

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) $\mu\text{g/g}$ 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 $\mu\text{g/g}$ 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 OR β protein than for the OR α 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,

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.

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1995), bone loss is prevented by dietary soyabean proteins (Arjmandi *et al.* 1996) and, more particularly, their IF (Arjmandi *et al.* 1998), by the two main soyabean glycoside IF, genistein or daidzein (Ishida *et al.* 1998), and by their respective 6''-*o*-succinylated products (Toda *et al.* 1999) or aglycone forms (Picherit *et al.* 2000). However, little is known about effective IF doses in such an animal model. The aim of the present study was therefore to investigate the dose-dependent bone-sparing effects of long-term daily IF consumption in adult ovariectomised rats.

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/Theix (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 anaesthetised 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 (OVX), 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 (1 ml water/g food) semi-purified diet without any addition. To prevent ovariectomy-induced hyperphagia,

the daily amount of diet distributed to each rat was adjusted to the mean level consumed by SH rats on the previous day. Thus, the food intake was constant during the 91 d experimental period and similar in all groups. Animals had free access to water and were weighed weekly to adjust IF doses to body weight. At 48 h before death, the body composition was estimated by dual-energy X-ray absorptiometry (Rose *et al.* 1998). On day –1 for IC or day 45 and 91 for other rats, the 24 h urine was collected for measurement of Ca and deoxyypyridinoline (DPD), a marker of bone resorption (Robins, 1994). Simultaneously, blood samples were harvested at 09.00 hours into ice-cooled heparinised plastic tubes containing 200 peptidase inhibitory units aprotinin (Iniprol; Choay, Paris, France)/ml blood, and centrifuged (3500 g, 5 min) immediately at 4°C. Plasma was then frozen at –20°C until required for measurement of phyto-oestrogens, Ca and osteocalcin (OC), a marker of osteoblast activity (Garnero & Delmas, 1999). On day 91, animals were killed by cervical dislocation. Left and right femurs were cleaned from adjacent tissues and collected for mechanical testing and bone mineral density (BMD) measurement, respectively. Successes of ovariectomy and IF consumption were confirmed by uterine weight and plasma phyto-oestrogen concentration, respectively.

Biochemical analysis

Plasma phyto-oestrogen concentrations. Plasma genistein, daidzein and equol concentrations were measured by ELISA (Bennetau-Pelissero *et al.* 2000; Le Houérou *et al.* 2000). The sensitivity was 35, 40 and 10 nm for genistein, daidzein and equol respectively. The intra- and inter-assay variations measured on ten different assays were 4.8 and 13.1 %, 5 and 12.8 %, and 5 and 13.6 % for genistein, daidzein and equol respectively.

Plasma osteocalcin concentrations. Plasma OC was measured by radioimmunoassay using rat ¹²⁵I-labelled OC, goat anti-rat OC antibody, and donkey anti-goat second antibody (Biochemical Technologies kit; Biochemical Technologies, Stoughton, MA, USA). The sensitivity was 0.01 nmol/l. The intra- and inter-assay variations were 6.8 and 8.9 % respectively.

Urinary deoxyypyridinoline excretion. Excretion of DPD was determined by competitive radioimmunoassay, using rat monoclonal anti-DPD antibody coated to the inner surface of a polystyrene tube, and ¹²⁵I-labelled DPD (Pyrilinks-D RIA kit; Metra Biosystems Inc., Mountain View, CA, USA). The sensitivity was 2 nmol/l. The intra- and inter-assay variations were 4 and 6 % respectively. Results were expressed as nmol DPD/mmol creatinine (Robins, 1994). The urinary creatinine assay, based on a modified Jaffés's method in which picric acid forms a coloured solution in the presence of creatinine (Cook, 1975), was used to adjust DPD values for variation in urine volume.

Plasma and urinary calcium concentrations. Plasma and urinary Ca concentrations were assessed by atomic absorption spectrophotometry, using a Perkin Elmer 400 spectrophotometer (Perkin Elmer, Norwalk, CT, USA). Samples were previously diluted with a La₂O₃ solution

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

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

* Sources of ingredients: casein, Union des caséinerie, Surgères, France; sucrose, Eurosucre, Paris, France; maize starch, Cerestar, Saint-Maur, France; cellulose, Durioux, Marne la Vallée, 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.

† 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 $\mu\text{mol/l}$ for 1 % of absorption. The limit of detection was 0.025 $\mu\text{mol/l}$.

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 GraphPadInStat

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 Kruskal–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

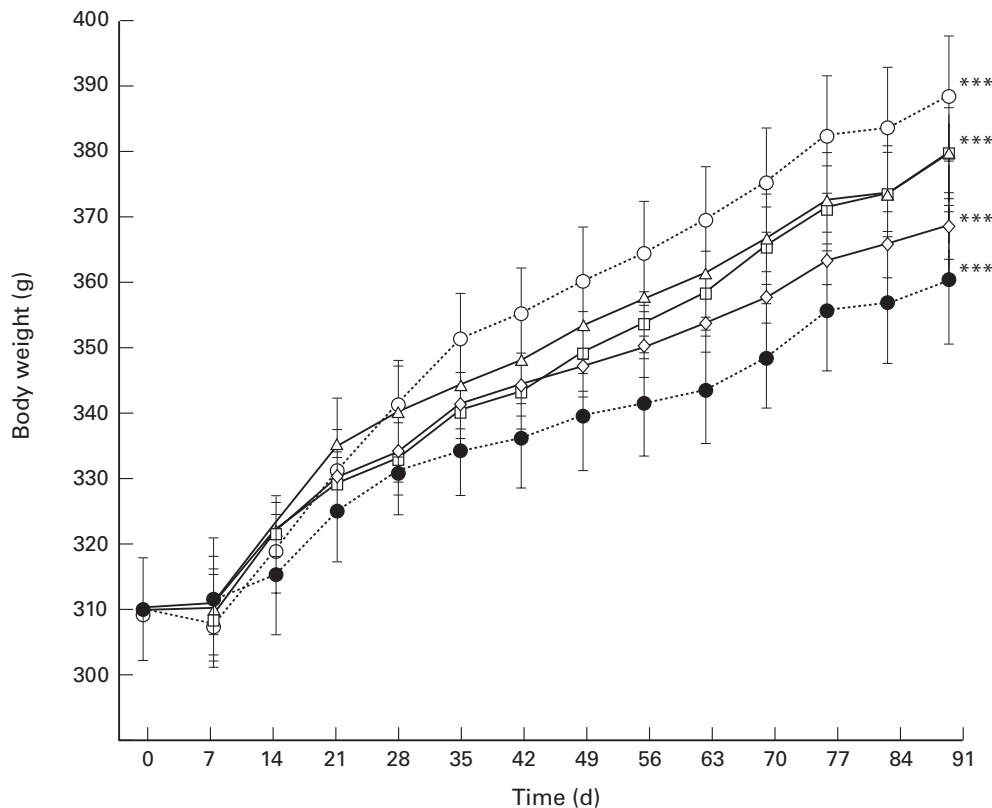


Fig. 1. Body weight changes in sham-operated (●) and ovariectomised rats without (○) or with isoflavones at 20 (□), 40 (△) or 80 (◇) $\mu\text{g/g}$ body weight per d. Values are means with their standard errors represented by vertical bars for ten rats. Mean values were significantly different from that on day 0: *** $P < 0.001$. For details of diets and procedures, see Table 1 and p. 308.

Table 2. Uterine weight measured in initial controls (IC), sham-operated (SH), and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) $\mu\text{g/g}$ body weight per d* (Mean values with their standard errors)

Treatment group	n	Uterine weight (g)	
		Mean	SE
IC†	6	0.77	0.10
SH‡	10	0.74 ^c	0.07
OVX‡	10	0.18 ^a	0.01
IF20‡	10	0.19 ^a	0.02
IF40‡	10	0.22 ^a	0.03
IF80‡	10	0.40 ^b	0.04

^{a,b,c}Mean values on day 91 with different superscript letters were significantly different ($P < 0.005$).

* For details of diets and procedures, see Table 1 and p. 308.

† Values on day -1.

‡ Values on day 91.

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 ($P > 0.05$). Phytoestrogen 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 ($P < 0.05$), indicating 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

Table 3. Plasma genistein, daidzein and equol concentrations measured in ovariectomised rats with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) $\mu\text{g/g}$ body weight per d† (Mean values with their standard errors)

Treatment group	n	Genistein ($\mu\text{mol/l}$)		Daidzein ($\mu\text{mol/l}$)		Equol ($\mu\text{mol/l}$)	
		Mean	SE	Mean	SE	Mean	SE
IF20	10	0.43	0.10	0.53	0.18	0.60	0.16
IF40	10	1.90*	0.42	2.53**	0.46	1.80*	0.34
IF80	10	4.04*	1.65	4.43**	1.45	2.68*	0.58

Mean values were significantly different from those for IF20 rats: * $P < 0.05$, ** $P < 0.01$.

† For details of diets and procedures, see Table 1 and p. 308.

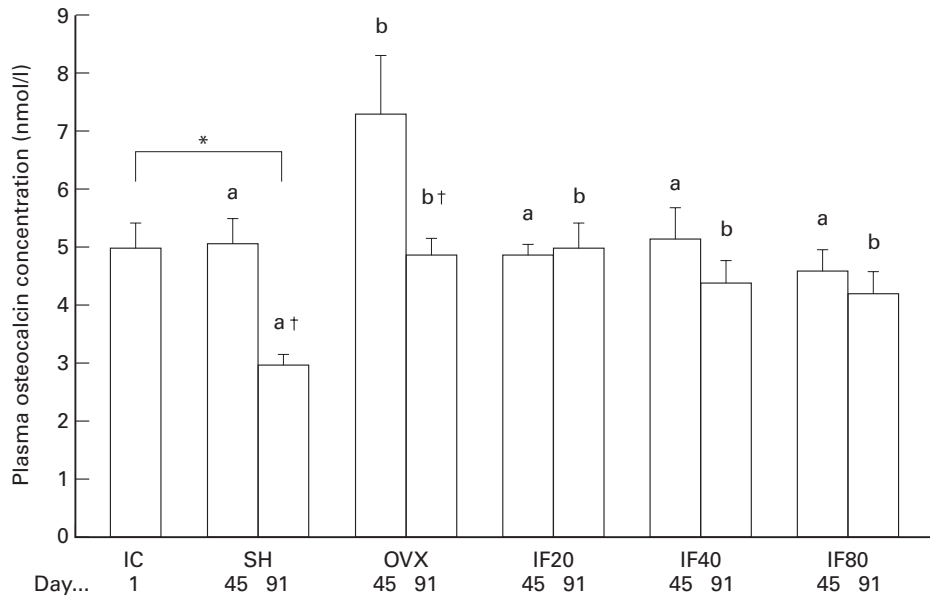


Fig. 2. Plasma osteocalcin concentrations measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) $\mu\text{g/g}$ body weight per d. Values are means with their standard errors represented by vertical bars for 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: $\dagger P < 0.05$. ^{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.

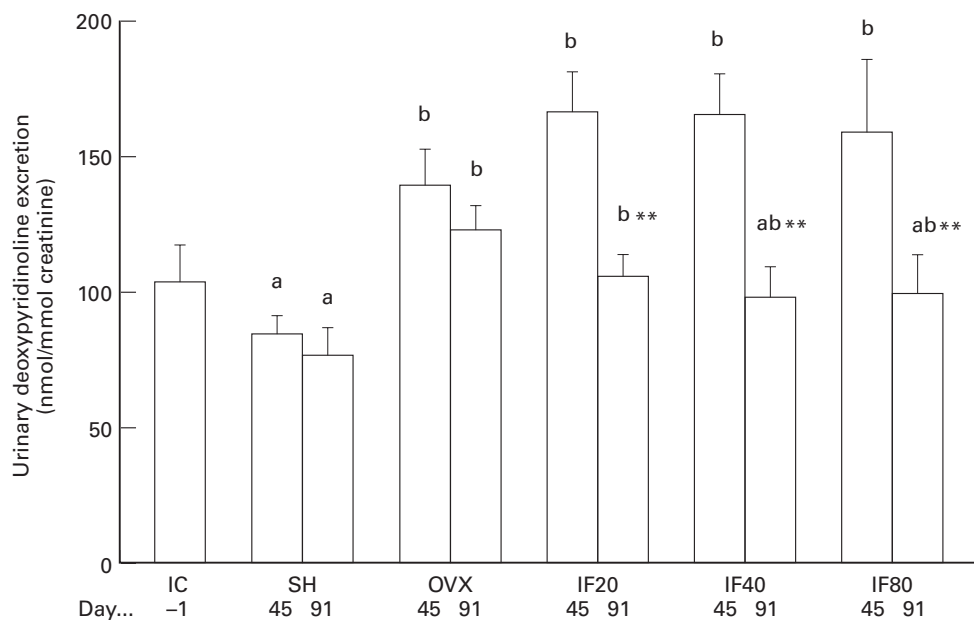


Fig. 3. Urinary deoxyypyridinoline (DPD) excretion measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) $\mu\text{g/g}$ body weight per d. Values are means with their standard errors represented by vertical 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.

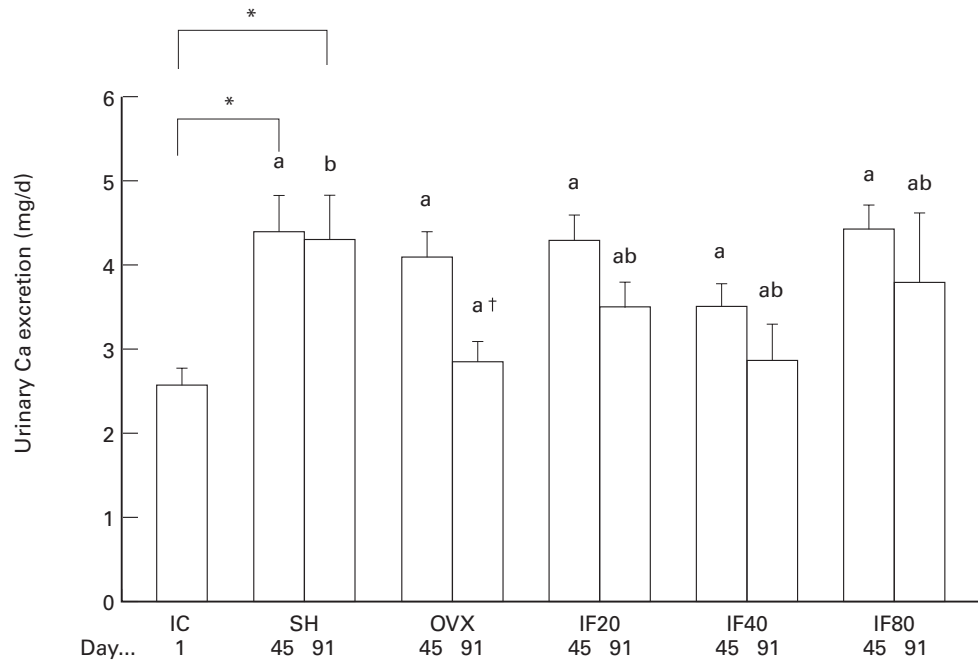


Fig. 4. Urinary calcium excretion measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) $\mu\text{g/g}$ body weight per d. Values are means with their standard errors represented by vertical bars for six (IC) 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: $\dagger P < 0.05$. ^{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.

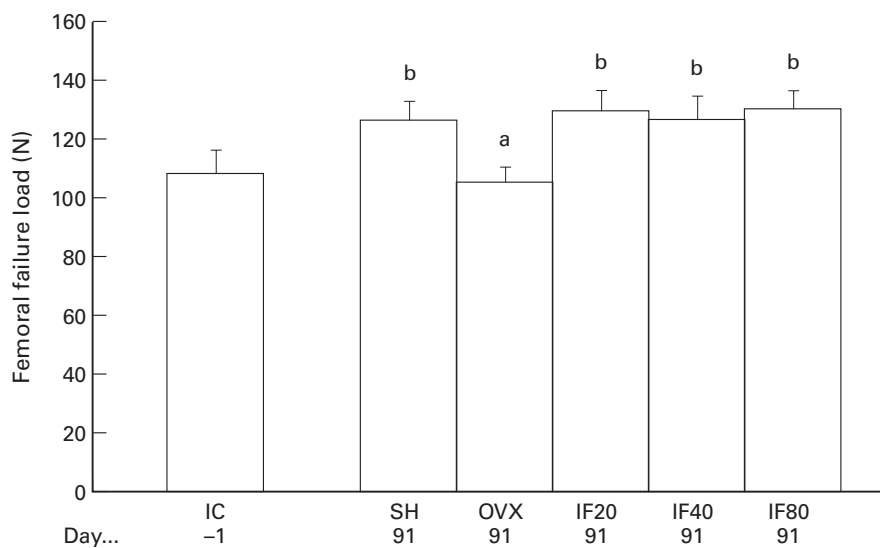


Fig. 5. Femoral failure load measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) $\mu\text{g/g}$ body weight per d. Values are means with their standard errors represented by vertical bars for six (IC) or 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.

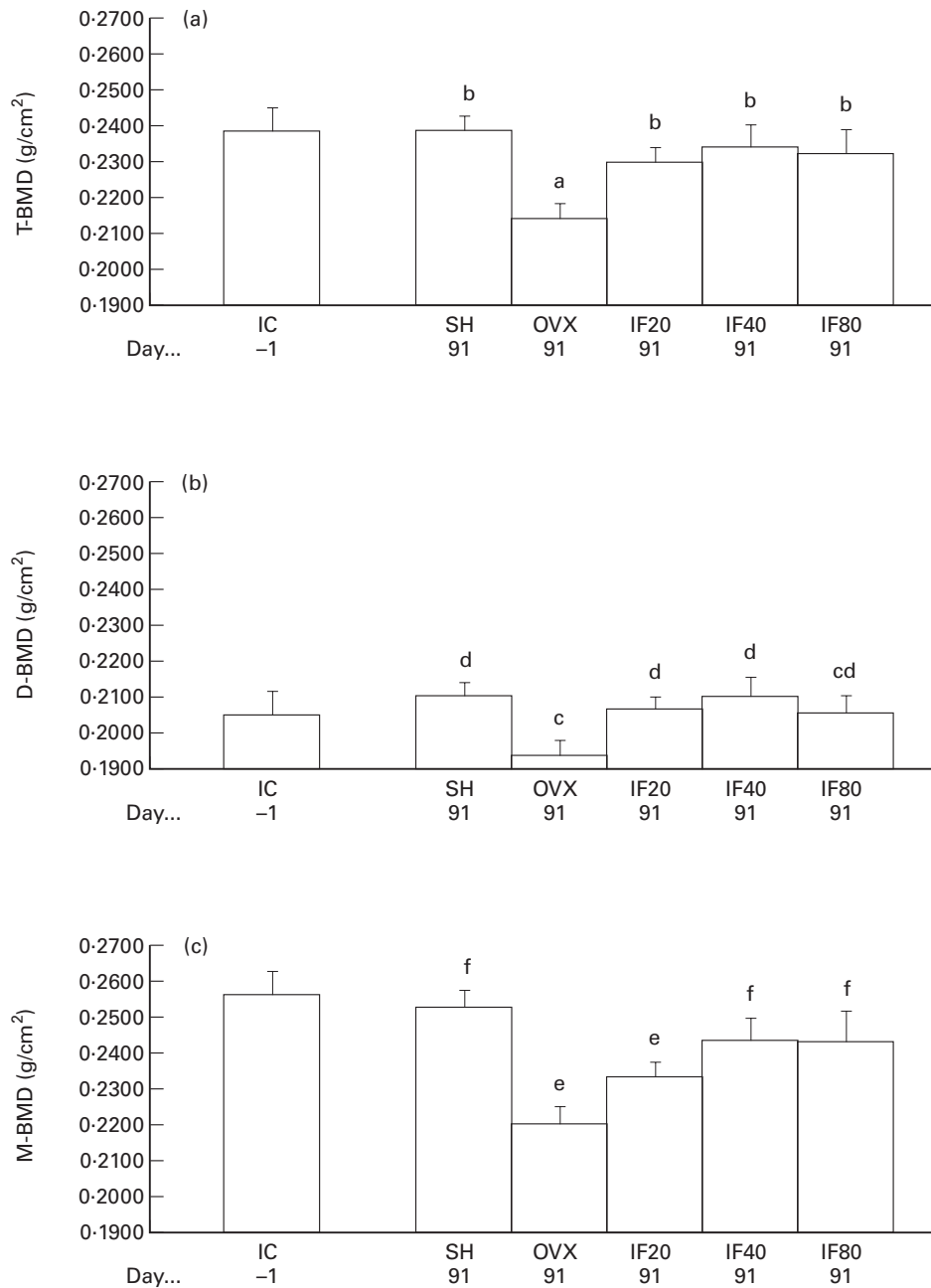


Fig. 6. (A) total femoral (T-BMD), (B) diaphyseal (D-BMD) and (C) metaphyseal (M-BMD) bone mineral densities measured in initial controls (IC), sham-operated (SH) and ovariectomised rats without (OVX) or with isoflavones at 20 (IF20), 40 (IF40) or 80 (IF80) $\mu\text{g/g}$ body weight per d. Values are means with their standard errors represented by vertical bars for six (IC) or ten (SH, OVX, IF20, IF40, IF80) rats. ^{a,b,c,d,e,f}Mean values on day 91 not sharing a common letter were significantly different: ^{a,b} $P < 0.005$, ^{c,d} $P < 0.01$, ^{e,f} $P < 0.001$. 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''-*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 oestradiol 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 oestradiol 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) $\mu\text{g/g}$ body weight per d. Moreover, in our experimental conditions cortical bone exhibited a greater response to IF than cancellous bone, as a diaphyseal BMD decrease was prevented by the lowest dose (IF20), while 40 or 80 $\mu\text{g/g}$ body weight per d were needed to elicit a protective effect at the metaphyseal level (Fig. 6). Thus, in contrast to the study of Anderson *et al.* (1998), which used genistein, we did not observe any biphasic effect of IF on cancellous bone. This discrepancy might be explained by the fact that rats received a mix of IF, which could exhibit synergetic actions.

The cancellous bone-sparing effects of IF might result from a reduced bone resorption, as shown by the lower urinary DPD excretion on day 91 than on day 45 in ovariectomised rats receiving IF; day 91 values in IF40 or IF80 were similar to those in SH (Fig. 3). In contrast, the IF consumption at 20 $\mu\text{g/g}$ body weight per d probably did not prevent the ovariectomy-induced bone resorption increase in the femoral metaphysis, as indicated by the day 91 urinary DPD excretion (Fig. 3). The bone-sparing effects of IF might also result from an increased osteoblast activity,

as indicated by plasma OC levels which were unchanged between days 45 and 91 in ovariectomised rats receiving IF; values in OVX or SH rats were depressed during this same period of time (Fig. 2). Indeed, it was recently reported that oral administration of ipriflavone, a synthetic IF inhibiting bone resorption, could also prevent ovariectomy-induced bone loss by increasing the bone formation rate (Arjmandi *et al.* 2000). Moreover, as the OC content in the human femur was higher in cortical bone than in cancellous bone (Magnusson *et al.* 1999), the increase in plasma OC might reflect activity in the cortical bone rather than in the cancellous bone, explaining partly why cortical bone was preserved.

In contrast with bone, the uterus exhibited a lower response to IF, as only high plasma phyto-oestrogen levels (IF80) weakly increased uterine weight (Table 2). These tissue-selective IF properties resulting in bone-sparing effects associated with a non-existent or modest uterotrophic activity were frequently observed in ovariectomised rodents. Effectively, uterine weight was not increased in animals given an oral dose of genistein ranging from 1.5 to 15 $\mu\text{g/g}$ body weight per d (Anderson *et al.* 1998; Picherit *et al.* 2000), genistein or 6''-*o*-succinyl-genistein at 50 $\mu\text{g/g}$ body weight per d (Ishida *et al.* 1998; Toda *et al.* 1999), daidzein 10 $\mu\text{g/g}$ body weight per d (Picherit *et al.* 2000), daidzein at 10 or 25 $\mu\text{g/g}$ body weight per d (Ishida *et al.* 1998), or genistein + daidzein at about 28 $\mu\text{g/g}$ body weight per d (Arjmandi *et al.* 1998), whereas bone was either modestly or greatly preserved, depending on which IF was used. Only higher dietary IF doses, e.g. 40 or 80 μg genistein (Santell *et al.* 1997), or 50 μg daidzein or 6''-*o*-succinyl-daidzein/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 $\mu\text{g/g}$ 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) $\mu\text{g/g}$ 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 OR α and OR β proteins were detected in the rat uterus (Hiroi *et al.* 1999), higher expression of OR α mRNA than OR β 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 OR β than for OR α (Kuiper *et al.* 1997, 1998). In rat bones, although it was recently reported that expression of both OR α and OR β mRNA was evident, with an OR β mRNA predominantly expressed in osteoblasts at the metaphyseal cancellous surface (Windahl *et al.* 2000), and that OR β mRNA expression was higher in cancellous bone than in cortical bone (Onoe *et al.* 1997), OR α :OR β 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 uterotrophic 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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