Dose-dependent bone-sparing effects of dietary isoflavones in the ovariectomised rat

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The dose-dependent bone-sparing effects of dietary isoflavones (IF) were investigated in adult (7-month-old) Wistar rats. Forty animals were ovariectomised, allocated into four groups of ten rats each, and immediately treated orally with IF at 0 (OVX), 20 (IF20), 40 (IF40) or 80 (IF80) mg/g body weight per d for 91 d; ten sham-operated (SH) controls received the same diet without added IF. Animals were killed on day 91. Both femoral failure load and total femoral, diaphyseal or metaphyseal bone mineral densities (BMD) were lower in OVX animals than in SH animals. Urinary deoxypyridinoline (DPD) excretion, a marker of bone resorption, and plasma osteocalcin (OC) levels, a marker of osteoblast activity, were higher in OVX animals than in SH animals. Total femoral and diaphyseal BMD and femoral failure load were similar in IF-treated rats and SH rats. Although metaphyseal BMD in IF40 or IF80 rats was similar to that in SH rats, its value was lower in IF20 rats than in controls. The day 91 urinary DPD excretion in IF40 and IF80 rats, but not in IF20 rats, was similar to that in SH rats. Day 91 plasma OC concentrations in IF-treated rats were similar to day 45 values, but were decreased in OVX and SH rats. Thus, daily IF consumption prevented ovariectomy-induced bone loss, both by depressing bone resorption and stimulating osteoblast activity. Moreover, as only the highest IF level induced a weak uterotrophic activity, the optimal IF dose which preserves both cancellous and cortical bone, but exhibits no oestrogen-like effects on the uterus, was 40 µg/g body weight per d.

Dietary isoflavones: Bone: Bone protective effects

The cessation of ovarian function, and more particularly oestrogen deficiency, is the major cause of post-menopausal osteoporosis (Albright et al. 1941). Although hormone-replacement therapy (HRT) is the only consistently effective treatment to prevent post-menopausal bone loss and reduce fracture risks, it may not be prescribed to all post-menopausal women because of undesirable side effects (Taylor, 1997). Selective oestrogen receptor (OR) modulators, which bind to the OR and exhibit either oestrogenic or antioestrogenic tissue-selective properties, might be suggested as alternatives to HRT. In the same way, phyto-oestrogens, which also bind to the OR (Miksicek, 1994) with a higher binding affinity for the ORβ protein than for the ORα protein (Kuiper et al. 1998), may act either as antioestrogens or weak oestrogens.

Phyto-oestrogens were identified as being responsible for widespread infertility in sheep grazing on Australian pastures containing Trifolium spp. clover (Bennetts et al. 1946; Braden et al. 1967). Epidemiological studies on disease prevalence have suggested positive associations between isoflavone (IF) consumption and decreased risk of cancer, cardiovascular diseases, and even osteoporosis, given the low mortality rates for such pathologies reported in most Asian countries (Bingham et al. 1998; Anderson et al. 1999). It was recently reported that soyabean proteins containing a high concentration of IF protected post-menopausal women against spinal bone loss (Potter et al. 1998). In the ovariectomised rat classically used as animal model for post-menopausal osteoporosis (Kalu, 1991; Wronski & Yen, 1991; Miller et al. 1995; Mosekilde, 1995), treated with IF at 20, 40 or 80 mg isoflavones/g body weight per d respectively; OC, osteocalcin; OR, oestrogen receptor; OVX, ovariectomised rats receiving no added dietary isoflavones; SH, sham operated.

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Abbreviations: BMD, bone mineral density; DPD, deoxypyridinoline; HRT, hormone-replacement therapy; IC, initial controls; IF, isoflavones; IF20, IF40, IF80, ovariectomised rats receiving 20, 40 or 80 µg isoflavones/g body weight per d respectively; OC, osteocalcin; OR, oestrogen receptor; OVX, ovariectomised rats receiving no added dietary isoflavones; SH, sham operated.
Materials and methods

Animals and diets

The study was conducted in accordance with current legislation on animal experiments in France. Fifty-six 195-d-old female Wistar rats from INRA Clermont-Ferrand/St Genès Champanelle, France) were individually housed in a metallic cage allowing separation and collection of urine, at 21°C, with 12 h–12 h light–dark cycles. Animals were fed a soyabean-protein-free powdered semi-purified diet (Table 1) from INRA Jouy en Josas (France) for 15 d. At 210 d of age (body weight 310 (SE 8) g), six rats designated as initial controls (IC) were killed, and fifty other rats were intraperitoneally anaesthesised with chloral hydrate (Fluka Chemie AG, Buchs, Switzerland; 80 g/l in saline solution (9 g NaCl/l); 0.4 ml/100 g body weight) and either sham-operated (SH; n 10) or ovariectomised (n 40). On the first day after surgery (day 0), ovariectomised animals were randomly allocated to four groups of ten rats each and given IF at 0 (OXY), 20 (IF20), 40 (IF40) or 80 (IF80) μg/g body weight per d for 91 d. The three IF diets were prepared by mixing a powdered soyabean-IF concentrate (Novasoy™ Isoflavone compound 152–400; Archer Daniels Midland Co., Decatur, IL, USA; containing 348 mg total IF (genistein 159, daidzein 156, glycitin 33)/g) with the semi-purified diet. SH and ovariectomised animals received the same humidified oils, Bailly, Aulnay sous Bois, France; peanut and rapeseed oils, Bailly, Aulnay sous Bois, France; vitamin mixture, Roche, Neuilly sur Seine, France; mineral mixture, Prolabo, Fontenay sous Bois, France; DL- methionine, Jerafrance, Jeufosse, France.

Table 1. Composition of the soyabean-protein-free powdered semi-purified diet

<table>
<thead>
<tr>
<th>Ingredient*</th>
<th>Content (g/kg)</th>
</tr>
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<tbody>
<tr>
<td>Casein</td>
<td>180.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>210.0</td>
</tr>
<tr>
<td>Maize starch</td>
<td>430.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>100.0</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>25.0</td>
</tr>
<tr>
<td>Rapeseed oil</td>
<td>25.0</td>
</tr>
<tr>
<td>Vitamin mixture†</td>
<td>10.0</td>
</tr>
<tr>
<td>Mineral mixture‡</td>
<td>18.5</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Sources of ingredients: casein, Union des caséineries, Surgères, France; sucrose, Eurosucrè, Paris, France; maize starch, Cerestal, Saint-Maur, France; cellulose, Durieux, Marne la Vallée, France; peanut and rapeseed oils, Bailly, Aunay sous Bois, France; vitamin mixture, Roche, Neully sur Seine, France; mineral mixture, Prolabo, Fontenay sous Bois, France; DL- methionine, Jerafrance, Jeufosse, France.
† With cholecalciferol 32·25 μg/kg.
‡ With (g/kg) Ca 2·3, P 1·6, Mg 0·42.
(1 g/l; Carlo Erba Reagenti, Val de Reuil, France). The sensitivity was 2 μmol/l for 1 % of absorption. The limit of detection was 0.025 μmol/l.

Physical analysis

Femoral failure load. Femoral length and mean diaphyseal diameter were measured using a precision caliper (Mitutoyo, Telford, Shropshire, UK). The femoral failure load was determined using a Universal Testing Machine (Instron 4501; Instron, Canton, MA, USA), according to a three-point bending test (Turner & Burr, 1993). The two lower supports were separated by a 20 mm distance to guarantee that 85–90 % of the bone flexure was due to bending. The cross-head speed was 0.5 mm/min.

Bone mineral density. BMD was assessed by dual-energy X-ray absorptiometry using a Hologic QDR-4500 A X-ray bone densitometer (Hologic, Massy, France). The total femur BMD, as well as BMD of two subregions, one corresponding to the diaphysis, which is rich in cortical bone, and the other to the distal femur metaphyseal zone, which is mainly cancellous bone, were determined (Pastoureau et al. 1995).

Statistical analysis

Results were expressed as mean with their standard errors (SE). All data were analysed using the GraphPad InStat software (Microsoft, San Diego, CA, USA). ANOVA was first performed to test for any significant differences among groups. When significant, the Student–Newman–Keuls multiple comparison test was used to determine the specific differences between means (Snedecor & Cochran, 1967). Parametric ANOVA was performed when data were sampled from populations with equal variance. If not, non-parametric methods were selected: a Kruskall–Wallis test was first performed; if it indicated a significant difference among groups, the Mann–Whitney U test was used to determine specific differences. Thus, analysis of uterine weight and plasma phyto-oestrogen concentrations was performed with non-parametric methods. The level of significance was $P < 0.05$ for all statistical tests.

Results

Body composition and uterine weight

Fig. 1 shows the body weight change during the experimental period. In each group, body weight was increased between day 0 and day 91 ($P < 0.001$); no significant difference was observed between groups ($P > 0.05$) on any experimental day. Fat mass (g/100 g body weight) was 23 (SE 2) in SH rats and 14 (SE 2) in IC rats ($P < 0.005$), and lean mass (g/100 g body weight) was 74 (SE 2) in SH rats and 83 (SE 2) in IC rats ($P < 0.005$). The day 89 values

![Fig. 1. Body weight changes in sham-operated (●) and ovariectomised rats without (○) or with isoflavones at 20 (●), 40 (△) or 80 (○) μg/g body weight per d. Values are means with their standard errors represented by vertical bars for ten rats. Mean values were significantly different from that on day 0: ***$P < 0.001$. For details of diets and procedures, see Table 1 and p. 308.](https://www.cambridge.org/core/core)
were similar between groups (fat mass 22 (SE 1), lean mass 75 (SE 1)).

As indicated in Table 2, uterine weight was not different between IC and SH groups (P > 0.05). On day 91, uterine weight measured in OVX, IF20, IF40 and IF80 rats was lower than in SH rats (P < 0.005). Moreover, although there was no significant change between OVX and IF20 or IF40 rats, uterine weight in IF80 rats was higher than that in OVX rats (P < 0.005).

### Biochemical analysis

As shown in Table 3, plasma genistein, daidzein and equol concentrations on day 91 were lower in IF20 rats than in IF40 or IF80 rats (P < 0.05). No significant difference was observed between IF40 and IF80 rats (P > 0.05). Phyto-oestrogen was not detected in plasma from IC, SH or OVX rats.

Fig. 2 shows plasma OC concentrations. No significant difference was observed between IC rats and the day 45 value for SH rats (P > 0.05). However, plasma OC concentrations in SH rats decreased between days 45 and 91 (P < 0.05), inducing that the day 91 value was lower than that for IC rats (P < 0.05). Similarly, plasma OC concentrations in OVX rats were lower on day 91 than on day 45 (P < 0.05), but there was no significant difference between values on days 45 and 91 in IF20, IF40 or IF80 rats (P > 0.05). Moreover, plasma OC concentrations on day 45 were significantly higher in OVX rats than in SH, IF20, IF40 or IF80 rats (P < 0.05). As a result, day 91 values were reduced in SH rats compared with ovariectomised rats with or without IF (P < 0.05).

Fig. 3 shows urinary DPD excretion. Values measured in SH rats on days 45 and 91 were similar, and not different from those in IC rats (P > 0.05). Moreover, no significant change was induced between days 45 and 91 in OVX rats (P > 0.05), while values in IF20, IF40 or IF80 rats were lower on day 91 than on day 45 (P < 0.01). Furthermore, urinary DPD excretion on days 45 and 91 was higher in OVX rats than in SH rats (P < 0.005), and values within 1 d were similar among ovariectomised rats. However, the urinary DPD excretion measured in IF40 or IF80 rats on day 91 was similar to that in SH rats (P > 0.05).

Fig. 4 shows urinary Ca excretion, which was lower in IC rats than the day 45 value measured in SH rats (P < 0.05). No significant difference was observed between days 45 and 91 in SH rats (P > 0.05), but the day 91 value was higher than that in IC rats (P < 0.05). Urinary Ca excretion in OVX rats decreased between days 45 and 91 (P < 0.05), while no significant change was apparent during this period of time in IF20, IF40 or IF80 rats (P > 0.05). Furthermore, all groups showed the same pattern on day 45 (P > 0.05). Thus, urinary Ca excretion on day 91 was lower in OVX rats than in SH rats (P < 0.05), with those of IF20, IF40 and IF80 rats being similar to those of OVX (P > 0.05) and SH rats (P > 0.05). There were no significant differences in plasma Ca concentration between groups (2.61 (SE 0.01) mmol/l).

### Physical analysis

Femoral sizes were similar in all groups (length 37.6 (SE 0.1) mm, diameter 3.70 (SE 0.03) mm). Moreover, as shown in Fig. 5, the femoral failure load was similar in IC and SH rats (P > 0.05). Day 91 values were lower in OVX rats than in SH rats (P < 0.05), while variables measured in IF20, IF40 and IF80 rats were higher than those in OVX rats and not different from those in SH rats.

Changes in BMD of the total femur and its diaphyseal and metaphyseal subregions are shown in Fig. 6 (A, B and C respectively). No significant difference was observed between IC and SH (P > 0.05). On day 91, BMD was lower in OVX rats than in SH rats (P < 0.01). Total BMD in IF20, IF40 and IF80 rats was higher than that in OVX rats, and even reached the same level as that in SH rats. The same pattern was observed for diaphyseal BMD in IF20 and IF40 rats. Diaphyseal BMD in IF80 rats was similar to...
Fig. 2. Plasma osteocalcin concentrations measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) µg/g body weight per d. Values are means with their standard errors represented by vertical bars for six (IC) or ten (SH, OVX, IF20, IF40, IF80) rats. Mean value for SH rats on day 91 was significantly different from that for IC rats: *P < 0.05. Within a group, mean value on day 91 was significantly different from that on day 45: †P < 0.05. a,b For day 45 and for day 91 mean values not sharing a common letter were significantly different P < 0.05. For details of diets and procedures, see Table 1 and p. 308.

Fig. 3. Urinary deoxypyridinoline (DPD) excretion measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) µg/g body weight per d. Values are means with their standard errors represented by vertical bars for six (IC) or ten (SH, OVX, IF20, IF40, IF80) rats. Within a group, mean value on day 91 was significantly different from that on day 45: ‡P < 0.01. a,b For day 45 and for day 91 mean values not sharing a common letter were significantly different (P < 0.005). For details of diets and procedures, see Table 1 and p. 308.
Fig. 4. Urinary calcium excretion measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) μg/g body weight per d. Values are means with their standard errors represented by vertical bars for six (IC) or ten (SH, OVX, IF20, IF40, IF80) rats. Mean value for SH rats was significantly different from that for IC rats: *P < 0.05. Within a group, mean value on day 91 was significantly different from that on day 45: †P < 0.05. *a,b For day 45 and for day 91 mean values not sharing a common letter were significantly different (P < 0.05). For details of diets and procedures, see Table 1 and p. 308.

Fig. 5. Femoral failure load measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) μg/g body weight per d. Values are means with their standard errors represented by vertical bars for six (IC) or ten (SH, OVX, IF20, IF40, IF80) rats. a,b Mean values on day 91 not sharing a common letter were significantly different (P < 0.05). For details of diets and procedures, see Table 1 and p. 308.
that in OVX ($P > 0.05$) and SH rats ($P > 0.05$). Finally, contrary to results obtained in IF20 rats, metaphyseal BMD in IF40 and IF80 rats was higher than that in OVX rats and similar to that in SH rats.

**Discussion**

Arjmandi *et al.* (1996) previously showed the bone-sparing effects of soyabean protein consumption in the young ovariectomised rat model for post-menopausal osteoporosis. Similarly, soyabean glycoside IF (Arjmandi *et al.* 1998), genistein or daidzein (Ishida *et al.* 1998), or their respective $6^\alpha$-o-succinylated products (Toda *et al.* 1999) were reported to protect the skeleton in the young ovariectomised rat. We recently showed that daily consumption of genistein or daidzein prevented the ovariectomy-induced osteopenia in adult rats (Picherit *et al.* 2000). However, the long-term dose–response effects
of dietary soyabean IF (including both genistein and daidzein) on bone loss in adult rats have never been investigated. The present study therefore reports for the first time the dose-dependent bone-sparing effects of a soyabean IF mix given orally for 91 d to 7-month-old ovariectomised rats.

Plasma phyto-oestrogen levels were increased in a dose-dependent manner (Table 3), confirming the three levels of IF consumption. Such concentrations were 1000–100 000-fold higher than plasma oestriadiol levels, depending on the diet, the phyto-oestrogen and the physiological status of rats. Effectively, except for a rise occurring late in dioestrus with a peak at mid-pro-oestrus (88 pg/ml), the basal plasma oestriadiol level throughout the 4 d oestrous cycle of the rat ranges from 17 to 37 pg/ml (Butcher et al. 1974). With regard to the effect of ovariectomy (confirmed by uterine atrophy 3 months later; Table 2) on bone, we observed a decrease in femoral BMD, both at cancellous and cortical sites (Fig. 6). The diaphyseal BMD reduction was also associated with an impairment of mechanical properties, as shown by the lower femoral failure load in OVX rats than in SH rats (Fig. 5). Bone loss probably resulted from an increase in bone turnover, as indicated by higher plasma OC concentration and urinary DPD excretion in OVX rats than in SH rats (Figs. 2 and 3). Pair-feeding minimised any effects of the hyperphagia and the increase in body weight that are associated with ovariectomy in rats (Kalu, 1991). As a result, fat mass was not greater in OVX rats than in SH rats. In these experimental conditions neither body weight nor body composition were influenced by daily IF consumption (Fig. 1).

As indicated in Fig. 6, IF prevented the ovariectomy-induced BMD decrease, both in the whole femur and its metaphyseal or diaphyseal subregions. Furthermore, diaphyseal BMD sparing effects were associated with a preservation of mechanical properties, as shown by the higher femoral failure load in rats receiving IF than in OVX rats (Fig. 5). These data are in accordance with previous studies, in which the oral dose of genistein and/or daidzein was about 28 (Arjmandi et al. 1998) or 50 (Ishida et al. 1998; Toda et al. 1999) µg/g body weight per d (Ishida et al. 1998; Toda et al. 1999), weakly increased uterine weight. When given subcutaneously a genistein dose of 25 µg/g body weight per d (Fanti et al. 1998) was required to induce uterotrophic activity, whereas 1 or 5 (Fanti et al. 1998) or about 20 (Ishimi et al. 1999) µg/g body weight per d did not increase uterine weight, while preventing bone loss. It is likely that OR is involved in the tissue-selective IF properties, which might depend on the predominance of one of the two OR subtypes in the target tissue. Effectively, although both ORα and ORβ proteins were detected in the rat uterus (Hiroi et al. 1999), higher expression of ORα mRNA than ORβ mRNA was recently reported in young intact rat uterus (Kuiper et al. 1997; Lim et al. 1999). Furthermore, both genistein and daidzein possess a greater affinity for ORβ than for ORα (Kuiper et al. 1997, 1998). In rat bones, although it was recently reported that expression of both ORα and ORβ mRNA was evident, with an ORβ mRNA predominantly expressed in osteoblasts at the metaphyseal cancellous surface (Windahl et al. 2000), and that ORβ mRNA expression was higher in cancellous bone than in cortical bone (Onoe et al. 1997), ORα:ORβ in cancellous or cortical bone is still unknown. Nevertheless, genistein effects might also result from various mechanisms of action, including both OR and non-OR-dependent pathways (Anderson et al. 1999).

In conclusion, the present study showed that daily IF consumption over a 91 d period in the adult ovariectomised rat model for post-menopausal osteoporosis provided bone-
sparing effects, both by depressing bone resorption and by stimulating osteoblast activity. Moreover, the optimal IF dose which prevented both cancellous and cortical bone loss, while not exhibiting any uterotropic activity, was 40 μg/g body weight per d, including about 18 μg daidzein/g body weight per d and 18 μg genistein/g body weight per d. Thus, although further data are required, the bone-protective effects of IF in oestrogen-deficient animals suggest that they might be a potential nutritional alternative to HRT in human subjects.

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