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## **Influence of lifelong soy isoflavones consumption on bone mass in the rat**

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## ABSTRACT

Soy isoflavones (IF) have shown a bone-sparing effect through epidemiological studies in the Asian population. However, there is no evidence if such a protection would result from a lifelong exposure. We investigated the impact of an early exposure to isoflavones on bone status. Sixty female Wistar rats were either fed a standard diet (n=30) or the same food enriched with isoflavones (0.87mg/g of diet) (n=30). After one month, they were allowed to mate and stayed on the same regimen during the whole gestation and lactation periods. At weaning, female pups were allocated to 4 nutritional groups: within each experimental group, the animals were split into 2 groups, fed either the standard or the IF rich diet. At 2, 3, 6, 12, 18 and 24 months after birth, respectively, 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 isoflavones-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. It was relevant with decreased bone biomarkers levels (osteocalcin and deoxypyridinoline) at 12 months. In animals perinatally exposed to IF, BMD continued to increase. Thus, at 24 months, femoral total ( $P<0.05$ ), metaphyseal ( $P<0.01$ ) BMD 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 bone status 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.

**Key words:** isoflavones exposure, rat, bone mass.

## **INTRODUCTION**

The bone mass of an individual depends on the peak attained during skeletal growth, and the subsequent rate of bone loss, as well. 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 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 due to the exposure of the foetus to isoflavones from soy products consumed during pregnancy by the mother might be expected (11). Indeed, the maternal-fetal intrauterine transfer of isoflavones in animals fed an isoflavones rich diet elicits high serum isoflavones levels in new-born rat pups, which are maintained throughout the suckling period by passage of isoflavones into maternal milk (12). Transfer of isoflavones into breast milk has been shown in feeding studies, in which lactating women were challenged with soyfoods. According to measurements of Franke *et al.* (13), with adjustment for body weight, isoflavonoid exposure is 4-6 times higher in infants fed soy-based formula than in adults eating a diet rich in soyfoods (approximately 30 g/d). 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 of human milk (14). On a body weight basis, the daily exposure of infants to isoflavones in soy infant-formulas is 6-11 fold higher than the dose that has hormonal effects in adult 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 isoflavones, 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 the report by Kohler *et al.* (23), an initial decrease in bone mineralization at three months was described in SBF-fed infants, in comparison with those fed mothers milk or cow milk-based formula. Further research is thus needed to determine the impact of phytoestrogens exposure via soy infant formula and soy-based dietary supplements early in life and throughout life on bone health. With respect to bone status and metabolism, no long term experiment targeting the effect of continued exposure to isoflavones through the perinatal and subsequent periods is available on bone mineral density (BMD) or biomechanical bone strength properties in humans or rats during aging.

Thus, the authors aimed at investigating 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 isoflavones during aging.

## **MATERIALS AND METHODS**

### **Animals and diets**

The study was conducted in accordance with the regional Ethics Committee on animal experiments, in France. Sixty female Wistar rats (F0 generation, 3 months old) were purchased from I.N.R.A. (Institut National de la Recherche Agronomique, Clermont-Ferrand/Theix, France). The animals were randomly divided into two groups (n=30 per group) and housed in plastic cages (8 animals/cage) at 25°C with relative humidity of 55% and under a 12:12h light dark cycle.

The F0 generation was fed *ad libitum* (dryfood 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 isoflavones (IF) (ADM: Novasoy Isoflavones compound 152-400; Archer Daniels Midland Company, Decatur, IL) (Table 1). The powdered soy IF concentrate contained 348 mg / g as total IF (genistein 159, daidzein 156, glycitin 33), that is to say 0.87 mg of isoflavones per gram of diet. As a consequence, the isoflavones intake was about 40 mg/ kg bw / d. 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 stayed 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 allocated to 4 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 2 months after parturition for the F0 generation, respectively, 10 animals in each group were intraperitoneally anaesthetized with chloral hydrate. Indeed, 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. 48 h before death, the body composition was estimated by dual energy X-ray absorptiometry (25). On day -1, the 24 h urines 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 heparinised plastic tubes containing 200 peptidase inhibitory units of aprotinin (Iniprol, Choay, Paris, France) per millilitre blood, and centrifuged immediately (3500 g for 5 min at 4°C). Then plasmas were frozen at -20°C until measurements of osteocalcin, 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 bone mineral density (BMD) measurement, respectively.

### Physical analysis

*Whole body composition:* Rats were anesthetised by intra peritoneal injection of chloral hydrate (80g / l in saline solution; 0.4 ml / 100g body weight). They were scanned with dual energy X ray absorpsiometry using Hologic QDR-4500 A (Hologic, Massy, France). Scans were analysed. Body fat mass, body lean mass and whole body bone mineral content were measured.

*Femoral bone mineral density (BMD):* Femoral BMD was assessed by dual-energy X-ray absorptiometry (DEXA), with the Hologic QDR-4500 A X-ray bone densitometer. The total right femur BMD (T-BMD), as well as the BMD of two subregions were 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 g/cm<sup>2</sup> (28). The intra- and inter-assay 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, using a 3-point bending test (29) with a Universal Testing Machine (Instron 4501, Instron, Canton, MA). An upper crosshead roller (diameter, 6 mm) was applied in front of the middle of the bone and advanced at 0.5 mm / minute until rupture was automatically determined by the apparatus. Load (Newtons) at rupture was recorded. This test method was previously validated by using plexiglas standard probes.

### Analysis

*Marker of osteoblastic activity:* Osteocalcin (OC) in plasma was measured by radioimmunoassay (RIA), using rat <sup>125</sup>I-labeled OC, goat anti-rat OC anti-body and donkey anti-goat second antibody (Biochemical Technologies kit, Stoughton, MA, USA). The sensitivity was 0.01 nmol/L. The intra- and inter-assay precisions were 6.8 and 8.9%, respectively, in the range of 1.70-14.00 nmol/L.

*Marker of bone resorption:* Deoxypyridinoline (DPD) in urine was determined by competitive radioimmunoassay, using rat monoclonal anti-DPD antibody coated to the inner surface of a polystyrene tube and <sup>125</sup>I-labeled DPD (Pyrilinks-D RIA kit, Metra Biosystems INCO, Mountain View, CA, USA). The sensitivity was 2 nmol/L. The intra- and inter-assay precisions were 3.9% to 5.3% and 6.7% to 7.8% respectively in the range of 42-163 nmol/L. Results were expressed as nmol DPD / mmol of creatinine (30). The urinary creatinine assay based on a modified Jaffés's method in which picric acid forms a coloured solution in the presence of creatinine was used to adjust DPD values for variation in urine volume (31).

### Statistical analysis

Results are expressed as means  $\pm$  SEM. All data were analysed using the Graphpad InStat software (Microsoft, SAN DIEGO, CA, USA). ANOVA was performed when data were sampled from populations with equal variance to test for any significant difference 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 2B).

During the experimental period, body weight increased in each F1 group (Fig. 2A). At 24 months all the rats that had been exposed to isoflavones (IF-C, C-IF and IF-IF) had a significant higher body weight compared to the control animals (C-C) ( $P < 0.01$ ).

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

Isoflavones supplemented mothers had a lower uterine weight compared to controls (g) ( $0.830 \pm 0.060$  vs  $1.050 \pm 0.081$ ,  $P < 0.05$ ) (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 to C-C and C-IF groups ( $P < 0.01$ ), whereas differences disappeared at 24 months of age.

### **Bone parameters.**

BMD values (g / cm<sup>2</sup>) of the total femur and its diaphyseal and metaphyseal subregions are shown in Fig. 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. Then, BMD steadied up to 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:  $0.250 \pm 0.005$ ; C-IF:  $0.249 \pm 0.007$  vs IF-C:  $0.264 \pm 0.005$ ; IF-IF:  $0.272 \pm 0.006$  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 with the age-matched F1 generation (i.e. 5 months).



Femoral size was similar in all groups (average length  $37.3 \pm 0.37$  mm, average diameter  $3.73 \pm 0.06$  mm) (data not shown). A progressive increase in the femoral failure load (N) (Fig. 3B) was recorded from 2 to 6 months in all animals (average value:  $38.26 \pm 1.68$  at 2 months vs  $104.21 \pm 2.74$  at 6 months,  $P < 0.0001$ ). Then, a steady state was reached at 6 months until 24 months of age in C-C and C-IF groups. On the contrary, in *in utero* IF exposed rats (IF-C and IF-IF), bone strength kept improving with age to reach higher values at 24 months compared to the 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 in all animals with aging. None consistent difference was observed among groups. A similar pattern was observed for DPD urinary excretion (Fig. 4B).

As far as the F0 generation was concerned, mothers had similar range of plasma osteocalcin concentrations with the age-matched F1 generation. Their level of resorption, as shown by DPD assessment (nmol / mmol creatinine), was much higher (IF:  $2.282 \pm 0.30$ , C:  $2.99 \pm 0.36$ ) than in 3 month-old ( $1.76 \pm 0.14$ ) or 6 month-old F1 generation rats ( $0.82 \pm 0.07$ ), respectively).

## **DISCUSSION**

Asian women are about half as likely as Caucasian 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 none 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 a biological activity. Indeed, even though such an 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 to the offspring from dams on a control diet (C-C and C-IF groups) (Fig. 3). Body weight and body fat mass were different from controls, as well, at this age (Fig. 2A and 2B). Indeed, as shown by Lamartinière *et al.* (34), isoflavones 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 foetus. In fact, the high levels of endogenous estrogens previously found in the newborn, with rapid decrease after delivery combined with the simultaneous increased metabolic capacity of the liver, suggest that these relatively high phytoestrogens concentrations can be well tolerated (35). Consequently phytoestrogens intake during pregnancy has physiological effects on the foetus. The transfer of isoflavones into breast milk was also demonstrated in feeding studies in which lactating women were challenged with soyfoods. Thus, given that absorption of soy isoflavones 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, like it is known for the endocrine and immune systems (38). Indeed, with regards to the body weight, all the rats that had been on an isoflavones-rich diet (whether it was only during gestation, after birth or during both) had a significant higher body weight at 24 months, compared to those that had never been exposed. Data on body composition are consistent with this pattern, as well. Same trends on bone health were demonstrated, i.e. a higher femoral BMD in late life in perinatally-exposed animals. Consequently, these developmental modifications may reflect physiological effects of phytoestrogens intake during pregnancy or lactation and it is likely that exposure to isoflavones during pregnancy may exert long-lasting effects. In the same way, a protective effect against DMBA induced cancer has been reported in rats when genistein was administered before puberty (39). Neonatal injections of genistein are also able 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 the body weight*

The animals that have been exposed to isoflavones, whether it was during gestation/lactation or after weaning (C-IF, IF-C and IF-IF groups), were characterised by a higher body weight at 24 months of age (Fig. 2A). Those data are quite difficult to understand because in the study conducted by Levy *et al.* (41) on pregnant rats birth weights of the offspring exposed to 25 mg of genistein/day were lower. Moreover, Dang & Lowik (42) have provided evidence of a modulation by genistein of PPAR $\gamma$  involved, among others, in adipogenesis, dyslipidemia or sensitivity to insulin, when systemic concentrations reach 1 $\mu$ M. Activation of this pathway is supposed to play a protective role and could explain why isoflavones 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 the second postnatal weeks in the rat (44). There is some evidence emphasizing that exogenous estrogens can alter in an age-specific manner, both uterine gland genesis and the number of glands per uterine section (45). Actually, in animals exposed neonatally with estrogens, a trophic uteri with impaired oestrogen responsiveness and reduced uterine gland number are usually observed (46). In this light, neonatal exposure to isoflavones 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 isoflavones *in utero* and *ex utero* appears to potentially affect the development of the genital tract. Basically, both ER $\alpha$  and ER $\beta$  proteins have been detected in the rat uterus (48). While estradiol binds with equal affinity to both ER $\alpha$  and ER $\beta$  (49), the phytoestrogens, genistein and daidzein, show greater selectivity towards binding to ER $\beta$  and can initiate an uterotrophic response in rodents (39,50). Fritz *et al.* (51) have shown that giving genistein to prepubertal rats increased uterine weights at day 21 postpartum while at day 50 there was no significant difference among treated and controls. Moreover, according

to Roberts *et al.* (52) data, gestational plus lactational exposure to genistein and 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 bone mineral density (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, in spite of being a quadruped, shares many similarities with regard to the behaviour of its skeleton to that of humans; a rapid bone formation and mineralization occurs 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 accomplish by the 12<sup>th</sup> month 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 isoflavones 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. Actually, Kohler *et al.* (23) and Steichen & 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 accordance 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 foetus. 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.

Moreover, we postulated the more complex theory that the bone effect of phytoestrogens might vary according to the menopause status. Given that phytoestrogens 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 ovariectomised rat (57). In our experimental conditions, postnatal exposure in intact animals did not improve bone mineral density. Consequently, the sex related differences in bone turnover and the role of sex hormones on the metabolism of bone in aging rodent need further work 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

oestrogen receptor (ER) and bind with equal affinity to both ER $\alpha$  and ER $\beta$  (58). ER $\alpha$  and ER $\beta$  mRNAs are expressed in osteoblasts, and the expression of ER $\beta$  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 also showed that ER $\beta$  mRNA was expressed predominantly in rat osteoblasts covering the metaphyseal bone trabecular surface <sup>(61)</sup>, and neither ER $\alpha$  nor ER $\beta$  mRNA was detected in rat cortical bone (60). Binding of oestrogen receptor to oestrogen response element (ERE) induces gene activation and is an important step in oestrogen-induced biological effects. Recently, a study by Kostelac *et al.* (61) has shown that estradiol and phytoestrogens induced an increase in ER binding to ERE in a concentration-dependant manner. Genistein (500  $\mu$ M) and daidzein (850  $\mu$ M) preferentially activate the binding of ER $\beta$  to ERE. The endogenous hormone 17 $\beta$  estradiol, as the daidzein metabolite equol, activate the binding of ER $\beta$  to ERE only slightly more effectively than the binding of ER $\alpha$  to ERE (61). So, exposure to IF may produce estrogenic effects in females. Here, the perinatal exposures appear to produce the most marked effects. However, non-estrogenic 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).

As a conclusion, lifelong exposure to phytoestrogens was seldom evaluated, even though it is highly relevant. This experiment demonstrates that rats born from mothers exposed to isoflavones during pregnancy and lactation exhibited improved total and metaphyseal femoral bone mineral density, as well as bone strength at 24 months. Thus, our data suggest that early programming events associated with phytoestrogens intake could be involved in bone mass variations during aging, since perinatal exposure to soy isoflavones lead to significant increase 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 argue for further studies in human, investigating 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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## **FIGURE LEGENDS**

### **Figure 1. Experimental design.**

### **Figure 2. Body weight (A), body fat mass (B) and uterine weight (C) changes during the experimental period.**

Control mothers at T0 (◇), isoflavones fed mothers at T0 (▲), F1 generation perinatally exposed to the standard diet and provided the standard diet (C-C) (◇) or the isoflavones-enriched diet (C-IF) (○) after weaning, F1 generation perinatally exposed to soy isoflavones and provided the standard diet (IF-C) (■) or the isoflavones-enriched diet (IF-IF) (▲) after weaning. Values are expressed as means±SEM.

A, body weight was significantly higher in all animals fed isoflavones compared to control groups (C-C) at 24 months (\*:  $P<0.01$ )

B, body fat mass was significantly higher in all animals fed isoflavones compared to control groups (C-C) at 24 months (\*:  $P<0.05$ )

C, uterine weight was significantly lower in isoflavones supplemented mothers (#:  $P<0.05$ ). Uterine weight was significantly higher in all animals perinatally exposed to isoflavones (IF-C and IF-IF) compared to the groups C-C and C-IF at 6, 12 and 18 months (†:  $P<0.01$ ).

### **Figure 3. Total (T-BMD), diaphyseal (D-BMD), metaphyseal (M-BMD) femoral bone mineral density (A) and femoral failure load (B) changes during the experimental period.**

Control mothers at T0 (◇), isoflavones fed mothers at T0 (▲), F1 generation perinatally exposed to the standard diet and provided the standard diet (C-C) (◇) or the isoflavones-enriched diet (C-IF) (○) after weaning, F1 generation perinatally exposed to soy isoflavones and provided the standard diet (IF-C) (■) or the isoflavones-enriched diet (IF-IF) (▲) after weaning. Values are expressed as means±SEM.

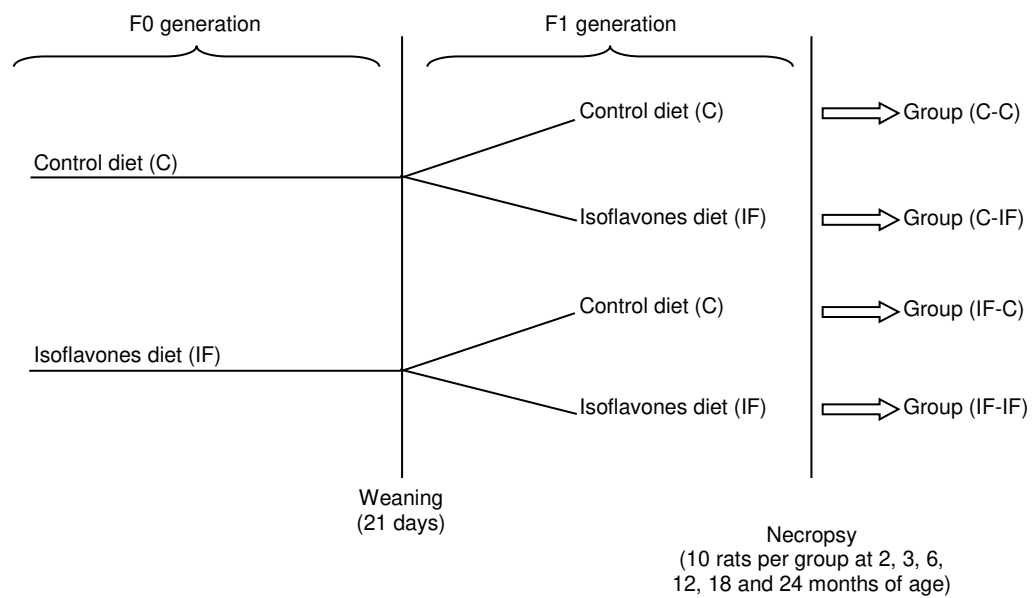
A, T-BMD was significantly higher in animals perinatally exposed to isoflavones compared to C-C and C-IF groups at 24 months (†:  $P<0.05$ ); D-BMD did not vary significantly at 24 months; M-BMD was significantly higher in perinatally exposed to isoflavones compared to C-C and C-IF groups at 24 months (†:  $P<0.01$ ); Femoral T-BMD, D-BMD and M-BMD were significantly lower in C-IF rats compared to C-C, IF-C and IF-IF animals at 12 and 18 months. ‡:  $P<0.05$ ).

B, femoral failure load was significantly higher in animals perinatally exposed to isoflavones compared to C-C and C-IF groups at 24 months (†:  $P<0.05$ )

### **Figure 4. Plasma osteocalcin concentraton (A) and urinary deoxypyridinoline excretion (B) changes during the experimental period.**

Control mothers at T0 (◇), isoflavones fed mothers at T0 (▲), F1 generation perinatally exposed to the standard diet and provided the standard diet (C-C) (◇) or the isoflavones-enriched diet (C-IF) (○) after weaning, F1 generation perinatally exposed to soy isoflavones and provided the standard diet (IF-C) (■) or the isoflavones-enriched diet (IF-IF) (▲) after weaning. Values are expressed as means±SEM.

### **Table 1. Composition of the soy protein-free powdered semipurified diet**



**Figure 1. Experimental design.**

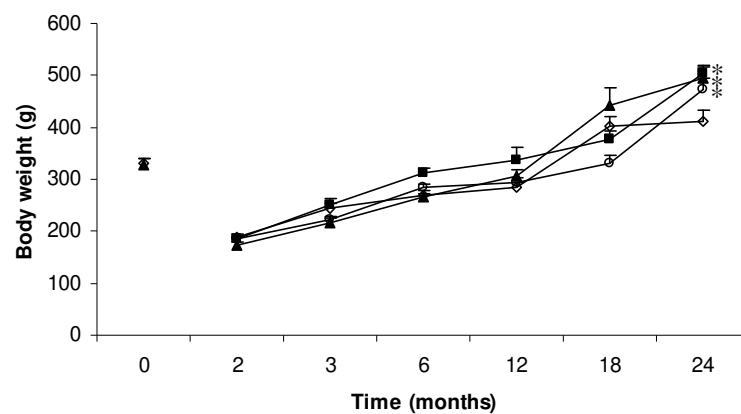
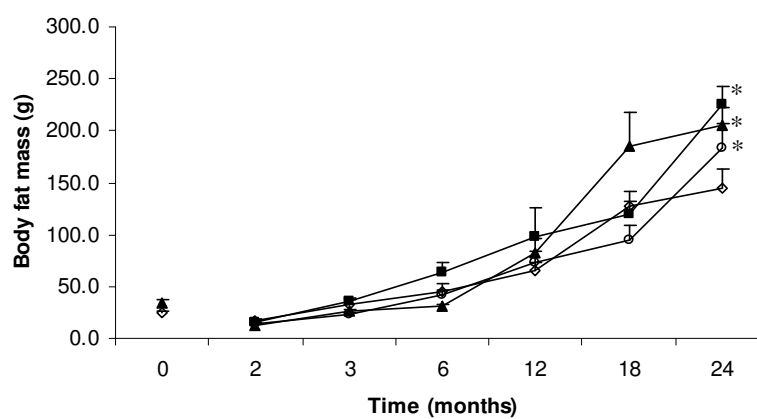
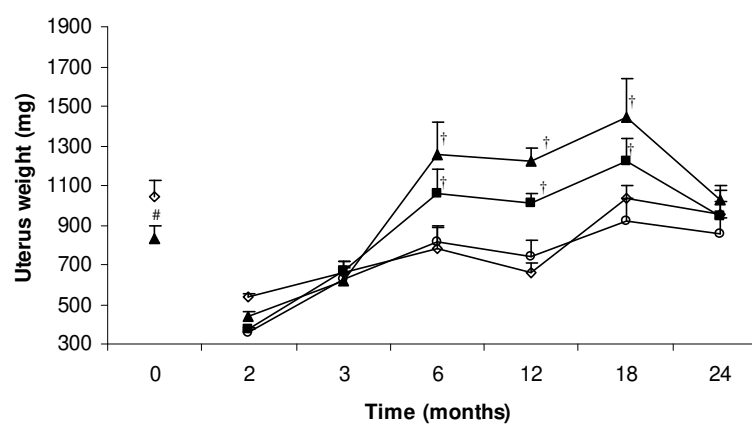
**A****B****C**

Figure 2. Body weight (A), body fat mass (B) and uterine weight (C) changes during the experimental period

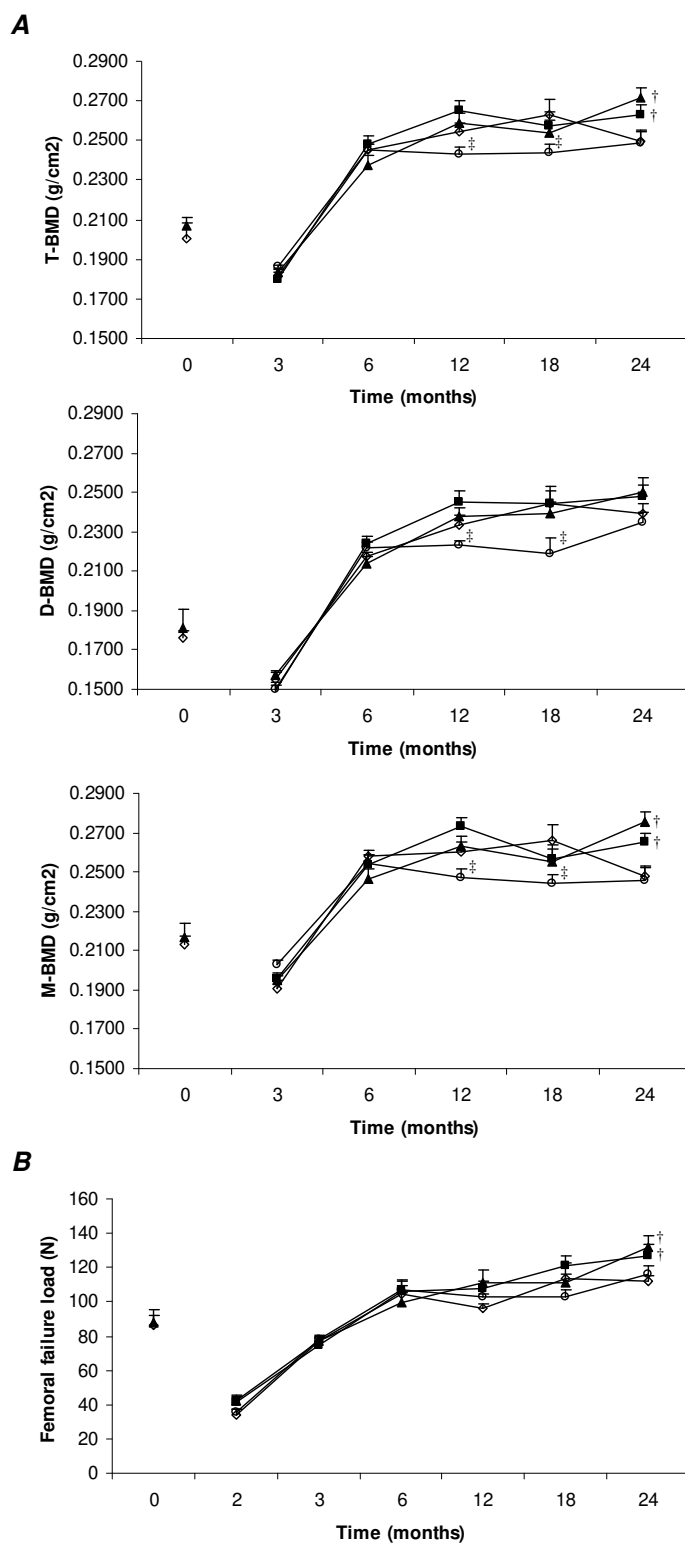


Figure 3. Total (T-BMD), diaphyseal (D-BMD), metaphyseal (M-BMD) femoral bone mineral density (A) and femoral failure load (B) changes during the experimental period.

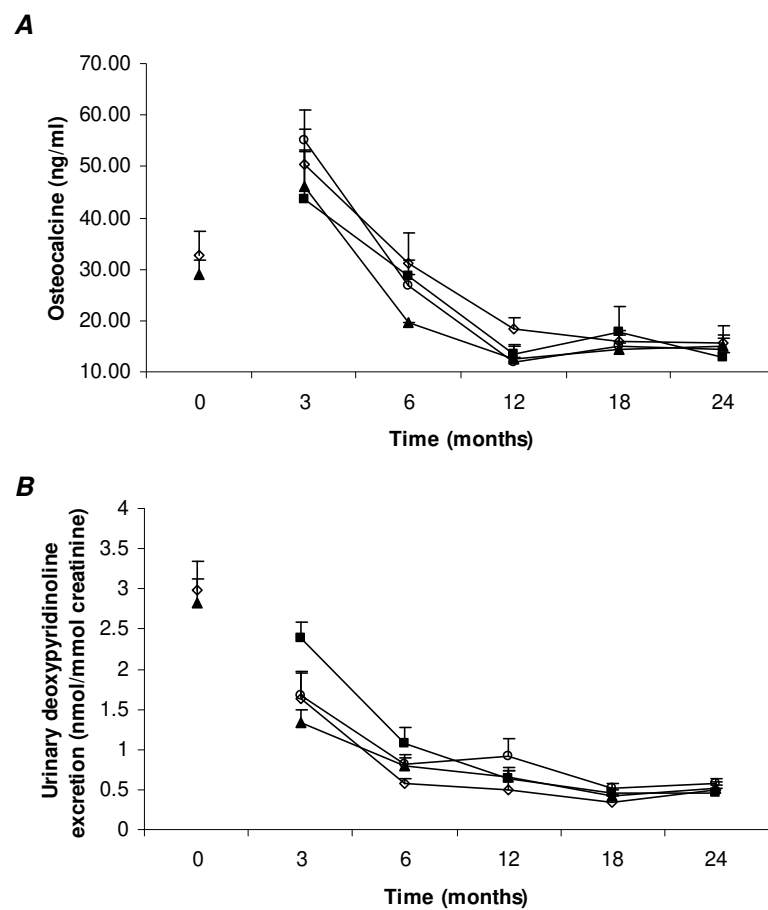


Figure 4. Plasma osteocalcin concentraton (A) and urinary deoxypyridinoline excretion (B) changes during the experimental period.

**Table 1. Composition of the soy protein-free powdered semipurified diet**

Ingredient <sup>1</sup>	g/kg
Casein	200
Sucrose	220
Cornstarch	440
Cellulose fiber	50
Peanut oil	25
Rapeseed oil	25
Vitamin mixture <sup>2</sup>	10
Mineral mixture <sup>3</sup>	25
DL-Methionine	3
Choline bitartrate	2

<sup>1</sup> Casein (Union des caséineries, Surgères, France), sucrose (Eurosucré, 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).

<sup>2</sup> Expressed in mg/kg of mixture : retinyl palmitate (250 IU/mg), 2000 ; cholecalciferol (400 IU/mg), 312 ; DL- $\alpha$ -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, 871.9g.

<sup>3</sup> Expressed in g/kg of mixture :  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , 308 ;  $\text{K}_2\text{HPO}_4$ , 194 ;  $\text{CaCO}_3$ , 146 ;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 109 ; NaCl, 168 ; MgO, 24.3 ;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 20.9 ;  $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ , 12.1 ;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 12.1 ;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.0005 ;  $\text{CoCO}_3$ , 0.0005 ;  $\text{Na}_2\text{SeO}_3$ , 0.0005.