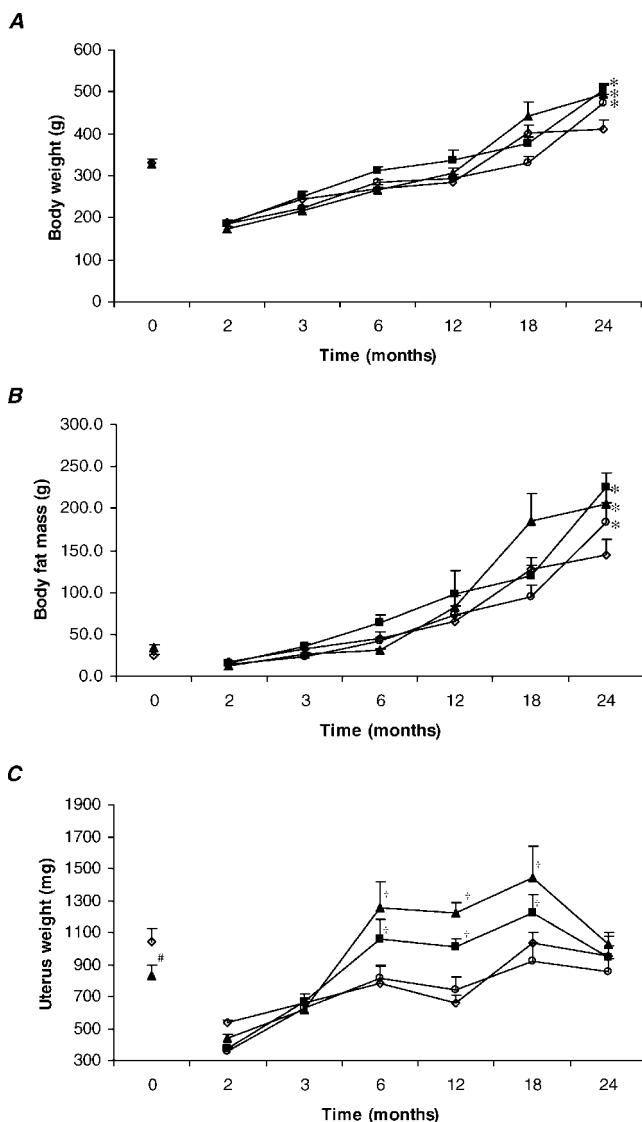


Figure 2. Body weight (A), body fat mass (B), and uterine weight (C) changes during the experimental period. Control mothers at T0 (open diamonds); IF-fed mothers at T0 (closed triangles); F1 generation perinatally exposed to the standard diet and provided the standard diet (C-C; open diamonds) or the IFs-enriched diet (C-IF; open circles) after weaning; F1 generation perinatally exposed to soy IFs and provided the standard diet (IF-C; closed squares) or the IF-enriched diet (IF-IF; closed triangles) after weaning. Values are expressed as means $\pm$ SEM. (A) Body weight was significantly higher in all animals fed IFs compared with control groups (C-C) at 24 months (* $P < 0.01$). (B) Body fat mass was significantly higher in all animals fed IFs compared with control groups (C-C) at 24 months (* $P < 0.05$). (C) Uterine weight was significantly lower in IF-supplemented mothers (# $P < 0.05$). Uterine weight was significantly higher in all animals perinatally exposed to IFs (IF-C and IF-IF) compared with the groups C-C and C-IF at 6, 12, and 18 months († $P < 0.01$).

Statistical Analysis. Results are expressed as means $\pm$ SEM. All data were analyzed using the Graphpad Instat software package (Microsoft Corp., San Diego, CA). Analysis of variance was performed when data were sampled from populations with equal variance to test for any significant differences among groups. When significant

($P < 0.05$), the Student-Newman-Keul's multiple comparisons test was used to determine the specific differences between means. If not parametric, a Kruskal-Wallis test was performed. If it indicated a significant difference among groups ($P < 0.05$), the Mann-Whitney U test was used to determine specific differences. The level of significance was set at $P < 0.05$ for all statistical tests.

Results

Body Weight, Fat Mass, and Uterine Weight.

Regarding the F0 generation, IF-supplemented and control females exhibited similar body weight and fat mass (Fig. 2A and B). During the experimental period, body weight increased in each F1 group (Fig. 2A). At 24 months, all the rats that had been exposed to IFs (IF-C, C-IF, and IF-IF) had a significantly higher body weight compared with control animals (C-C) ($P < 0.01$).

With regards to fat mass (Fig. 2B), a progressive increase was observed with aging in all rats (225.2 ± 17.7 g at 24 months vs. 15.9 ± 2.4 g at 2 months). At 24 months, IF-C (225.2 ± 19.1 g), IF-IF (205 ± 23.3 g) and C-IF (183.3 ± 18 g) rats had a higher body fat content than control animals (143.8 ± 17.7 g) ($P < 0.05$).

IF-supplemented mothers had a lower uterine weight compared with control animals (0.830 ± 0.060 g vs. 1.050 ± 0.081 g; $P < 0.05$) (Fig. 2C). In the F1 generation, uterine weight consistently increased from 3 months of age. From 6 to 18 months, heavier uterus were observed in IF-IF and IF-C animals compared with C-C and C-IF groups ($P < 0.01$), whereas differences disappeared at 24 months of age.

Bone Parameters. BMD values (g/cm^2) of the total femur and its diaphyseal and metaphyseal subregions are shown in Figure 3A. T-BMD, D-BMD, and M-BMD significantly increased between 3 and 6 months. Peak bone mass was achieved between 6 and 12 months, and did not differ between groups. BMD then stabilized approaching 24 months in the offspring born from control mothers (C-C and C-IF), whereas it continued to increase in *in utero* IF-exposed rats (IF-C and IF-IF). At 24 months, the animals from IF-fed mothers exhibited a higher BMD than the rats from control mothers (C-C, 0.250 ± 0.005 ; C-IF, 0.249 ± 0.007 vs. IF-C, 0.264 ± 0.005 ; IF-IF, 0.272 ± 0.006 for T-BMD at 24 months). This was significant for both total ($P < 0.05$) and metaphyseal ($P < 0.01$) levels, while only a trend was recorded at the diaphysis. Furthermore, after 12 and 18 months, a significant decrease ($P < 0.05$) in T-BMD, D-BMD, and M-BMD was observed in the C-IF group. In the mothers, the experimental diet did not elicit any significant difference in bone parameters. Indeed, they had similar BMD to the age-matched F1 generation (i.e., 5 months).

Femoral size was similar in all groups (average length, 37.3 ± 0.37 mm; average diameter, 3.73 ± 0.06 mm) (data not shown). A progressive increase in the femoral failure load (Fig. 3B) was recorded from 2 to 6 months in all animals (average value, 38.26 ± 1.68 at 2 months vs.

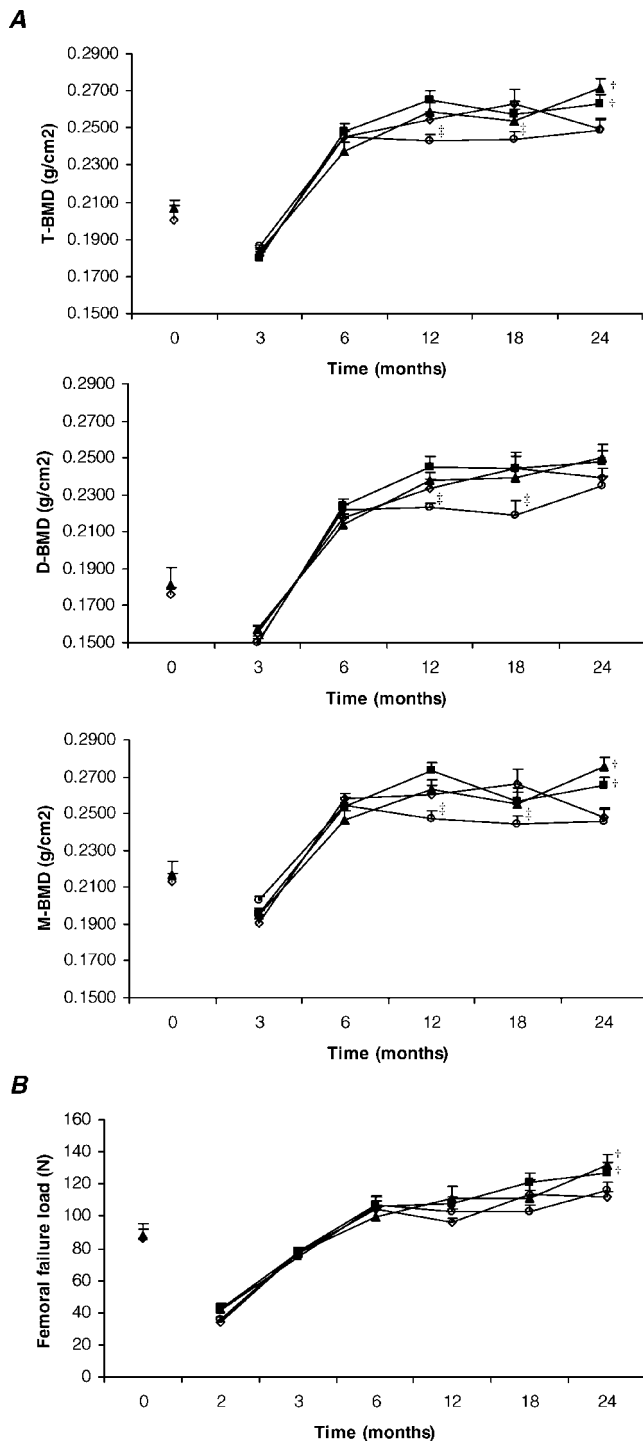


Figure 3. Total (T-BMD), diaphyseal (D-BMD), metaphyseal (M-BMD), femoral BMD (A) and femoral failure load (B) changes during the experimental period. Control mothers at T0 (open diamonds); IF-fed mothers at T0 (closed triangles); F1 generation perinatally exposed to the standard diet and provided the standard diet (C-C; open diamonds) or the IF-enriched diet (C-IF; open circles) after weaning; F1 generation perinatally exposed to soy IFs and provided the standard diet (IF-C; closed squares) or the IFs-enriched diet (IF-IF; closed triangles) after weaning. Values are expressed as means $\pm$ SEM. (A) T-BMD was significantly higher in animals perinatally exposed to IFs compared with C-C and C-IF groups at 24 months ($\dagger P < 0.05$). D-BMD did not vary significantly at 24 months. M-BMD was significantly higher in animals perinatally exposed to IFs

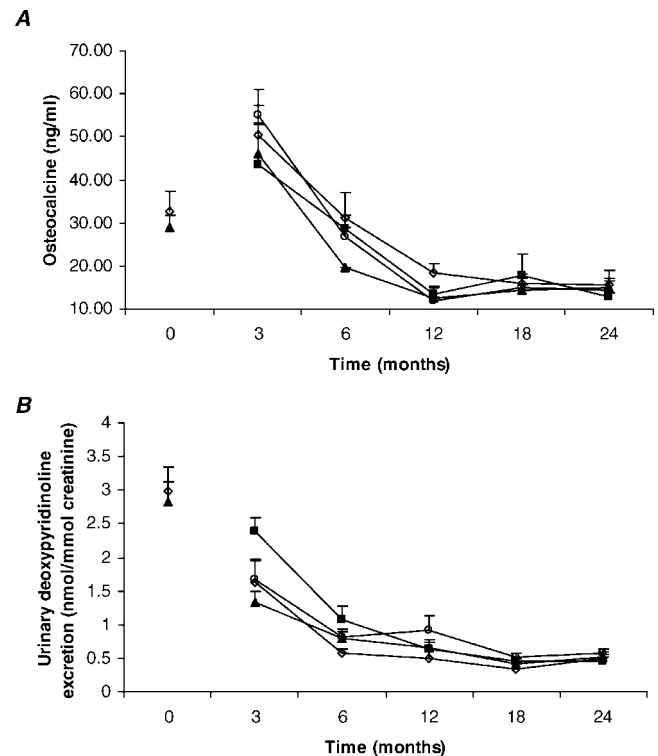


Figure 4. Plasma OC concentration (A) and urinary deoxypyridinoline excretion (B) changes during the experimental period. Control mothers at T0 (open diamonds); IF-fed mothers at T0 (closed triangles); F1 generation perinatally exposed to the standard diet and provided the standard diet (C-C; open diamonds) or the IF-enriched diet (C-IF; open circles) after weaning; F1 generation perinatally exposed to soy IFs and provided the standard diet (IF-C; closed squares) or the IFs-enriched diet (IF-IF; closed triangles) after weaning. Values are expressed as means $\pm$ SEM.

104.21 $\pm$ 2.74 at 6 months; $P < 0.0001$). A steady state was reached at 6 months through 24 months of age in C-C and C-IF groups. In contrast, in *in utero* IF-exposed rats (IF-C and IF-IF), bone strength continued to improve with age, reaching higher values at 24 months compared with animals from mothers fed the control diet (C-C, 111.9 $\pm$ 3.2; C-IF, 116.0 $\pm$ 5.0 vs. IF-C, 128.1 $\pm$ 6.3; IF-IF, 131.8 $\pm$ 6.5; $P < 0.05$).

Markers for Bone Metabolism. Plasma OC concentrations (ng/ml) (Fig. 4A) decreased with age in all animals. No consistent difference were observed among groups. A similar pattern was observed for DPD urinary excretion (Fig. 4B).

As far as the F0 generation was concerned, mothers had a similar range of plasma OC concentrations to that of the age-matched F1 generation. Their level of resorption, as shown by DPD assessment (nmol/mmol creatinine), was

← compared with C-C and C-IF groups at 24 months ($\dagger P < 0.01$). Femoral T-BMD, D-BMD, and M-BMD were significantly lower in C-IF rats compared with C-C, IF-C, and IF-IF animals at 12 and 18 months ($\ddagger P < 0.05$). (B) Femoral failure load was significantly higher in animals perinatally exposed to IFs compared with C-C and C-IF groups at 24 months ($\dagger P < 0.05$).

much higher (IF, 2.282 ± 0.30 ; C, 2.99 ± 0.36) than in 3-month-old (1.76 ± 0.14) or 6-month-old F1 generation rats (0.82 ± 0.07).

Discussion

Asian women are about half as likely as white women to experience a hip fracture (32). Understanding the reasons for this striking ethnic difference in osteoporosis, or even other degenerative diseases, has led to the hypothesis of a beneficial effect of Eastern lifestyle. Actually, ecological studies point to a high-soy diet as one attractive explanation (33). This is why the use of dietary phytoestrogens as a possible option for the prevention of osteoporosis has raised considerable interest. However, translating those data to the Western situation has been challenging, and, thus far, no long-term study has attempted to tease out the influence of lifelong exposure to phytoestrogens on bone. Indeed, whether early exposure can explain a reduced risk of fracture is uncertain, as no such prospective studies have been performed to date. To our knowledge, little information is available regarding the effect of *in utero* and lifelong exposure on peak bone mass acquisition and subsequent bone health.

Physiological *In Utero* Effects of Phytoestrogens. In our experimental conditions, we have shown that perinatal exposure to phytoestrogens was able to elicit biological activity. Indeed, even though such exposure was not associated with significant changes early in life (no difference among groups at 3 months), bone status was improved later in life in IF-C and IF-IF groups (at 24 months) compared with the offspring from dams on a control diet (C-C and C-IF groups) (Fig. 3). Body weight and body fat mass were also different from control animals at this age (Fig. 2A and B). Indeed, as shown by Lamartinière *et al.* (34), IFs are able to cross the placental barrier. Actually, those authors have provided evidence that plasma concentrations of phytoestrogens are similar in the pregnant female and the fetus. In fact, the high levels of endogenous estrogens previously found in the newborn, with rapid decrease after delivery combined with the simultaneous increase in metabolic capacity of the liver, suggest that these relatively high phytoestrogens concentrations can be well tolerated (35). Consequently, phytoestrogen intake during pregnancy has physiological effects on the fetus. The transfer of IFs into breast milk was also demonstrated in feeding studies in which lactating women were challenged with soy foods. Thus, given that absorption of soy IFs from the intestinal tract is efficient in the neonates (36), neonatal exposure may also explain such effects.

Programming Effects of Phytoestrogens. It is acknowledged that any disturbance of the environment provided by the mother can modify early fetal development, with possible long-term outcomes, as demonstrated by extensive work on programming (37). In fact, programming sets the pattern of how the host responds to biochemical

effectors. We suggest that changes observed in our experimental conditions may have occurred as a consequence of programming effects, as is known for the endocrine and immune systems (38). Indeed, with regard to body weight, all the rats that had been on an IF-rich diet (whether it was only during gestation, after birth, or during both) had a significantly higher body weight at 24 months compared with those that had never been exposed. Data on body composition are also consistent with this pattern, and the same trends were demonstrated in bone health (i.e. a higher femoral BMD in late life in perinatally exposed animals). Consequently, these developmental modifications may reflect physiological effects of phytoestrogen intake during pregnancy or lactation, and it is likely that exposure to IFs during pregnancy may exert long-lasting effects. In the same way, a protective effect against dimethylbenz[a]anthracene (DMBA)-induced cancer has been reported in rats when genistein was administered before puberty (39). Neonatal injections of genistein also serve to suppress the development of DMBA-induced mammary adenocarcinomas in rats (DMBA administration occurring at 50 days postpartum) (40). As a result, early programming events modulated by phytoestrogens could be involved in bone mass changes observed during aging.

Biological Effects of Phytoestrogens on Body Weight. The animals that were exposed to IFs, whether it was during gestation/lactation or after weaning (C-IF, IF-C and IF-IF groups), were characterized by a higher body weight at 24 months of age compared with control animals (Fig. 2A). Those data are difficult to interpret, because, in a study conducted by Levy *et al.* (41) on pregnant rats, birth weights of the offspring exposed to 25 mg of genistein/day were lower than those of control animals. Moreover, Dang and Lowik (42) have provided evidence of a genistein-induced modulation of peroxisome proliferator-activated receptor (PPAR) γ , involved in adipogenesis, dyslipidemia, or sensitivity to insulin (among others processes), when systemic concentrations reach 1 μM . Activation of this pathway is thought to play a protective role, and could explain why IFs have been shown to lower fat deposition in SHR/N-cp rats, an animal model of obesity (43).

Biological Effects of Phytoestrogens on the Uterus. In our study, at 6 months, uterine weight in perinatally exposed animals was significantly higher than in the offspring from control dams (Fig. 2C). Normal uterine gland formation occurs between the first and second postnatal weeks in the rat (44). There is some evidence emphasizing that exogenous estrogens can alter both uterine gland genesis and the number of glands per uterine section in an age-specific manner (45). Actually, in animals exposed neonatally with estrogens, trophic uteri with impaired estrogen responsiveness and reduced uterine gland number are usually observed (46). In this light, neonatal exposure to IFs can alter concentrations of endogenous sex hormones. Indeed, according to Nagao *et al.* (47) and Awoniyi *et al.* (24), *in utero*, lactation, and early neonatal exposure to

dietary genistein adversely affects reproductive processes in the adult female rat. These events likely explain the maintenance of a higher uterus weight from 6 to 18 months in IF-C and IF-IF rats. Consequently, the exposure to IFs *in utero* and *ex utero* appears to potentially affect the development of the genital tract. Basically, both estrogen receptor (ER) α and ER β proteins have been detected in the rat uterus (48). While estradiol binds with equal affinity to both ER α and ER β (49), the phytoestrogens, genistein and daidzein, show greater selectivity towards binding to ER β , and can initiate a uterotrophic response in rodents (39, 50). Fritz *et al.* (51) have shown that giving genistein to prepubertal rats increased uterine weights at Postpartum Day 21, while at Postpartum Day 50 there was no significant difference among treated and control animals. Moreover, according to data published by Roberts *et al.* (52), gestational plus lactational exposure to genistein, with subsequent dietary exposure to genistein, are not likely to elicit adverse effect on gametogenic function in male rats.

Biological Effects of Phytoestrogens on the Skeleton. According to our data, femoral BMD (T-BMD) (Fig. 3A) increased with skeletal maturation and achieved its peak between 6 and 12 months of age. Thereafter, a steady state was reached in the offspring born from the mothers raised on a control diet. This parameter was correlated with bone strength (Fig. 3B). In this light, the rat, despite being a quadruped, shares many similarities with humans in regard to the behavior of its skeleton: rapid bone formation and mineralization occur during the first few months of life (53), and these processes continue during the first year of life (54). As a matter of fact, Schapira *et al.* (55) showed that, in the Wistar rat, the building of bone mass occurs during the first year of life; bone mineral densities and mineralization peaks occur by 12 months of age—and most of these values are already attained at the age of 8 months.

As far as phytoestrogens consumption is concerned, all animals displayed the same BMD at 6 months, whether they were exposed to IFs or not (Fig. 3A). Nevertheless, the offspring that has been perinatally exposed exhibited a higher BMD at 24 months. This was the case at both total and metaphyseal levels, while only a trend was observed at the diaphysis. In contrast, Kohler *et al.* (23) and Steichen and Tsang (22) reported that soy formula-fed children displayed a slower mineralization and maturation of bone during the first 6 months of life. On the other hand, our results are in accord with those of Adlercreutz *et al.* (35), who showed that daidzein and genistein passed the placental barrier, the levels in the mother being similar to those in the fetus. Moreover, they showed that the fetal liver is able to carry out glucuronidation. Those data could explain why IF-C and IF-IF rats have a higher femoral T-BMD than the other rats.

In addition, we postulated the more complex theory that the bone effect of phytoestrogens might vary according to the menopause status. Given that phytoestrogen receptor

interactions will necessarily compete with those of the cognate ligand, the result will likely partly depend on concentrations of endogenous sex steroids (56). Indeed, the bone-sparing effect of phytoestrogens has been consistently demonstrated in the ovariectomized rat (57). In our experimental conditions, postnatal exposure in intact animals did not improve BMD. Consequently, the sex-related differences in bone turnover and the role of sex hormones on the metabolism of bone in the aging rodent need further investigation and clarification. Estrogens play a critical role in growth, development, and maintenance of a diverse range of tissues. They exert their physiological effects *via* the ER and bind with equal affinity to both ER α and ER β (58). ER α and ER β mRNAs are expressed in osteoblasts, and the expression of ER β mRNAs is higher in cancellous bone of the rat distal femoral metaphysis and lumbar vertebrae than in cortical bone of the femoral diaphysis (59). Again, recent studies have also shown that neither ER α nor ER β mRNA was detected in rat cortical bone (60), and ER β mRNA was expressed predominantly in rat osteoblasts covering the metaphyseal bone trabecular surface (61). Binding of ER to estrogen response element (ERE) induces gene activation and is an important step in estrogen-induced biological effects. Recently, a study by Kostelac *et al.* (61) showed that estradiol and phytoestrogens induced an increase in ER binding to ERE in a concentration-dependant manner. Genistein (500 μ M) and daidzein (850 μ M) preferentially activate the binding of ER β to ERE. The endogenous hormone, 17 β -estradiol, similar to the daidzein metabolite, equol, activates the binding of ER β to ERE only slightly more effectively than the binding of ER α to ERE (61). As such, exposure to IF may produce estrogenic effects in females. Here, the perinatal exposures appear to produce the most marked effects. However, nonestrogenic mechanisms, or other properties of soy, may also play a role. For example, enhanced intestinal absorption of soy-derived calcium could contribute to the bone-conserving effects of soy (62).

In conclusion, lifelong exposure to phytoestrogens has seldom been evaluated, even though it is highly relevant. This experiment demonstrates that rats born from mothers exposed to IFs during pregnancy and lactation exhibited improved total and metaphyseal femoral BMD, as well as bone strength at 24 months of age. Thus, our data suggest that early programming events associated with phytoestrogen intake could be involved in bone mass variations during aging, since perinatal exposure to soy IFs lead to significant increases in bone status later in life. Early exposure to dietary phytoestrogens may provide a preventive strategy that optimizes peak bone mass and bone strength, thereby potentially decreasing the risk of developing osteoporosis and fragility fracture later in life. Our observations indicate the need for further studies in humans to investigate the potential effects of prolonged fetal, neonatal, or adult exposure to phytoestrogens, notably on change in sexual development and in bone mass or markers of bone loss.

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