Dietary phyto-oestrogens and bone health

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The use of dietary phyto-oestrogens as a possible option for the prevention of osteoporosis has raised considerable interest because of the increased concern about the risks associated with the use of hormone-replacement therapy. However, the evidence in support of a bone-sparing effect in post-menopausal women is still not sufficiently convincing. Most studies have been performed on soyabean isoflavones (genistein and daidzein), either in the purified form or as a soyabean-based product or extract. In vitro studies using primary cell cultures or stabilised cell lines indicate that treatment with genistein may lead to a reduction in bone resorption, but effects on bone formation have also been shown. Investigations using animal models have provided convincing evidence of major improvements in bone mass or bone turnover following soyabean feeding. Cross-sectional observations in South-East Asian populations with moderately high intakes of soyabean isoflavones (50 mg/d) have shown that women in the high quartile of intake have higher bone mineral density (BMD) and reduced bone turnover, an effect that has not been shown in populations with low average intakes. Human trials have given an indication of a possible effect on lumbar spine BMD, although they have been either short term (<6 months) or methodologically weak. Unresolved issues are: the optimal dose compatible with safety; the individual differences in response that can be related to diet and genotypes; the duration of exposure. Furthermore, there needs to be an evaluation of the relative biological effects of phyto-oestrogens other than isoflavones (lignans, resorcylic acid lactones, flavanols, coumestans) that are also present in European diets.

Phyto-oestrogens: Osteoporosis: Soyabean

The increase in life expectancy has led to bone health becoming a major concern. Femur or vertebral fractures are likely events that may severely affect the quality of life of the elderly. Hormone-replacement therapies (HRT) have been the first choice for treatment of hormone-related osteoporosis, but they have major side effects that preclude their universal use: uterine bleeding and hyperplasia; cardiovascular disease; gall bladder disease; oestrogen-induced endometrial cancer; increase in the risk of breast cancer. Since the Writing Group for the Women’s Health Initiative Investigators (2002) observed a 15 % increase in invasive breast cancer in women taking oestrogen plus progestogen for <5 years and a 53 % increase in those taking it for >5 years, suitable alternatives have been sought.

The observation that South-East Asian women (Hong Kong, Indonesia, Korea, Malaysia, the Philippines, Singapore and Taiwan) report lower occurrence of hot flushes and sweating has led to the hypothesis that a protective role might be played by dietary factors, particularly soyabean phyto-oestrogens (Eden, 1998). In those countries a lower prevalence of chronic diseases such as cardiovascular disease, breast cancer and osteoporosis has also been observed. Thus, the use of dietary phyto-oestrogens has been considered as a possible option for the prevention of osteoporosis and, in view of their perceived natural origin, this approach is receiving considerable public attention. However, while many scientific panels have now reached a consensus on the effect of phyto-oestrogens on cardiovascular disease prevention, the same conclusion has not been reached for osteoporosis.

The purpose of the present review is to describe the rationale for the biological effects of phyto-oestrogens on bone and to analyse the evidence from in vitro and in vivo studies in animals and human subjects.

Chemical structure and presence in food

Phyto-oestrogen is a general definition that has been applied to “… any plant substance or metabolite that induces biological responses in vertebrates and can mimic or
modulate the actions of endogenous oestrogens usually by binding to oestrogen receptors’ (Food Standards Agency, 2002). Three main classes have been identified: flavonoids; lignans; coumestans. A fourth class, resorcylic acid lactones, should also be added, although they are less relevant for human nutrition. The main compound is in fact zearalenone, which is synthesised by Fusarium moulds that develop during long-term storage of maize and is regarded as a contaminant. The common characteristic of these four classes is that they are diphenolic compounds with structural similarities to natural and synthetic oestrogens and anti-oestrogens (an aromatic A ring with one hydroxyl group and a second hydroxyl group on the same plane of the A ring; Fig. 1). Phyto-oestrogens are present in food both as aglycones and glucosides.

The main phyto-oestrogens currently recognised are soya-bean isoflavones, mainly genistein, daidzein and glycitein (mainly contained in soyabean germ) and their glycosides (genistin, daidzin, glycitin). The oestrogenic properties of other flavonoids present in marked amounts in several plant products have also been recognised: flavonols, e.g. quercetin and its glycosylated derivative rutin (quercetin-3-O-glucose rhamnose) widely present in vegetables such as onions or apples; flavanols, e.g. luteolin and apigenin contained in black olives; flavanones, e.g. hesperetin and naringenin contained in red oranges. Lignans (lariciresinol, isolariciresinol, secoisolariciresinol, matairesinol) are mainly present in wholemeal cereals, linseed and wild berries (Mazur et al. 2000).

Fig. 1. Chemical structures of the four major classes of phyto-oestrogens, compared with those of the oestrogens and four additional classes of flavonoids commonly present in food. Isoflavones (e.g. genistein, daidzein, glycitein, formononetin, biochanin A) are present in soyabeans, chick peas and lentils. Lignans (e.g. lariciresinol, isolariciresinol, secoisolariciresinol, matairesinol) are present in wholemeal cereals, linseed and wild berries. Resorcylic acid lactones (e.g. zearalenone) are present in maize contaminated by Fusarium moulds. Coumestans (e.g. coumestrol) are present in young sprouting legumes, clover (Trifolium spp.) and lucerne (Medicago sativa) sprouts. Flavonols (e.g. quercetin, myricetin, kaempferol) are present in onions, broccoli, fennel, apples and cherries. Flavanones (e.g. naringenin, hesperidin) are present in citrus fruits and prunes. Flavones (e.g. apigenin, luteolin) are present in parsley, celery and black olives. Catechins (e.g. epicatechin, galloocatechin) are present in tea, apples and cocoa. Anthocyanidins (e.g. pelargonidin, malvidin, cyanidin) are present in cherries and grapes (Ross & Kasum, 2002).
Absorption

Absorption of isoflavones requires hydrolysis of the sugar moiety by intestinal β-glucosidase (Setchell et al. 2002b). Aglycones are then absorbed by diffusion through the enterocyte and across the intestinal wall. They undergo an extensive phase II metabolism (mainly glucuronidation and sulfation) both in the upper small intestine (gastro-intestinal mucosa) and in the liver (Setchell et al. 1998). Deconjugation takes place at the level of the target tissue (Rowland et al. 2003). Absorption is very rapid; plasma isoflavones are detected at 15–30 min post-ingestion and reach a peak at between 3 and 7 h. Lignans are absorbed more slowly; they appear in plasma at approximately 8.5 h post-ingestion (Morton et al. 1997).

Effects of phyto-oestrogens at the molecular level

The effects of phyto-oestrogens are related not only to the interaction with the oestrogen receptors (ER), but also to other actions not mediated by the ER in a similar fashion to oestrogens. Hall et al. (2001) indicate that, in addition to the classical ligand-dependent action, there are three non-classical actions: ligand-independent; DNA binding-independent; cell-surface (non-genomic) signalling. The non-classical actions seem particularly important in explaining the selective action of phyto-oestrogens on bone.

The classical action is dependent on the binding of oestrogens with ER, which are ligand-activated nuclear transcription factors, members of the nuclear receptor family of transcription factors. Two different ER have been described, ERα and ERβ, which differ in both their ligand-binding properties (C-terminal domain) and their transactivation activity (N-terminal domain). Up to five isoforms have been detected for each receptor (Nilsson et al. 2001). Different tissues have different proportions of the two receptors, with a predominance of ERα in reproductive system tissues (Food Standards Agency, 2003). Both receptors are present in bone tissue. Cells of osteoblastic origin express both ERα (Braidman et al. 1995) and ERβ (Braidman et al. 2001). Preosteoclasts express ERα, but ERα expression is lost during osteoclast maturation (Orefó et al. 1999). Indirect immuno-peroxidase staining of fracture callus biopsies has shown that expression of ERα and ERβ is similar for all cells in males and females <40 years of age, but there is a decreased proportion of osteocytes expressing ERα and ERβ proteins in women >40 years of age (Batra et al. 2003). In addition, three ER-related receptors, formerly termed ‘orphan’ receptors, have been described, but only one of them (ER-related receptor α) has been identified in osteoblastic cells and in osteoclasts in vivo and in vitro (Bonnelly et al. 2002). The two ER signal in different ways depending on both ligand and response element activities (Paech et al. 1997). Binding with different compounds induces different conformational changes in the ER. Thus, phyto-oestrogens can act as pure agonists, as partial agonists or as pure antagonists. For example, genistein binds to ERβ in the same way as 17β-oestradiol, but the transactivation helix adopts a different position such that the action is that of a partial agonist (Pike et al. 1999). ERα and ERβ can form homodimers or heterodimers with different transcription activities. ERα homodimers have a higher transcription activity than ERβ homodimers, while the heterodimers have a transcription activity intermediate between those of the α/α and β/β homodimers. The presence of different proportions of α and β receptors may lead to different combinations and this factor could also explain the tissue differences in their action.

As far as the non-classical actions are concerned, the ligand-independent action involves growth factors, such as insulin-like growth factor (IGF) 1. The modification of the phosphorylation state of the ER by cellular kinases may serve as an important mechanism of ligand-independent activation (Hall et al. 2001). The cell-surface (non-genomic) signalling involves regulation of enzymes implicated in signal transduction, such as protein tyrosine kinase, mitogen-activated protein kinase and inhibition of DNA topoisomerase II. In human mammary epithelial cells genistein has been shown to inhibit cell proliferation through the p38 mitogen-activated protein kinase pathway (Frey & Singletary, 2003). Phyto-oestrogens may also act through the phosphatidylinositol-3 kinase or serine/threonine protein kinase pathways. Phospho-serine/threonine protein kinase is also localised in the nucleus of cultured cardiomyocytes after exposure to 17β-oestradiol or genistein (Camper-Kirby et al. 2001).

Finally, phyto-oestrogens can also modulate the action of endogenous oestrogens through the regulation of ER expression and the metabolism and bioavailability of oestrogens. Pharmacological, but not physiological, concentrations of genistein can modulate sex steroid receptor expression in the rat uterus (Cotroneo et al. 2001). In vitro phyto-oestrogens inhibit the formation of 17β-[3H]oestradiol from [3H]androstenedione, although this activity has not been confirmed in vivo (Saarinen et al. 2001). A study in post-menopausal women indicated that a diet rich in phyto-oestrogens and complex carbohydrates was associated with a 25 % increase in sex hormone-binding globulin in the intervention group (Berrino et al. 2001).

The oestrogenic potency of the different compounds has been measured in competitive-binding assays, in which the relative binding affinity for ERα and ERβ is measured, as well as in transactivation assays. If the former is used (Kuiper et al. 1997) the affinity for ERα is E2 >> coumestrol > zearalenone > genistein > daidzein and the affinity for ERβ is E2 = coumestrol >> zearalenone > genistein > apigenin = kaempferol > daidzein > naringenin. If instead a transactivation assay is carried out, then for ERα E2 >> zearalenone = coumestrol > genistein > apigenin = phloretin > biochanin A = kaempferol > naringenin = formononetin = ipriflavone = quercetin = chrysin and for ERβ E2 >> genistein = coumestrol = zearalenone = daidzein = biochanin A = apigenin = kaempferol = naringenin = phloretin = ipriflavone = quercetin = chrysin.

The proliferation of several cell systems has also been used to compare the different phyto-oestrogen compounds. In a proliferation assay that used MCF-7 cells coumestrol, daidzein, luteolin and quercetin exerted a proliferation-stimulating activity as strong as that of oestradiol, but they suppressed the induction of the proliferation-stimulating activity of environmental oestrogens (Han et al. 2002). An in vitro assay that used BT-474 human breast cancer cells
demonstrated that biochanin A and genistein had the most potent oestrogenic activity. Of the other flavonoids, luteolin and naringenin displayed the strongest oestrogenicity, while apigenin had a relatively strong progestinetic activity (Zand et al. 2000).

In conclusion, the action of phyto-oestrogens varies according to the type, combination and concentration of compounds, the type of tissue and factors affecting ER presence, such as age. Phyto-oestrogens have a role as partial agonists, as they may also inhibit the action of oestrogens. Finally, androgenic and progestinetic effects should also be considered in addition to the oestrogenic effects. Thus, it would probably be more appropriate to refer to these compounds as selective oestrogen receptor modulators of plant origin.

**Effects of phyto-oestrogens on bone cells**

Genistein is the phyto-oestrogen that has been most investigated for its effect on the proliferation and differentiation of a number of cell types. Through its effect on tyrosine kinase, genistein is able to modulate cell cycle progression in the S phase, to induce G2/M arrest and to induce apoptosis (Matsukawa et al. 1993). Genistein is also able to inhibit the growth of breast cancer cell lines (Lamartiniere et al. 2002), to inhibit angiogenesis (Fotsis et al. 1993) and to modulate lymphocyte activation and proliferation (Guo et al. 2001).

Specific effects on bone cells have been shown both in osteoblasts and osteoclasts. In osteoblasts genistein stimulates a concentration-dependent increase in alkaline phosphatase activity. In osteoblastic MC3T3-E1 cells genistein or daidzein at concentrations of $10^{-7–10^{-5}}$ M increase protein content, DNA content and alkaline phosphatase activity (Yamaguchi & Sugimoto, 2000). This effect has also been demonstrated in tissue cultures. In femoral metaphyseal tissue from elderly female rats genistein ($10^{-5–10^{-3}}$ M) increases Ca content and alkaline phosphatase activity. This increase is blocked by tamoxifen, indicating an ER-mediated pathway, and also by cycloheximide, suggesting it is mediated by overall increased protein synthesis (Yamaguchi & Gao, 1997, 1998). On the other hand, genistein can also cause apoptosis of osteoblasts by activating caspase-3 and cleaving adhesion molecules such as cadherins and catenins (Hunter et al. 2001). In normal fetal osteoblast cells genistein increases the progesterone receptor and alkaline phosphatase gene expression and inhibits osteopontin and interleukin 6 gene expression (Rickard et al. 2003).

In osteoclasts an effect has been observed on osteoclast differentiation as well as function. Genistein reduces osteoclast differentiation from pre-osteoclasts via non-ER-related signalling pathways. Gao & Yamaguchi (1999) observed that genistein is able to reduce the formation of osteoclast-like mononuclear cells induced by dibutyryl cAMP (cAMP pathway), but not the formation of mononuclear cells induced by phorbol 12-myristate 13-acetate (protein kinase C pathway). Phyto-oestrogens may also act on osteoclast production through inhibition of macrophage colony-stimulating factor (Srivastava et al. 1998). Hughes et al. (1996) showed that oestrogens cause osteoclasts to undergo apoptosis through increased production of transforming growth factor-$\beta_1$ by osteoblasts. In porcine bone marrow cells daidzein was able to reduce $1,25$-dihydroxycholecalciferol-stimulated osteoclast formation in vitro, promote caspase-8 and caspase-3 cleavage and DNA fragmentation of monocyctic bone marrow cells and reduce the area resorbed by mature osteoclasts (Rassi et al. 2002). Lieberherr et al. (2003) have put forward the hypothesis that phyto-oestrogens exert oestrogen-like actions on bone cells through the IGF system. By increasing IGF-binding protein 4 proteolysis phyto-oestrogens may increase IGF bioavailability and induce osteoblast proliferation. Osteoblasts would, in turn, express transforming growth factor-$\beta_1$, which either directly or by an IGF-binding protein 3-mediated action would induce osteoclast apoptosis. Lorenzetti et al. (2002) have demonstrated the involvement of IGF-I, although the presence of osteoblasts was not required. The system used was a murine monocyte-macrophage cell line (RAW264.7) that differentiates into osteoclasts in the presence of receptor activator nuclear factor $\kappa B$ ligand. At $50 \mu M$ genistein blocks cell proliferation, while the addition of IGF-I inhibits this effect. At physiological concentrations both IGF-I and phyto-oestrogens inhibit osteoclast differentiation in a similar way to oestradiol. High concentrations of genistein induce increased osteoclast differentiation, an effect that is further increased by the addition of IGF-I. Genistein reduces cell adhesion and stimulates apoptosis via inhibition of topoisomerase II (Gao & Yamaguchi, 1999). Genistein ($10^{-5–10^{-7}}$ M) inhibits the increase in glucose consumption and lactic acid production induced by parathyroid hormone or prostaglandin E$_2$ in tissue culture (Yamaguchi & Gao, 1998). S. Lorenzetti (personal communication) cultured human peripheral blood mononuclear cells in vitro with soluble receptor activator nuclear factor $\kappa B$ ligand and macrophage colony-stimulating factor. Preliminary results showed that zearalenone, as well as oestradiol, are able to inhibit the secretion of matrix metalloproteinases, which are responsible for bone resorption and known markers of osteoclast differentiation.

In *in vitro* studies indicate that phyto-oestrogens such as genistein are able to stimulate osteoblastic activity and inhibit osteoclast formation and action at a range of concentrations ($10^{-5–10^{-7}}$ M) consistent with the levels observed in human subjects after ingestion of genistein. In the study of Setchell et al. (2001) administration of a single bolus dose of $50 \text{ mg}$ genistein indicated that the concentration that gives a maximum response is $1.26$ (SD 0.27) $\mu \text{mol/L}$.

Cell studies make a valuable contribution to the understanding of the mechanism of action of phyto-oestrogens and to the explanation of some of the *in vivo* observations. However, adequate characterisation of such models is required, particularly as far as the ER status is concerned.

**Effects of phyto-oestrogens on bone: animal models**

Coxam (2003) has recently reviewed twenty-two studies involving ovariectomised rats, six studies of other rat models (including male rats) one study of mice and two studies of ovariectomised monkeys. The studies, which used various doses, different phyto-oestrogen-rich products and
purified compounds (soyabean, soya milk, soyabean protein, genistein, daidzein, rutine, zearalenone and ipriflavone, as well as several phyto-oestrogen-rich medications used in traditional medicine) and different end points (BMD, bone strength, bone mass, histomorphometry and bone formation and resorption markers), all showed an effect in rats (except in one case) but not in monkeys.

Mühlbauer & Li (1999) have demonstrated that consumption of a variety of salad vegetables, herbs and cooked vegetables commonly found in the human diet can increase bone mineral content, mean cortical thickness and mineral density of trabecular bone in male rats, and they attributed this effect to flavonoids.

Although when compared with ageing human bone the ovariec-tomised-rat model has limitations related to the characteristics of bone remodelling (bone growth slows down but does not completely stop in the ageing rat), it is a recognised model (Food and Drug Administration, 1994). This rat model is used in preclinical studies of bone quality, for comparison of foods and compounds and to establish mechanisms of action in the whole organism, particularly in relation to bone tissue composition, mechanical properties and bone tissue physiology.

The rat studies demonstrated the effects of vegetable flavonoids on the thickness of bone trabeculae (Mühlbauer & Li, 1999) and those of rutin (Molteni & Coxam, 2001), flavonoids on the thickness of bone trabeculae (Mühlbauer & Li, 2003) and enterolactone in osteoporotic rats (Greendale et al., 2001) on femoral failure load. Soyabean protein increased bone formation in tibial periosteal cortical bone of castrated rats (Hegsted et al., 1999).

Coxam (2003) concludes that ‘… a substantial body of work in animal models was attained in the past few years, and has provided convincing data for significant improvements of bone mass or other endpoints following soy feeding.’

Cross-sectional observations in human populations

Several cross-sectional observations have been carried out in population groups consuming soyabean isoflavones, both in South-East Asian countries and in the Western world (Table 1). Only papers published in an extended form have been included in the review, so that details of the methodology can be appreciated. The studies differ widely in their specific objectives and methodologies. First, they targeted different population groups. Seven of the eleven studies examined were conducted in post-menopausal women, one in pre- and post-menopausal women and two in perimenopausal women. The sample size is also extremely variable, ranging from thirty to about 3000. Second, most studies looked at the current intake of soyabean isoflavones using food-frequency questionnaires (FFQ; Tsuchida et al., 1999; Ho et al., 2001; Mei et al., 2001; Greendale et al., 2002) or weighed records (3–7 d), and only Kritz-Silverstein & Goodman-Gruen (2002) looked at the usual consumption of foods. Although lifelong exposure was seldom evaluated, ethnicity can be considered to be a relevant marker. In most cases estimates of isoflavone intake were obtained by applying food composition tables that mainly comprise soyabean-based foods (e.g. tofu and soya milk) or bean sprouts and soya sauce. However, soyabean is an ingredient

### Table 1. Human observational studies on phyto-oestrogens and bone health

<table>
<thead>
<tr>
<th>Reference</th>
<th>Menopausal status</th>
<th>No. of subjects</th>
<th>Ethnicity of subjects</th>
<th>Isoflavone intake (mg/d)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-intake populations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tsuchida et al. (1999)</td>
<td>Peri-menopausal</td>
<td>995</td>
<td>Japanese</td>
<td>12.6 g soyabean protein</td>
<td>+ Metacarpal BMD</td>
</tr>
<tr>
<td>Somekawa et al. (2001)</td>
<td>Post-menopausal</td>
<td>478</td>
<td>Japanese</td>
<td>1.4 v. 15</td>
<td>+ BMD</td>
</tr>
<tr>
<td>Ho et al. (2001)</td>
<td>Premenopausal</td>
<td>132</td>
<td>Hong Kong Chinese</td>
<td>18 v. 47</td>
<td>Post-menopausal: + BMD-LS and Ward’s triangle, ↓ PTH, osteocalcin, N-telopeptide</td>
</tr>
<tr>
<td>Mei et al. (2001)</td>
<td>Premenopausal</td>
<td>293</td>
<td>Japanese</td>
<td>32</td>
<td>NS BMD NS-ALP</td>
</tr>
<tr>
<td>Nagata et al. 2002</td>
<td>Post-menopausal</td>
<td>357</td>
<td>Japanese</td>
<td>20–150</td>
<td>NS BMD; ↓ enterolactone in osteoporotic subjects</td>
</tr>
<tr>
<td>Kim et al. 2002</td>
<td>Post-menopausal</td>
<td>75</td>
<td>Korean</td>
<td>20–150</td>
<td></td>
</tr>
<tr>
<td>Low-intake populations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kardinaal et al. (1998)</td>
<td>Post-menopausal</td>
<td>70</td>
<td>Dutch</td>
<td>0.35</td>
<td>NS rate of bone loss</td>
</tr>
<tr>
<td>Di Leo et al. (2002)</td>
<td>Post-menopausal</td>
<td>30</td>
<td>Italian</td>
<td>0.35; 17.5</td>
<td>NS Tr BMD; To BMD; stress strain index</td>
</tr>
<tr>
<td>Greendale et al. (2002)</td>
<td>Peri-menopausal</td>
<td>1003</td>
<td>Caucasian</td>
<td>0.5–10.7 mg genistein</td>
<td>+ BMD-FN; BMD-LS in Japanese women</td>
</tr>
<tr>
<td></td>
<td></td>
<td>497</td>
<td>African-American</td>
<td></td>
<td>NS BMD in other ethnic groups</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>Chinese</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>227</td>
<td>Japanese</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BMD, bone mineral density; BMD-LS, BMD at the lumbar spine; BMD-FN, BMD at the femoral neck; Tr BMD, trabecular BMD; To BMD, total BMD; DPD, deoxypyridinoline; ALP, alkaline phosphatase; PTH, parathyroid hormone; +, higher at higher intakes; ↓, lower at higher intakes; NS, no significant difference at higher intakes.
of many industrial products and this factor is sometimes not recognised. Only in some cases (Kardinaal et al. 1998; Somekawa et al. 2001) were more objective measures of isoflavone intake, such as urinary isoflavones, obtained. The outcome variables measured were BMD at the lumbar spine, femoral neck or metacarpal and bone formation or resorption markers. Finally, only some of the studies controlled for confounding factors (age, height, weight, years since menopause, smoking, intake of Ca and other nutrients, alcohol consumption, HRT usage etc.). It is therefore difficult to attempt to pool the data from the different studies and a more qualitative evaluation is required.

Of the two studies conducted among premenopausal women, the first one, a 3-year prospective study conducted in 30–40-year-old women (Ho et al. 2001), reported significant differences \( (P<0.05) \) in spinal BMD in the highest (mean 15 mg isoflavones/d)- and lowest (mean 1-4 mg/d)-soyabean-intake groups, while the second study (Mei et al. 2001) reported no association between dietary phyto-oestrogen intake and BMD.

Among the studies of post-menopausal women, of the six studies conducted in countries with high isoflavone intake four indicated better bone health in the groups with the highest intake. Mei et al. (2001) found significant differences between the highest (mean 47.4 mg/d) and lowest (mean 28.9 mg/d) isoflavone intakes in the lumbar spine BMD \( (0.820 (SD 0.145) \text{ vs. } 0.771 (SD 0.131) \, \text{g/cm}^2; P<0.05) \) and Ward’s triangle BMD \( (0.450 (SD 0.142) \, \text{g/cm}^2; P<0.05) \). Women with the highest isoflavone intake had significantly lower levels of serum parathyroid hormone, osteocalcin and urinary N-terminal telopeptides. Tsuchida et al. (1999) found an association between weekly Ca intake from soyabean and BMD of the metacarpal. Horiiuchi et al. (2000) also found an association between soyabean protein intake and the z score of BMD. Somekawa et al. (2001) found that early post-menopausal women in the highest quartile of intake (mean 83.3 mg/d) had 7.9 % higher lumbar spine BMD than women in the lowest quartile of intake (mean 28.9 mg/d). Two studies failed to find an association; there was no significant association between intake and BMD or alkaline phosphatase (Nagata et al. 2002), or between total urinary phyto-oestrogen excretion and BMD (Kim et al. 2002).

In the four studies conducted in populations with lower intakes no association was found between isoflavone intake and bone BMD or bone turnover, except in the subgroups with the highest lifelong intakes, i.e. Asian immigrants (Greendale et al. 2002; Kritz-Silverstein & Goodman-Gruen, 2002). In the study of Greendale et al. (2002) African-American and Caucasian women reported intakes of 0.5 and 1.4 mg genistein/d respectively, while Kardinaal et al. (1998) reported urinary isoflavone levels indicative of an intake of 0.35 mg/d and the women on a typically ‘Mediterranean’ diet had intakes of 0.35 mg total phyto-oestrogens/d (Di Leo et al. 2002).

**Table 2. Human intervention trials on phyto-oestrogens and bone health in post-menopausal women**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study design</th>
<th>No. of subjects</th>
<th>Isoflavone (IF) dose (mg/d)</th>
<th>IF source</th>
<th>Duration (months)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short term (&lt; 6 months)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murkies et al. (1995)</td>
<td>Parallel-arm</td>
<td>58</td>
<td>74*</td>
<td>Soyabean flour</td>
<td>3</td>
<td>↓ Urinary hydroxyproline</td>
</tr>
<tr>
<td>Washburn et al. (1999)</td>
<td>Crossover</td>
<td></td>
<td>34*</td>
<td>Soyabean-protein isolate</td>
<td>1.5</td>
<td>↓ Serum alkaline phosphatase</td>
</tr>
<tr>
<td>Dalais et al. (1998)</td>
<td>RCT</td>
<td>52</td>
<td>53*</td>
<td></td>
<td>3</td>
<td>NS BMD, ↓ 5.2 % BMC</td>
</tr>
<tr>
<td>Wangen et al. (2000)</td>
<td>RCT, crossover</td>
<td>17</td>
<td>8, 65, 130*</td>
<td></td>
<td>3</td>
<td>NS bone markers</td>
</tr>
<tr>
<td>Scambia et al. (2000)</td>
<td>RCT, crossover</td>
<td>39</td>
<td>50*</td>
<td></td>
<td>1.5</td>
<td>NS serum osteocalcin</td>
</tr>
<tr>
<td>Scheiber et al. (2000)</td>
<td>Single group, not PC</td>
<td>42</td>
<td>60</td>
<td>Soyabean foods</td>
<td>3</td>
<td>NS Osteocalcin, ↓ urinary N-telopeptide</td>
</tr>
<tr>
<td>Uprmalis et al. (2000)</td>
<td>RCT, double-blind</td>
<td></td>
<td>50*</td>
<td></td>
<td>3</td>
<td>NS serum osteocalcin, urinary N-telopeptide</td>
</tr>
<tr>
<td>Uesugi et al. (2002)</td>
<td>RCT, crossover</td>
<td>23</td>
<td>61.8*</td>
<td></td>
<td>1</td>
<td>↓ Urinary DPD</td>
</tr>
<tr>
<td>Lucas et al. (2002)</td>
<td>RCT double-blind</td>
<td>36</td>
<td>(40 g/d)</td>
<td>Flaxseed</td>
<td>3</td>
<td>NS bone resorption markers</td>
</tr>
<tr>
<td>Medium term (≥ 6 months)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potter et al. (1998)</td>
<td>RCT double-blind</td>
<td>66</td>
<td>90*</td>
<td></td>
<td>6</td>
<td>↑ Lumbar BMD 2.2 %</td>
</tr>
<tr>
<td>Alekel et al. (2000)</td>
<td>RCT double-blind</td>
<td>69</td>
<td>4.4, 80.4*</td>
<td></td>
<td>6</td>
<td>↑ Lumbar spine bone loss</td>
</tr>
<tr>
<td>Clifton-Bligh et al. (2001)</td>
<td>RCT double-blind</td>
<td>46</td>
<td>28, 57, 85*</td>
<td>Red clover†</td>
<td>6</td>
<td>↑ 3.4 % BMD proximal radius and ulna</td>
</tr>
<tr>
<td>Hsu et al. (2001)</td>
<td>RCT</td>
<td>37</td>
<td>150*</td>
<td></td>
<td>6</td>
<td>NS calcaneous BMD</td>
</tr>
<tr>
<td>Lydekting-Olsen et al. (2002)</td>
<td>RCT</td>
<td>89</td>
<td>IF-rich soya milk</td>
<td></td>
<td>24</td>
<td>↓ BMD loss</td>
</tr>
<tr>
<td>Chiuchi et al. (2002)</td>
<td>RCT</td>
<td>187</td>
<td>Soya-rich diet</td>
<td></td>
<td>6</td>
<td>↓ Serum osteocalcin</td>
</tr>
<tr>
<td>Morabito et al. (2002)</td>
<td>RCT</td>
<td>90</td>
<td>54</td>
<td>Genistein</td>
<td>12</td>
<td>↓ BMD, ↓ excretion of PYD and DPD, ↑ BALP</td>
</tr>
</tbody>
</table>

NS, difference not significant from baseline; ↑, increase from baseline; ↓, decrease from baseline; RCT, randomised controlled trial; PC, placebo controlled; BMD, bone mineral density; BMC, bone mineral content; DPD, deoxypyridinoline; PYD, pyridinoline; BALP, bone alkaline phosphatase.

*Total IF.
†Trifolium pratense.

**Intervention trials**

Human trials are probably the most indicative measure of the effect of phyto-oestrogens. Table 2 lists the main studies conducted so far in post-menopausal women. Again, studies
only appearing in abstract form that have not given details of the protocol have not been included, except one that has been quoted in an extended paper, because it is the only study that has achieved a 24-month period of supplementation (Lydeking-Olsen et al. 2002).

Seven of these studies were of <6 months duration. Murkies et al. (1995) recruited women who were 5–6 years post-menopausal and assigned them to a daily intake of either 45 g soybean flour or 45 g wheat flour. After 3 months urinary hydroxyproline was increased in both groups, but significantly more in the wheat-flour group. Washburn et al. (1999) found that women given a soybean-protein isolate providing 34 mg isoflavones/d had lower serum alkaline phosphatase levels than women given the same isolate with a lower isoflavone content. Dalais et al. (1998) conducted a 3-month randomised controlled trial (RCT) with fifty-two post-menopausal women who received diets containing soybean (providing 53 mg total isoflavones/d), linseed or wheat. A significant difference from baseline was observed only in the soybean-diet group, with a 5.2% increase in bone mineral content. Wangen et al. (2000) carried out a crossover RCT with seventeen post-menopausal women, each undertaking one of three 3-month periods with 8, 65 or 130 mg isoflavones/d. In the high-isoflavone period the excretion of deoxypyridinoline and cross-linked C-telopeptides, both resorption markers, was lower than that in the baseline period, but the difference was not statistically significant. Scambia et al. (2000) conducted a RCT with thirty-nine post-menopausal women aged 29–63 years, who were supplemented with 50 mg total isoflavones/d for 6 weeks. Bone markers did not show significant changes in osteocalcin levels. Upmalis et al. (2000) found no difference for serum osteocalcin or urinary cross-linked N-telopeptides after 12 weeks of supplementation with 50 mg genistein/d. Uesugi et al. (2002) conducted a RCT with twenty-three peri-menopausal women (age-range 40–62 years) for 4 weeks, and observed a reduction in bone resorption (urinary pyridinoline and deoxypyridinoline) in the group given 61.8 mg total isoflavones/d. In another study (Lucas et al. 2002) post-menopausal women received a flaxseed supplement containing lignins, which had no effect on biomarkers of bone metabolism.

Seven more studies of ≥6 months duration have been published, of which five were conducted for 6 months. Potter et al. (1998) carried out a double-blind placebo-controlled RCT with sixty-six post-menopausal women. In this study 90 mg isoflavones/d induced a 2.2% increase in lumbar spine BMD compared with baseline. However, the group showing the effect had the lowest baseline BMD. Alekel et al. (2000) conducted a RCT with sixty-nine peri-menopausal women comparing isoflavone intakes of 4 and 80 mg/d and showed a 1.28% reduction in lumbar spine BMD in the low-intake group, while the high-intake group was protected. Clifton-Bligh et al. (2001) performed a double-blind study of forty-six post-menopausal women receiving daily 28, 57 or 85 mg of a red clover (Trifolium pratense)-isoflavone preparation (Rinostil) containing genistein, daidzein, formononetin and biochanin. The two higher-intake groups showed an increase in BMD of 3–4% at the proximal radius and ulna. Hsu et al. (2001) carried out a study of thirty-seven post-menopausal women receiving 150 mg isoflavones/d. No effect was observed in calcaneal BMD. Chiechi et al. (2002) conducted a RCT with 187 post-menopausal women (age-range 39–60 years) comparing a soyabean-rich diet with HRT; the isoflavone intake is not clear from the report. There was an increase in serum osteocalcin levels and a non-significant reduction in hydroxyproline excretion in the soyabean-diet group, while the group receiving HRT showed a significant reduction in hydroxyproline excretion. A reduction in trabecular BMD was observed in the control group, while the groups receiving the soyabean diet and HRT showed no significant reduction.

Two studies were carried out for >6 months. In the study of Morabito et al. (2002) ninety post-menopausal women were randomly assigned to receive 54 mg genistein/d, HRT or a placebo and followed for 12 months. In the groups receiving genistein and HRT they observed an increase in BMD at the femoral neck, Ward’s triangle and lumbar spine, and reduced pyridinoline and deoxypyridinoline excretion, at 6 and 12 months. Interestingly, they also observed increased serum bone alkaline phosphatase and osteocalcin levels at 6 and 12 months in the genistein-treated group. The study of Lydeking-Olsen et al. (2002) was carried out for 24 months, but the findings are unfortunately only available in an abstract. Some data have also been published in Setchell et al. (2002a). Eighty-nine post-menopausal women (age-range 41–75 years) were randomly assigned to receive isoflavone-rich soya milk (100 mg/d) and natural transdermal progesterone (25 mg/d) individually or in combination, a placebo and a progesterone-free cream. Lumbar spine BMD and bone mineral content did not differ from baseline in the groups receiving soya-milk (+1.1% and +2.0% respectively) and transdermal-progesterone (−1.1% and +0.4% respectively), but there was a loss in both variables in the groups receiving the placebo (−4.2% and −4.3% respectively) and the progesterone-free cream (−2.8% and −2.4% respectively). There were no significant changes in femoral neck BMD or bone mineral content in any of the groups.

Again, it is difficult to pool the data from the different studies. First there was an inconsistency in the objectives of the studies. Valtueña et al. (2003) suggested that studies should address: the bone-sparing effect in post-menopausal women; the selection of compounds, dose and mode of delivery; the size of the effect and its sustainability; the timing of the intervention (before/after menopause); the mechanisms of action (reduced bone breakdown, increased formation, or both); the sites of the skeleton receiving the largest benefit; the relevance in terms of fracture risk reduction. These factors have not been addressed by most of the studies. The main weakness of human trials is, however, their duration. Long-term studies that cover a minimum of one remodelling cycle (30–80 weeks) should be conducted. In fact, many studies have been conducted for other purposes, e.g. cardiovascular disease or menopausal symptom relief.

Despite methodological weaknesses and inconsistencies, the conclusion that can be drawn from human studies is indicative of a positive effect of soyabean isoflavones on bone health. In observational studies the results for post-
menopausal women indicate that intake levels similar to those observed in South-East Asian populations (mean 50 mg isoflavone/d) allow the preservation of bone mass in menopause, possibly through a decrease in bone resorption. All studies have looked at ethnic groups with lifelong intakes, and it is difficult to use observational data to obtain indications for Western populations. Among the intervention trials, short-term studies are not consistent in indicating an impact on either BMD or bone turnover. Among the longer-term studies, six of the seven studies showed an effect, although moderate. The only negative study (Hsu et al. 2001) was underpowered.

Public health implications and unresolved issues

Research in the field is progressing rapidly, as is consensus among the scientists. The National Institutes of Health (2000) Consensus Conference stated, ‘There is a great deal of public interest in natural estrogens, particularly plant-derived phytoestrogens. These compounds have weak estrogen-like effects, and although some animal studies are promising, no effects on fracture reduction in humans have been shown’. Only 3 years later another panel of experts convened by the UK Food Standard Agency (2002) concluded that ‘Clinical data on the effects of phytoestrogens on bone density are limited but results of short-term human studies suggest small protective effects in the lumbar spine. The data for protective effects at other sites are equivocal’. Since this latter statement only the paper by Morabito et al. (2002) has made a further contribution, but little can be added, although two major trials are currently being undertaken (one in Europe (the PHYTOS project) and one in the USA (the OPUS project)). There is, however, still a knowledge gap. Unresolved issues include: the optimal dose compatible with safety; the individual differences in response related to diet and genotypes; the duration of exposure; lifelong exposure as opposed to post-menopausal exposure only. Furthermore, the relative biological effects of phyto-oestrogens other than isoflavones (lignans, resorcylic acid lactones, flavonols, coumeastans) that are also present in European diets need to be evaluated.

If an effect of phyto-oestrogen on bone health in menopause is demonstrated, then the question arises of how to deliver the compounds, i.e. with products containing high amounts (e.g. soyabean), with supplements or with enriched foods. There is a large difference in the dietary intakes of soyabean isoflavones between South-East Asia and the Western World. In USA and Europe it is of the order of 1 mg/d (Kuhnau, 1976). van Erp-Baart et al. (2003) obtained an estimate of isoflavone intake within the framework of the Vegetal Estrogens in Nutrition and the Skeleton (VENUS) project. They used a purposely-developed database and then applied it to the national dietary intakes obtained in Republic of Ireland, Italy, The Netherlands and the UK. The intake in Europe is also of the order of 1 mg/d, and ≤6–10 mg/d among consumers of soyabean foods. A UK study of a small group of vegetarians (n 35) pointed to an intake of 12 mg genistin and daidzein/d (Food Standards Agency, 2002). In South-East Asia isoflavone intake is on average 50 mg/d, but it can also be as high as 100 mg/d. Achieving such an intake would involve substantial changes in Western dietary habits, even beyond the current intake of some population groups (i.e. vegetarians). The second option is the enrichment of commonly-used Western foods with soyabean isoflavones or with other phyto-oestrogens. In order to establish the most appropriate fortification levels, a decision should be reached on an appropriate dose. The US Consensus Panel (2000, unpublished results), reviewing the available literature at the time, concluded that 40–60 mg aglycones/d are required for cardiovascular risk reduction, 60 mg aglycones/d for relief of menopause symptoms, 90 mg aglycones/d to achieve bone health benefits and 50–110 mg aglycones/d for cancer prevention. Thus, the panel concluded that the recommended isoflavone intake might range from 60 to 100 mg aglycones/d, with the lower dose considered as ‘reasonable and responsible’. Since the diet contains negligible amounts of isoflavones, they would have to be added. Furthermore, the level of fortification would have to be low enough to prevent the intake of excessively high doses.

The third option would be to consider whether other compounds already present in Mediterranean food might be suitable for use in replacing soyabean isoflavones in relation to their bone-sparing effect. Hertog et al. (1997) estimated the intake of flavonoids and flavones in The Netherlands to be 23 mg/d, and Leth & Justesen (1998) estimated the intake of flavonol, flavones and flavonanes in Denmark to be 28 mg/d. In Southern European countries this estimate might be even higher, since the intake of fruit and vegetables is approximately 500 g/d for Italy, France and Spain. It is not known whether the effects of the different compounds are synergistic or whether they compete for mechanisms of action, or indeed cooperate in the modulation of such response mechanisms. However, the impact of high intake levels of other flavonoids should be considered and the recommended doses of isoflavone adjusted accordingly.

References


Molteni & Coxan V (2001)


