Prevention of osteopaenia by phyto-oestrogens: animal studies

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Osteoporosis has become a major public health problem. Because the biggest culprit in the process of bone loss is oestrogen deficiency, hormone replacement therapy remains the mainstay for prevention, but prophylaxis by this means is limited. Phyto-oestrogens deserve special mention because emerging data support the suggestion that these weakly oestrogenic compounds, present in plants, may prevent bone loss associated with the menopause and thus represent a potential alternative therapy for a range of hormone-dependent conditions, including postmenopausal symptoms. A substantial body of work in animal models in the past few years has provided convincing data for significant improvements in bone mass and other endpoints following feeding with soya. Thus, phyto-oestrogens appear to have potential promise for maintaining or modestly improving bone mass of human subjects when consumed at optimal dosages. However, we must appreciate the limits of the information reached before extrapolating to man and we need to gather more data before health professionals can actively advocate the increased consumption of soya. Indeed, it will be important further to characterise the physiological effects of phyto-oestrogens and their margins of safety.

Phyto-oestrogens: Bone: Animals

Introduction

A substantial body of work in animal models in the past few years has provided convincing evidence for significant improvements in bone mass and other endpoints following feeding with soya (Anderson & Garner, 1997). These studies point strongly towards this bone-sparing effect of soya, attributed to its isoflavones component (Arjmandi et al. 1996). However, because we are constrained by the systems with which we study the ageing skeleton, we must appreciate the limits of the information gained (Rubin et al. 1999).

Indeed, since the best model for the study of man is man, there was no real pressure to seek an animal model for the disease. However, it is clear that, as a result of ethical and other constraints, sole reliance on human subjects for studying osteoporosis limits the ability to test new hypotheses and to evaluate new potential therapies (Kalu, 1999). The development and application of appropriate animal models of human bone loss are thus legitimate components of the quest to understand, manage and possibly prevent the occurrence of osteoporosis in the future (Kalu, 1999). Given the high degree of genetic homology among mammals suggesting strong, intricate ties, it seems reasonable that animal models of human bone disease would provide valuable insights into the pathogenesis and aetiology of the ageing skeleton. However, it is essential to approach these models with a strong sense of their limitations (Rubin et al. 1999). First, in considering a preventive strategy for osteopaenia, the complexities inherent in the ageing processes of the skeleton (a natural condition of deterioration rather than a disease *per se*) may be difficult to study through an experimental perturbation in the animal. Second, animals do not exhibit any spontaneous fractures that are the hallmark of osteoporosis in man (even if they live out a normal life span), although ovariectomy is routinely performed on dogs, cats, pigs, cows, etc. Additional differences include the absolute size and architecture of the bone (Hartke, 1998). Furthermore, most animals used as models are quadrupeds and therefore have different loading patterns on the bone; thus, as the prevailing mechanical stimuli applied to the bone exert a potent control on modelling and remodelling, bone metabolism may not be similar to that of man.

Keeping in mind these difficulties, an animal model of the ageing human skeleton can be defined as a living

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animal in which the characteristics of bone loss and its sequelae resemble those found in man in one or more respects (Kalu, 1991). Consequently, good animal models share at least four characteristics: convenience (refers to ease), relevance (comparability of phenomenon, reproducibility), predictability (capability of the model to foretell the anticipated outcome) and appropriateness (availability, facilities required, response time, environmental considerations, etc.; Frenkel, 1969; Kalu, 1991, 1999; Rodgers et al. 1993).

Taking into account these conditions, the United States Food and Drug Administration (FDA) has laid down guidelines that advise the testing of anti-osteoporotic drugs using a rodent model and a large animal model, the most suitable species being the ewe, the pig or the non-human primate (FDA, 1994).

Rat

The ovariectomised (OVX) rat model is, by far, the most widely used animal model of ageing bone loss because it fulfils the four desired criteria referred to previously. This is why the 1994 FDA guidelines for pre-clinical and clinical evaluation of agents used in the treatment or prevention of postmenopausal osteoporosis suggest that the OVX rat must be used as the primary model system for pre-clinical investigation (FDA, 1994).

The advantages of the rat are numerous. Studies with rats can be carried out easily under the standardised conditions of the laboratory. They are rather inexpensive and a response can be induced in a short time. Their shorter life span enables studies on old animals. Finally, although rodents do not experience a natural menopause, ovariectomy has been used to produce an artificial menopause and OVX rats consistently and predictably lose bone at specific sites of the skeleton (Saville, 1969). Indeed, numerous reports have proved that there are many similarities with the human skeleton, such as:

1. the development of osteopaenia, primarily in cancellous bone, with a rapid phase of bone loss during the early stages of oestrogen deficiency;
2. increased bone turnover associated with the initial, rapid step of bone loss after castration; and
3. oestrogen treatments preventing bone loss by depressing bone turnover in oestrogen-deficient rats and human subjects (Wronski & Yen, 1991).

In addition, similar responses to therapy with parathyroid hormone, calcitonin, tamoxifen, bisphosphonates and exercise are strong evidence that the OVX rat bone loss model is suitable for studying problems that are relevant to postmenopausal bone loss (Kalu, 1991).

While the rat may be an effective initial screening tool to monitor the efficacy of potential therapeutic interventions in the ageing human skeleton, we must accept the limitations in extrapolating perturbations of bone growth to the slow, progressive degeneration of bone morphology (Rubin et al. 1999). It must be emphasised that the rat skeleton is unusual in that growth may slow following a few months, but never actually ceases (Riesenfeld, 1981). It is thus difficult to extrapolate from a model of slowed growth to the human

condition of rapid decline. However, in every instance where rats have been studied carefully over a prolonged period, linear bone growth has been reported to slow down considerably and even stop at advanced age (Kalu et al. 1984, 1989). The generally held view that rats do not normally experience a progressive age-related decline in bone mass is contradicted by studies (Kalu et al. 1984; Gaumet et al. 1994) showing that a loss of cancellous bone in the appendicular skeleton occurs with ageing in rats, while cortical bone is more resistant (Wronski et al. 1989). Perhaps the most serious indictment of the rat as an inappropriate model of human bone loss is the misconception that the mature rat skeleton does not remodel. The paucity of haversian systems contributed to this notion (Kalu, 1999), but even early literature reported resorption and formation phases of a typical bone modelling unit (Baron et al. 1984). Actually, cortical bone in the rat has latent remodelling capacity, intracortical remodelling being found in older animals (Kalu, 1991). This process increases with age and accounts for over 90 % of turnover activity in vertebral cancellous bone and 66 % in proximal tibial metaphysis (Erben, 1996). In fact, haversian remodelling does not appear to be affected by oestrogen deficiency in man (Villanueva et al. 1966), as cortical bone loss occurs mainly at the endocortical surface (Keshawarz & Recker, 1984).

Despite these limitations, the OVX rat is considered as an appropriate model for investigating issues relevant to human postmenopausal bone loss, and the recognition of this by the FDA has given a tremendous boost to studies using this model (Kalu, 1999).

Because bone balance is the amount of resorption relative to formation, to make progress in developing therapeutics it is also important to consider 'the resorption or osteoclastic model' from the 'formation–deficit model' (Hartke, 1998). The first model, which works well for measuring anti-resorptive efficacy, is synonymous with a high turnover. It can be induced through Ca deficiency, immobilisation or, of course, oestrogen deficiency. Thus, because of increased osteoclastic activity, there is perforation of trabeculae, and endosteal resorption from the interior surface of the cortical bone. On the contrary, to look at agents that accelerate synthesis, the formation–deficit model induced by glucocorticoid administration is more appropriate (Hartke, 1998). In addition, since the FDA guidelines stipulated that pre-clinical studies of bone quality should be performed in two species (one being the OVX rat), other animal models of ageing bone loss have been required.

Mouse

The mouse has a long history of being exploited in studies of age-related osteopaenia. Indeed, the senescence accelerated mouse strain is currently being used to study the aetiological mechanisms in senile osteoporosis because fractures occur spontaneously due to an accentuated bone loss with ageing (Takeda et al. 1991). However, with regard to postmenopausal osteoporosis, oestrogen therapy prevents bone loss in mice mainly by increasing bone

formation, while this therapy is known to inhibit bone resorption in man (Bain et al. 1993).

Guinea pig

The guinea pig is a popular research mammal owing to its short reproductive cycle, but, because there are no consequences on bone volume in OVX adult guinea pigs (Vanderschueren et al. 1992), this species seems unlikely to be a useful model for human osteoporosis.

Ferret

The OVX ferret exhibits oestrogen-deficiency osteopaenia and haversian-based skeletal remodelling, as well as age-related bone loss. However, difficulties include a seasonal poly-oestrous reproductive cycle and induced ovulation (Hartke, 1998). Moreover, bone mass is negatively influenced by short light cycles and unbred females are prone to anaemia due to prolonged oestrus (Kalu, 1999).

Dog

In many respects, the adult dog skeleton has often proved an excellent analogue of human bone. The ratio of cortical to cancellous bone is similar in both species. Haversian and cancellous osteons remodel in a manner morphologically similar to that in man, although at a faster rate (Kimmel & Jee, 1982).

However, dogs are seasonally mono-oestrus and this obvious departure should be considered (Miller et al. 1995). Moreover, the role of the beagle in studying oestrogen-depletion bone loss seems limited, because of the small skeletal effect of oophorectomy. Thus, the adult beagle remains a good experimental model for studies requiring haversian remodelling, when oestrogen is not a factor (Kimmel, 1991).

Pig

Pigs have skeletal characteristics that make them potentially a good large animal model for skeletal research. These advantages include their gastrointestinal physiology and the nature of the oestrus cycle. They have lamellar bone and remodelling based on both trabecular and intracortical bone modelling units. However, they exhibit a much higher bone mass and denser trabecular network than man, inducing a different loading pattern of the skeleton. Moreover, to optimise loss of bone after osteopaenia, mild Ca restriction is required (Mosekilde et al. 1993).

Sheep

The sheep has been used as a model for postmenopausal osteoporosis as the microstructure of the bone, assessed by histomorphometry, is similar to that of man together with many biological parameters. In addition, ovariectomy induces bone loss (Hornby et al. 1995). Finally, a decline in bone volume in the iliac crest due to ageing indicates that the ewe may be a model for senile as well as postmenopausal osteoporosis (Turner et al. 1993). However, the sheep skeleton undergoes seasonal variations, with depressed bone formation in the winter months, a phenomenon seen in only human subjects living in the Northern Hemisphere. Furthermore, the sheep is a herbivorous ruminant animal, engendering some potential differences in the metabolism of vitamins, dietary minerals and other micronutrients (especially phyto-oestrogens).

Non-human primates

Non-human primates have been described as a suitable model (Jerome et al. 1994), as their organ systems most closely resemble the human ones (i.e. gastrointestinal tract, endocrine system and bone metabolism; Newman et al. 1995). Because many primates maintain upright body posture, their bone biomechanics may be more similar to that of man. Female macaques cycle monthly and have hormonal patterns similar to those of the human female (Hodgen et al. 1977), and, although spontaneous menopause does not occur in most non-human primates, they exhibit similar skeletal response to cessation of ovarian function (castration; Mann et al. 1990). However, peak bone mass is not reached until 9 years of age (in cynomolgus monkeys); therefore, unless using old animals, ovariectomy in the skeletally immature primate seems an inappropriate model (Newman et al. 1995). Moreover, due to the very stringent controls applied to the use of these species (risk of zoonotic diseases) and the costs of such a study, without the considerations of emotive pressures, the primate becomes less favourable as a model (Ford & Hornby, 1996).

In conclusion, although the rat is used more often than other species, its major limitations demand that other species be considered as potential models. A logical approach may be to use the rat for preliminary screening of new pharmacological agents or therapeutic modalities, followed by verification in other species before undergoing clinical trials in human patients (Rodgers et al. 1993).

Effects of phyto-oestrogens on animal bone

Experimental models

Table 1 lists the published effects of phyto-oestrogens on bone metabolism in animal models. The animal model used most commonly for studying the potential anti-osteoporotic properties of natural phyto-oestrogens has been the OVX rat (Omi et al. 1992, 1994; Anderson et al. 1995; Arjmandi et al. 1996, 1998b, 2000; Blair et al. 1996; Draper et al. 1997; Fanti et al. 1998; Ishida et al. 1998; Horcajada-Molteni et al. 2000a; Picherit et al. 2000). Genistein has been reported to elicit a bone-sparing effect in OVX lactating rats (Anderson et al. 1998), in elderly female rats (Gao & Yamaguchi, 1998) and in OVX mice (Ishimi et al. 1999), while a diet based on soya proteins (14%) does not seem to improve bone quality (assessed by histomorphometry) in intact female rats (Hegsted et al. 1999).

Nevertheless, bone-sparing effects of ipriflavone, a synthetic isoflavone (Brandi, 1996), have been demonstrated

Table 1. List of published effects of phyto-oestrogens on bone metabolism in animal models

Animal model	Compound tested	Dose	Effect	Endpoint	Reference
OVX rat	soyabean milk	22·7 g soy protein/100 g diet for 35 d	positive	BMD, bone strength, Ca absorption	Omi et al. (1994)
	soya protein (with IF/without IF)		positive effect of IF	BMD, ALP, TRAP	Goyal et al. (1995); Arjmandi et al. (1998a,b)
	soyabean	22·7 g soy protein/100 g diet for 35 d	positive	BMD, ALP, TRAP	Arjmandi et al. (1996)
	genistein	44 μmol/d for 30 d	inhibits osteo-clastic activity	BMD	Blair et al. (1996)
	environmental oestrogens	0·1, 1, 10 or 30 mg/kg per d for 5 weeks and 4 d	oestrogen-like effect	BMD	Dodge et al. (1996)
	coumestrol or zearalanol phyto-oestrogens	1·5 μmol or 1 mg/2 weeks; 131, 25 mg/week for 6 weeks	positive	BMD, phyto-oestrogens, biochemistry	Draper et al. (1997)
	traditional Chinese (Kampo) medicine	4·5–5 g/d for 7 weeks	positive	BMD, morphometry (SEM)	Hidaka et al. (1997)
	soya protein	22 % for 4 weeks	positive	ash weight, Ca content, ALP, bone ALP	Harrison et al. (1998)
	daidzein–genistein	10 mg/kg per d for 3 months	daidzein is more efficient	bone density, strength, DPD, osteocalcin	Desir et al. (1998); Picherit et al. (2001a,b)
	daidzin–genistin	50 mg/kg per d for 4 weeks	positive with daidzein	bone density, strength, ash weight, Ca content, DPD, PYD	Ishida et al. (1998)
	soya protein (curative effect)	22·7 g soya protein/100 g diet for 65 d	no curative effect	histomorphometry, ALP, OH-proline, IGF-I, IGFBP-3	Arjmandi et al. (1998a)
	genistein	1, 5 or 25 mg/g bw subcutaneously for 21 d	positive	BMD, histomorphometry, DPD, osteocalcin	Fanti et al. (1996, 1998)
	8-isopentenylnaringenin	30 mg/kg per d for 2 weeks	positive	OH-proline, DPD, PYD	Miyamoto et al. (1998)
	soya protein (14 %)	1·2 mg genistein/g protein for 3 months	positive	histomorphometry	Hegsted et al. (1999)
	6-O-acyl succinylated IF glycosides	50 mg/kg per d for 4 weeks	positive		Toda et al. (1999)
	prune (curative effect)	5, 15 or 25 % for 60 d in diet	positive curative effect	BMD, bone strength, Ca absorption	Deyhim et al. (1999)
	mixture of Chinese herbs	1 g/kg per d for 30 d	positive	bone mass, histomorphometry	Cui et al. (1999)
	ipriflavone	100 mg/kg per d for 35 d	positive	density, histomorphometry, ALP, IGF-I, IGFBP	Arjmandi et al. (2000)
	IF soya	20, 40 or 80 mg/kg per d for 3 months	positive	bone density, strength, DPD, osteocalcin	Picherit et al. (2001a,b)
	IF soya	20, 40 or 80 mg/kg per d for 3 months (curative)	no curative effect	bone density, strength, DPD, osteocalcin	Picherit et al. (2001a,b)
	linseed	10 % of the diet	increases bone strength	bone density, strength, DPD, osteocalcin	Horcajada-Molteni et al. (2000b)
	rutine	0·25 % of the diet	positive	bone density, strength, DPD, osteocalcin	Horcajada-Molteni et al. (2000a)
OVX lactating rat	genistein	0·5, 1·6 or 5·0 mg/d for 2 weeks	biphasic effect	ash weight, morphometry (SEM)	Anderson et al. (1998)
OVX running rat	soya IF	40 mg/kg bw per d for 3 months	positive, no additive effect	BMD, histomorphometry	Malochet et al. (1999)
Elderly female rats	genistein	100–200 mg/kg bw for 3 d	positive	ALP activity, Ca content of DNA	Gao & Yamaguchi (1998)
Orchidectomised rat	soya protein	dose ?, for 65 d	protective	bone strength	Juma et al. (1996)
Male rat	different vegetables	1 g of the dried test foodstuff for 4 weeks	onion is the most efficient	tetracycline excretion	Mühlbauer & Li (1999)
	ipriflavone	200–400 mg/kg bw per d for 1 month	positive	bone density, ash weight, Ca, P and Mg content, ALP, telopeptide	Civitelli et al. (1995)
OVX mice	genistein	0·1–0·7 mg/d	positive effect	bone density, trabecular bone volume	Ishimi et al. (1999)
OVX monkeys	IF-rich soya	28 mg/animal for 2 years	none	BMC, ALP, TRAP	Jayo et al. (1996)
	soya protein isolate	equivalent to 148 mg IF/d for 7 months	none	histomorphometry	Lees & Ginn (1998)

OVX, ovariectomised; IF, isoflavones; bw, body weight; BMD, bone mineral density; ALP, alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; SEM, scanning electron microscopy; DPD, deoxypyridinon-line; PYD, pyridinonline; OH-proline, hydroxyproline; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; BMC, bone mineral content.

in other animal models of bone loss: the prednisolone-treated rat (Yamazaki *et al.* 1986), the streptozotocin diabetic rat (Shino *et al.* 1986), the vitamin D-deficient rat (Takenaka *et al.* 1986), the immobilised rat (Foldes *et al.* 1988) and also in OVX animals (Cecchini *et al.* 1997). Intact male rats have been used to study the effect of vegetables (Mühlbauer & Li, 1999) and ipriflavone (Civitelli *et al.* 1995), a protective effect of soya protein also being shown in orchidectomised animals (Juma *et al.* 1996).

In contrast to the rodent model, surgically postmenopausal monkeys given about 112 nmol isoflavones/d (28 mg/d) for 2 years failed to demonstrate any positive effect on bone parameters (Jayo *et al.* 1996). Similarly, Lees & Ginn (1998) found that soya protein consumption by OVX cynomolgus monkeys (providing an isoflavone dose equivalent to 592 nmol/d (148 mg/d) for a woman) for 7 months did not prevent ovariectomy-induced higher bone resorption, the only significant effect ($P<0.01$) being a very slight increase in bone formation at the endocortical surface (% bone-forming rate:bone volume (standard error of the mean, SEM); 2·43 (SEM 0·77) *v.* 0·37 (SEM 0·26) in casein-fed animals). Consequently, non-human primates seem to behave differently and thus may not be a good model for such a study.

The seeds of osteoporosis can be sown during youth because it can result from inadequate achievement of peak bone mass or more rapid bone loss following skeletal maturity. Indeed, assuming that two people lose bone density at the same annual rate, the individual with the lower initial bone density will cross the threshold of abnormality at an earlier age. Although heredity is the strongest determinant, peak bone mass can be optimised by providing adequate nutrition. In any animal, growth is a polygenic phenomenon, creating many problems for analysis. However, working on growing animals can be useful and was actually chosen by Pointillarta *et al.* (unpublished results) to study the effect of soy isoflavones (8 nmol (2 mg)/kg body weight for 42 d, per os) on 15-d-old female piglets. Under these conditions, phyto-oestrogens consumption was devoid of any skeletal effect.

Effects of phyto-oestrogens: from food to molecules

Isoflavones are one of the most common plant metabolites. They occur almost ubiquitously in the fruits, vegetables and beverages of the human diet as well as in several important medicinal plants. Use of such molecules in folk medicine is based to a large extent on empiricism, since this praxis is much older than the science of chemistry (Kuhnau, 1976).

Hidaka *et al.* (1997) have demonstrated a preventive effect of traditional Chinese (Kampo) medicines on experimental osteoporosis induced by ovariectomy in rats. The administration of Unkei-to, Hachimi-jio-gan and Juzen-taiho to OVX rats clearly restored bone mineral density (BMD) to the level of sham-operated (SH) rats. This was strengthened by results from scanning electron microscopy, as the porous or erosive appearance in OVX rats was corrected by the administration of such treatments. These gynaecological medicines are supposed to be based on phyto-oestrogens

and have been used to treat ovary function failure and lower back pain during the climacteric period and also after oophorectomy (Tsumura & Company, 1990; Koyama, 1991). Cui *et al.* (1999) showed that a mixture of herbs (containing *Epinedium sagitanum maxim*, *Astragalus mimbranaecus* and *Rhizoma atractylodis macrocephalac*) completely prevented glucocorticoid-induced cancellous bone loss in young male rats by stimulation of bone formation and depression of bone resorption. Again, in OVX rats, the daily dose of 1 g/kg body weight for 30 d almost completely prevented induced-bone loss (BMD; +22 % *v.* OVX and − 3% *v.* SH animals).

Flavonols. Mühlbauer & Li (1999) have demonstrated that consumption of a variety of salads, herbs and cooked vegetables common in the human diet can alter bone metabolism in the rat. Bone mineral content, mean cortical thickness and mineral density of trabecular bone increased by 17·4 (SEM 6·4)%, 14·8 (SEM 7·6)% and 13·5 (SEM 3·1)% relative to controls, respectively, in male rats fed 1 g of dry onion/d for 4 weeks. A mixture of lettuce, tomato, cucumber, arrugula, onion, garlic, wild garlic, common parsley, Italian parsley and dill (100 mg each) elicited a higher prevention of bone resorption, indicating an additive effect. In OVX females, onion consumption inhibited bone loss in a dose-dependent manner (30–1500 mg/d). Onion extracts also prevented cancellous and cortical bone loss induced by a combination of low protein intake and diet-mediated mild hyperparathyroidism in rats (Ingold *et al.* 1998). Onions are rich in flavonoids, with quercetin being the main component (Justesen *et al.* 1998).

These results could be explained at least partly by the work of Horcajada-Molteni & Coxam (2001), which demonstrated that 3 months' ingestion of a diet containing 0·25 % rutine (a quercetin glycoside: quercetin-3-*O*-glucose rhamnose) improved bone quality in OVX rats. Indeed, femoral trabecular and total BMD were increased by such a diet (+6·1 % and +4·2 %, respectively, *v.* OVX). Femoral failure load was even higher in these animals than in SH rats, this protective effect resulting from slowing down resorption (decreased urinary excretion of deoxypyridinoline) and increasing osteoblastic activity (high plasma levels of osteocalcin).

Flavanones. Miyamoto *et al.* (1998) showed that 8-isopentenylnaringenin qualified as 'a new class of non-steroidal phyto-oestrogens'. When given subcutaneously at a daily dose of 30 mg/kg body weight for 2 weeks to 11-week-old OVX rats, it completely suppressed osteopaenia as assessed by BMD (mg/cm^2; 141·9 (SEM 1·7) *v.* 132·1 (SEM 0·9) in OVX and 139·1 (SEM 1·3) in SH animals), urinary excretion of deoxypyridinoline/creatinine (pmol/ μmol; 27·1 (SEM 1·1) *v.* 55·9 (SEM 1·6) in OVX and 41·0 (SEM 2·4) in SH animals) or hydroxyproline (μg/g body weight per d; 1·01 (SEM 0·04) *v.* 1·62 (SEM 0·06) in OVX and 1·18 (SEM 0·13) in SH animals). This molecule binds to oestrogen receptors with an affinity of 0·7 % that of oestradiol.

Isoflavones. Kalu *et al.* (1988) suggested that the enhanced Ca intestinal absorption, along with modulation of parathyroid hormone, might provide a partial explanation for the beneficial effect of consumption of soya

foods on bone health. Later, Omi *et al.* (1992) reported that soyabean milk is an excellent source for increasing BMD and mechanical bone strength in OVX rats. However, as the mechanism was unclear and the component in soya milk that elicited the effect unknown, they performed a new experiment in the same animal model with two soya milk fractions prepared according to their molecular weight (a high- and a low-molecular-weight powder including soyabean milk peptides). BMD of lumbar spine, tibial proximal metaphysis and tibial diaphysis were increased by both diets, as was the mechanical strength of the femur. Again, this effect could actually be explained by a higher intestinal Ca absorption rate but it was still unclear what kind of molecule affected bone metabolism (Omi *et al.* 1994).

Arjmandi *et al.* (1996) reported the prevention of bone loss in 90-d-old OVX rats consuming soyabean protein isolate (22·7 %) instead of casein for 30 d. The animals had significantly higher mean BMD of the right femur and the fourth lumbar vertebra, the effect being more important on trabecular bone than on cortical bone. However, this protective effect of the soya diet could not be explained by an inhibition of bone turnover because serum concentrations of both alkaline phosphatase and tartrate-resistant acid phosphatase were significantly greater in the OVX and OVX plus soya groups than in SH animals. These results suggested that formation exceeded resorption because BMD was higher in soya-fed rats, despite the high remodelling level. In a similar experiment carried out for 1 month in OVX rats fed a 22 % soyabean protein diet, Harrison *et al.* (1998) failed to demonstrate any decrease in the markers of bone turnover, while femur and tibia ash and Ca contents were increased.

The active components in soya protein with respect to this bone-sparing effect are the isoflavones. In an experimental study, 95-d-old OVX rats were given soya protein (22·7 %) with either normal (mg isoflavones/kg soy protein isolate: genistin 1462, genistein 25·1, daidzin 590, daidzein 11·3) or reduced isoflavone content (only 10 % of the standard one) for 1 month. The OVX group fed soya isoflavones had significantly greater femoral bone density (1·497 (SEM 0·03)g/cm^3) than the control OVX group (1·449 (SEM 0·044)g/cm^3), whereas those OVX animals fed soya depleted in isoflavones (1·452 (SEM 0·030) g/cm^3) were not different from controls. Histomorphometry revealed that the greater bone formation induced by OVX was not corrected by soya (Goyal *et al.* 1995; Arjmandi *et al.* 1998*a*), because bone formation rate related to bone surface was not significantly different in OVX and soya-fed animals. These data are supported by the recent work of Jeffery *et al.* (2000), in which animals were treated for 3 months with casein plus isoflavones, soya or isoflavones-depleted soya. In contrast, Juma *et al.* (1996) found protection against the decrease in bone elasticity induced by orchidectomy in 90-d-old rats by soya consumption for 65 d, regardless of its isoflavone content.

Soya isoflavone concentrate (348 mg isoflavones/g; genistin 159 mg, daidzin 156 mg, glycitin 33 mg) has been given at doses of 80, 160 and 320 nmol (20, 40 and 80 mg)/kg body weight per d for 3 months to 195-d-old OVX rats (Picherit *et al.* 2001*a*). Such a diet prevented osteopaenia induced by OVX because the BMD of the total femur and of its diaphyseal and metaphyseal subregions was similar to that of SH controls, but higher than values in OVX animals. This was associated with the preservation of mechanical properties, as, again, the femoral failure load in OVX rats receiving isoflavones was not different from that of SH animals. In contrast to the findings of Arjmandi *et al.* (1998*a,b*), this bone-sparing effect could be attributed to a stimulated osteoblastic activity, as indicated by plasma osteocalcin levels (Picherit *et al.* 2000).

The protective effects observed may depend not only on the amount of isoflavones but also on their composition. Of two isoflavone concentrates containing roughly the same amount of daidzin but different genistin:glycitin, the one exhibiting more genistin was the most efficient in preventing OVX-induced bone loss in terms of mineral density and bone strength (C Picherit and V Coxam, unpublished results). Draper *et al.* (1997) demonstrated no bone-sparing effects with isoflavones isolated from clover (100 mmol (25 g) isoflavones incorporated into 10 kg of rodent diet) given for 6 weeks to 6-month-old OVX rats. However, this observation may be explained by the fact that biochanin A and formononetin (the main isoflavones in clover) are very weak, exerting respectively <0·006 and 0·0006 times the potency of oestradiol on alkaline phosphatase activity in a human endometrial cell lines, compared with potencies of 0·084 for genistein, 0·061 for daidzein and 0·202 for coumestrol (Markiewicz *et al.* 1993). In a similar study to demonstrate an effect on bone, a 14 % soya protein diet with 1·2 mg genistein/g protein was given for 3 months to 11-month-old OVX or SH rats (Hegsted *et al.* 1999). It increased bone formation in tibial periostal cortical bone, but only in castrated animals. Percentage of double-labelled periostal surface was 14·2 % for soya-fed OVX rats *v.* 2·27 % in casein-fed OVX rats and 1·57 % in SH animals fed the soya diet. Similarly, the percentage of mineralising surface was 19·35 % for soya-fed OVX rats compared with 5·05 % for casein-fed OVX rats and 2·01 % for casein-fed OVX rats implanted with oestrogen pellets (Hegsted *et al.* 1999).

OVX lactating rats, raised on a low-Ca diet treated with a genistein-rich soyabean isoflavone preparation (2, 6·4 or 20 nmol (0·5, 1·6 or 5 mg)/d) over a period of 14 d, retained significantly more bone mass than vehicle-treated controls and even more than with a physiological dose (5 μg/d) of conjugated oestrogens (Premarin; Anderson *et al.* 1995, 1998).

The effects of purified compounds have been studied. In OVX rats weighing 200 g, purified genistein (44 μmol/d for 30 d) promoted a similar improvement in the femur, femoral weight being 12 % greater than that of controls (Blair *et al.* 1996). These authors also demonstrated an inhibition of osteoclastic activity via a mechanism independent of cellular attachment, at doses approximating those inhibiting tyrosine kinase autophosphorylation *in vitro*. In an additional experiment, 2-month-old OVX rats were injected daily with genistein (4, 18 or 92 pmol (1, 5 or 25 μg)/g body weight, subcutaneously) for 21 d. There was a dose-dependent improvement in skeletal

retention of both trabecular and cortical bone associated with a higher bone formation rate per tissue volume and a trend towards more osteoblasts per bone perimeter; the parameters of resorption were not affected (Fanti *et al.* 1996, 1998). OVX mice given 0·37 or 2·59 μmol genistein (0·1 or 0·7 mg) per d subcutaneously, using a mini-osmotic pump, for 2-4 weeks restored the trabecular bone volume of the femoral distal metaphysis as shown by histological analysis (Ishimi *et al.* 1999).

The ability of daidzein to prevent bone loss was investigated in comparison to genistein (Desir *et al.* 1998; Picherit *et al.* 2000). Twelve-month-old OVX rats received diets containing purified genistein or daidzein at the dose of 38 nmol/g body weight per d for 3 months. In the daidzein group, BMD (measured in lumbar vertebrae, the femur and its metaphyseal and diaphyseal zones) was not different from that of SH animals, whereas in genistein-treated rats, only the diaphyseal bone mass was similar to that of SH controls. Image analysis performed in the distal femur metaphysis revealed that the cancellous bone area was similar in SH and daidzein-treated animals. Therefore, daidzein was more efficient than genistein in preventing osteopaenia. These results corroborated the data from Ishida *et al.* (1998) with the glycosylated forms of these two isoflavones. Furthermore, in a recent study, Ishida *et al.* (2000) showed that the lower femoral density and breaking strength induced by OVX in 11-week-old rats was largely prevented by daidzein or glycitein (190 nmol/kg body weight per d) but not by the same dose of genistein.

Coumestans. Dodge *et al.* (1996) showed that oral dosing for 5 weeks (starting 1 month after ovariectomy) with coumestrol (0·37, 3·7, 37 and 112 μmol (0·1, 1, 10 and 30 mg)/kg body weight) effectively spared the bone loss associated with ovariectomy in 6-month-old rats, resulting in BMD (measured by quantitative computer tomography) similar to that of intact animals but higher than that of OVX animals. In similar studies (Draper *et al.* 1997), 1·5 mmol of coumestrol was injected intramuscularly as cottonseed oil twice weekly for 6 weeks to 6-month-old OVX rats. This treatment reduced oophorectomy-induced bone loss in those skeletally mature rats. The fall in BMD from baseline to 6 weeks post-ovariectomy was significantly greater in the control OVX than in the coumestrol OVX group. This was associated with lower urinary Ca:creatinine and deceased urinary excretion of deoxypyridinoline (28·2 (SEM 6·8 *v.* 48·2 (SEM 27·1) nmol/mmol creatinine). Consequently, this prevention of bone loss was due, at least in part, to a decrease in bone resorption (Draper *et al.* 1997).

Lignans. In an experiment carried out on 3-month-old OVX rats (Horcajada-Molteni *et al.* 2000*b*), a 10 % flaxseed diet, given for 3 months, improved femoral failure load (N; 118·1 (SEM 4·8) *v.* 104·1 (SEM 5·0) in OVX animals and 100·1 (SEM 1·6) in SH animals), without any effect on bone density. This process resulted from a decreased bone resorption (reduced deoxypyridinoline excretion and lower calciuria). As mineral density stayed unchanged, the higher biomechanical parameters may have resulted from an effect on micro-architecture rather than on mineralisation.

Myco-oestrogens. Zearalanol, a resocyclic acid lactone, given intramuscularly twice weekly for 6 weeks to 6-month-old OVX rats at the dose of 1 mg prevented bone loss as assessed by BMD, compared with control OVX animals, at all skeletal sites measured (whole body, femur or spine; Draper *et al.* 1997). Dodge *et al.* (1996) also demonstrated that zearanol spares the bone loss associated with ovariectomy.

Effects of phyto-oestrogens: influence of the molecular form

All soyabean proteins and food currently available for human consumption contain significant amounts of isoflavones either as the aglycone or as different types of glycoside conjugate (Setchell, 1998). However, little is known about the biological activity of the individual glycosidic conjugates. Even if they are readily hydrolysed by intestinal bacteria (Setchell *et al.* 1984), the role of conjugation may be important in influencing the bioavailability of the aglycone structures because of their preferential absorption (Setchell, 1998). Direct consequences on bone health have been assessed and, although not addressed in the same study, a similar response was elicited by genistin and daidzin (Ishida *et al.* 1998) compared with genistein and daidzein (Picherit *et al.* 2000).

Oral administration for 4 weeks at a dose of 50 mg/kg body weight per d of conjugated forms of daidzein (7-*O*-β-(6″-*O*-succinyl)-D-glucoside) or genistein (7-*O*-β-(6″-*O*-succinyl)-D-glucoside), isolated from soybeans fermented with *Bacillus subtilis* (natto), prevented bone loss in OVX rats fed a Ca-deficient diet. Indeed, both were as effective as daidzin and genistin molecules (Toda *et al.* 1999).

Curative effect v. preventive effect

In the area of pre-clinical testing of potential drugs for osteoporosis therapy, the bone loss of the OVX rat has received the most attention. Using this experimental model, two main types of study can be carried out: preventive therapy to stop the progression of bone loss and restorative therapy to rebuild bone in established osteoporosis. Arjmandi *et al.* (1998*a,b*) investigated the bone-sparing effect of soya proteins in a curative way. They demonstrated a slight reversal of cortical bone loss, partially through higher femoral insulin-like growth factor-1 mRNA transcripts resulting from both soya and soya with reduced isoflavone content: 35 d after surgery, the OVX rats that started a soya diet for 65 d tended to have higher (albeit not significant) mean femoral densities than did the OVX group. In adult OVX rats, daily consumption of isoflavones for 3 months did not reverse established osteopaenia, although it decreased bone resorption (as shown by urinary deoxypyridinoline excretion; Picherit *et al.* 2001*a*). This diet was able to prevent bone loss (assessed by BMD) if given right after castration (Picherit *et al.* 2001*b*).

Safety issues

Evidence from studies of various animal species has demonstrated that ingestion of high levels of phyto-oestrogens can produce adverse effects on reproductive endpoints including fertility. Studies in laboratory animals have also shown that exposure to high doses during development can adversely affect brain differentiation and reproductive development in rodents (Humfrey, 1998). Consequently, before advising soya supplementation in postmenopausal women, we have to investigate further the safety issues of current consumption of such nutrients.

Conclusion

The overriding conclusion is that, when provided at optimal dosages, phyto-oestrogens have modest, positive effects on bone tissue.

As human studies looking at the effect on BMD are very sparse (Dalais *et al.* 1998; Potter *et al.* 1998; Alekel *et al.* 2000), as are those investigating bone turnover (Bonacorsi *et al.* 1997; Wangen *et al.* 2000), such animal experiments provide very useful data. Thus, phyto-oestrogens appear to have potential promise for maintaining or modestly improving bone mass of human subjects when consumed at optimal dosages, as previously suggested by Anderson & Garner (1997). However, because we are constrained by the system with which we study the ageing skeleton, we must appreciate the limits of the information reached before extrapolating to man and we need to gather more data before health professionals can actively advocate the increased consumption of soya. It will be important further to characterise their physiological effects and margins of safety. It is not always clear whether those molecules, often given as single compounds and at high levels, that appear to influence disease risk in animals are functional to the same degree or in the same manner in human subjects consuming realistic doses as part of a habitual diet (Lampe, 1999).

Questions and issues that remain to be resolved include optimal dosages, possible gender differences in response to phyto-oestrogens, demonstration that observed health benefits can be attributed directly to phyto-oestrogens rather than to other components of soya and phyto-oestrogen-rich foods, and the relative impact of different compounds within the phyto-oestrogen family that fall into the broad categories of isoflavones and lignans (Tham *et al.* 1998). Despite the inherent ability of phyto-oestrogens to protect bone tissue, we need to investigate the range of their potential actions fully, in order to rule out unknown metabolic effects with undesirable consequences (Whitten *et al.* 1995).

Acknowledgements

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