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Review Article

Hylonome

Isoflavones and bone: Animal and human evidence of efficacy

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Abstract

Previous reports of soy extracts and isoflavone-enriched preparations studied in animals and humans have found that these molecules, when given at appropriate doses, have positive effects on the skeleton, including improvements in bone mineral content (BMC) and bone mineral density (BMD). A reduction in fracture risk of human subjects has not yet been shown in a prospective trial. Isoflavones, which exist in significant amounts only in soybeans, exert estrogen-like effects in human bone cells because of their unique organic structures that are similar to that of estradiol. The discovery of the b isoform of the estrogen receptor (ER) suggests that the molecular regulation of bone remodeling by estrogens, or estrogen-like molecules, including isoflavones, is more complex than previously thought. Depending on the type of ER present in a particular tissue, isoflavones may act as weak estrogen agonists or as weak estrogen antagonists. For example, isoflavones act as weak estrogen agonists in osteoblasts, but in reproductive cells, such as in the breast and uterus, they behave as weak estrogen antagonists. Weak agonistic effects of the isoflavones include stimulation of osteoblast proliferation and differentiation and increasing the production of cytokines that may inhibit osteclastic activity. The selective beneficial effects of estrogen-like molecules in bone tissues, compared to the anti-estrogenic effects in cells of reproductive tissues, make isflavones attractive for the promotion of bone health. Relatively greater values of BMC and BMD of Asian populations with high consumption of soy isoflavones throughout life, compared to those with lower intakes, indirectly support the skeletal benefits of this pattern of intake of these estrogen-like molecules.

Keywords: Isoflavones, Bone, Estrogen Receptors, Osteoporosis

Introduction

Isoflavones represent one major group of phytoestrogens. These non-steroidal compounds derived from plants were discovered because of their ability to affect fertility in grazing animals[1,2]. Isoflavones interact with estrogen receptors (ERs), and, therefore, they have estrogen-like properties, similar to environmental estrogens or xenoestrogens. Typically, isoflavones are less potent then estrogen. The naturally occurring isoflavones have polyphenolic structures, which function in plants mainly as antioxidants[3]. Isoflavones, such as genistein and daidzein, are found in relatively large amounts in soy foods. The isoflavones have beneficial effects on the skeleton, but fairly high doses are required (see below). These phytomolecules also have other health-promoting benefits on other tissues, such as cardiovascular tissues and blood lipids[4].

The purpose of this review is to provide current evidence for the benefits of isoflavones, especially genistein, on bone tissue, including measurements of bone mineral density (BMD) and bone markers, from human investigations and animal studies. Since isoflavones in concentrated forms have only recently become available to investigators, ambiguities and uncertainties remain about their general utility in human subjects. Most evidence has been accrued for genistein. This phytomolecule, whether consumed as the glycone (with glucose) or not (see below), may in the future

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Food Item	Genistein (mg per 100 g)	Daidzein (mg per 100 g)
Soybeans, raw	111	84
Soybeans, roasted	87	56
Soy flour	81	23
Tempeh	32	27
Tofu	16	15

Adapted from various sources, including Wang and Murphy (1994)[5].

Table 1. Content of Genistein and Daidzein in Soy Products (mg per 100 g dry weight).

be approved as an alternative therapeutic agent to estrogen. The following topics are covered in this report: major sources of isoflavones, structures and metabolism of the isoflavones, mechanisms of action of isoflavones, physiological handling of isoflavones, and studies of the effects of isoflavones on bone in animals, humans and cells. Also, a brief section on concerns about safety and toxicity of isoflavones is given.

Major sources of isoflavones

Foods and supplements (mixtures) derived from soybeans contain isoflavones in varying amounts, depending on the extraction procedure (Table 1)[5]. For example, tofu contains only approximately 50% of the amount of isoflavones compared to roasted soybeans. A few other legumes, such as various beans, also contain isoflavones, but at amounts typically 10 to 100-fold lower than in soybeans. Most of the 5g of soy consumed each day by North Americans is from the use of soy meal mixed in small amounts with other flours in breads and other baked goods, and smaller amounts may be obtained from tofu and a few other soy-containing products. Many Asians, however, consume 30-50 g of soy or modified soy foods each day. The consumption of soy products, especially by postmenopausal women in western nations, appears to be increasing, but it is not likely that sufficient amounts can be easily consumed from foods alone to reduce the risk of osteoporosis (see below under Questions and uncertainties). Supplements will, therefore, be needed.

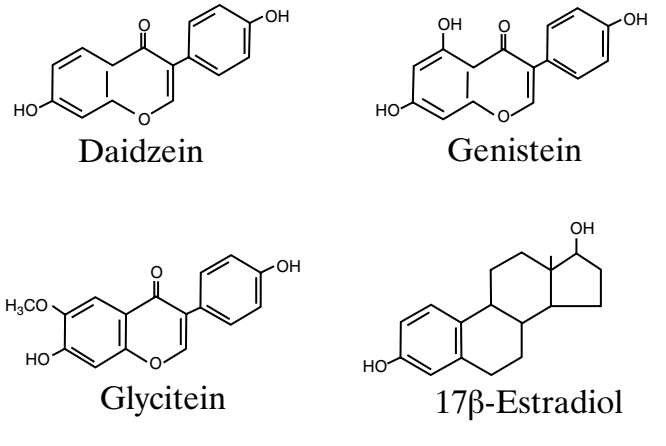

Figure 1. Structures of isoflavones (daidzein, genistein, and glycitein) in comparison to estradiol. The A benzene ring of isoflavones (left) is attached to the heterocyclic pyrane ring; the B benzene ring is to the right.

For practical reasons, the soybean and other soy products are the major sources of isoflavones. The composition of the major soy isoflavones are: genistein (~60%), daidzein (~25%), and glycitein (~15%). The contents of isoflavones in food sources and supplements (mixtures) derived from soybeans vary depending on the extraction and preparation procedures. Table 1 shows the distribution of isoflavones in foods. Isoflavone levels in soybean or products derived from soy are about 1-3 mg/g protein[5]. Because a large difference in intakes of dietary soybean or soy products exists between east Asian nations (e.g., China, Japan, and Korea) and western countries, the serum levels of isoflavones in east Asians are much higher than those in western nations[6,7].

Structures and metabolism of isoflavones

The chemical structures of genistein and other isoflavones are compared to the structure of estradiol in Figure 1. The basic structure of isoflavones consists of two benzene rings, which are linked by a heterocyclic pyrane ring. Also, one hydroxy group (-OH) is found attached to each benzene ring. Genistein and daidzein differ by one hydroxyl group on the A ring of the isoflavone structure. The structures of isoflavones have two characteristics that are similar to the structure of 17β-estradiol, one of the most potent estrogens: (1) both 17β-estradiol and isoflavones have an aromatic ring with a hydroxyl group; (2) a nearly identical distance exists between two hydroxyl groups in both 17β-estradiol and isoflavones[8]. It is not surprising, then, that isoflavones can bind to the estrogen receptor because of their structural similarity to estradiol. However, the isoflavones have much lower binding affinities to estrogen receptors than estradiol. In addition, they have been shown to have lower potential to stimulate alkaline phosphatase production in bone cells compared to estradiol, as shown in Table 2[9-11]. The structural distinctions between estrogens and isoflavones may explain the selective effects of isoflavones in different estrogen-responsive tissues. This point is discussed in the following sections.

Metabolic transformations of the glycated (or glycosylated) isoflavones, also called glycones, to the functional aglycone molecules, such as genistein and daidzein, are illustrated with genistein in Figure 2. The glucose molecules are enzymatically removed from the glycones within the gut lumen to yield the "free" aglycone, i.e., genistein and daidzein. The isoflavones exist predominantly as glycones in the soybean. An advantage of the bound glucose is to keep these molecules in storage and inactive

Molecule	Relative binding affinity		Relative potency †
	ERα*	ERβ◆	
17β-estradiol	1	1	1.0
genistein	0.0025	0.15	0.0008
daidzein	0.0010	NA■	0.00013

* Adapted from Miksicek (1994)[9], ◆ Adapted from Routledge (2000)[11],
† Adapted from Markiewicz et al. (1993)[10]; ■ Not available.

Table 2. Relative binding affinity to ERα and ERβ and relative potencies in inducing alkalinephosphatase between 17β-estradiol and isoflavones.

until needed for their roles as antioxidants in plant tissues. A recent study by Setchell et al. (2001) reported the pharmacokinetic behavior of naturally occurring isoflavones tested in 19 healthy women[12]. In this study, time to reach peak plasma concentrations for the aglycones was found to be only about half of the time as for glycones. This finding is consistent with the extra time needed for the enzymatical cleavage of glucose molecules from the glycones to make the aglycones bioavailable.

Mechanisms of action of isoflavones on bone cells

Estrogen receptors (ERs) respond to both true estrogens and isoflavones, as well as to other environmental estrogen-like molecules. The isoflavones have higher binding affinities for the ERs in mammalian cells than most other estrogen-like molecules but lower than for estradiol (see Table 2). When isoflavones reach the target tissue, isoflavones cross the cell membrane by passive diffusion; they then bind to ERs in the cytosol and form an isoflavone-ER complex; this complex then translocates into the nucleus for activation of the estrogen response element (ERE), which is involved in the regulation of DNA-directed mRNA synthesis and the production of new proteins.

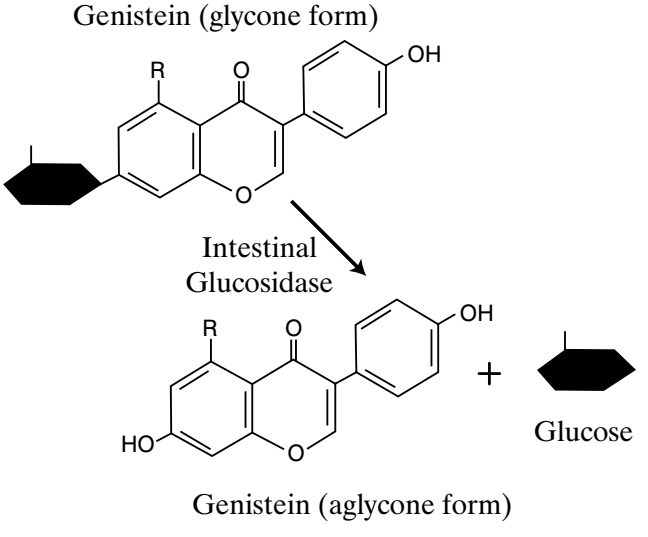

Figure 2. Metabolism of genistein. Conversion of genistein, a glycone, to genistein, an aglycone. In addition, biochanin A can also be converted in the gut to genistein. Genistein may be further metabolized to para-ethylphenol in the gut lumen (not shown here).

ERs are found in osteoblasts[13,14], but their presence in mammalian osteoclasts remains controversial. Besides secreting bone formation-related proteins, such as alkaline phosphatase and osteocalcin, osteoblasts are also capable of synthesizing many other cytokines. These cytokine products (eg., interleukin-6 and osteoprotegerin, etc.) have been demonstrated to have critical roles in the regulation of osteoclast differentiation and activities[15,16]. Isoflavones may, therefore, have indirect effects on osteoclasts by mediating cytokine production in osteoblasts. The net result of these actions of isoflavones is inhibition of osteoclast differentiation and bone resorption activities, including reduction of their numbers.

The discovery of a new type of estrogen receptor, ERβ, has raised a number of questions regarding the respective physiological roles that these two receptors, ERα and ERβ may play in an estrogen-responsive tissue[17]. This new receptor may help explain the selective effects of estrogen-like molecules in different tissues. A recent report indicates that genistein may differentially bind to ERα and ERβ receptors, acting as a partial agonist with ERβ receptors[18]. At this point, understandings of the relationship between genistein stimulation and ER expression remain limited, and the roles of ERs in the activation of gene transcription in osteoblasts may be cell type- and promoter-dependent, as suggested previously[19]. Because of the different binding affinity to ERα and β, isoflavones may act differently on the two estrogen receptors. In addition, not all isoflavones are equally effective. A recent report suggested that daidzein is more effective than genistein in rodent studies[20].

Besides this ER-dependent pathway, some isoflavones, such as genistein, can also bind with membrane receptors and function as a tyrosine kinase inhibitor[21], which regulates protein phosphorylation. In this way, isoflavones influence cell cycle and metabolism through second messengers in the cytoplasm (Figure 3).

Isoflavone Content in animal diet	Time	Effects on Bone retention	Reference
1 mg/day *	28 days	positive	Blair et al., 1996[25]
0.5 mg-5.0 mg/d	14 days	biphasic effects	Anderson et al., 1998[26]
50 mg/kg/d	28 days	positive	Ishida et al., 1998[27]
5-25mg/gbw/d (inject)	21 days	positive	Fanti et al., 1998[28]
NA$^{\omega}$	30 days	positive	Arjmandi et al., 1998α and b[29,30]
0.1-0.7 mg/d	14-28 days	positive	Ishimi et al., 1999[31]
50 mg/kg/d	28 days	positive	Toda et al., 1999[33]
10 mg/kg/d	90 days	positive	Picherit et al., 2000[20]
0.7-5 mg/d	28 days	positive	Ishimi et al., 2000[32]

* Pure genistein; $^{\omega}$ Not available.

Table 3. Effects of isoflavones on BMD and BMC in ovariectomized rats or mice.

Physiological handling of the isoflavones

After digestion of the glycosylated forms (glycones) by non-specific glycosidases within the gut lumen or on the brush border membrane surfaces, the free isoflavones are rapidly absorbed at the surfaces of the intestinal absorbing cells and, then, within the cells the isoflavones are further modified. Genistein, for example, is conjugated with either glucuronic acid (glucuronate) or sulfuric acid (sulfate) in the gut cells prior to release to the venous circulation that is collected by the hepatic portal vein. The major metabolites found in circulating blood are conjugated rather than free. It is presumed that peripheral tissues (or capillaries) contain the deconjugating enzymes that allow the free isoflavones to enter cells and exert their actions[22]. The isoflavone molecules have metabolic half-lives of several hours to days, and they are excreted in bile or urine. A few metabolites derived from intraluminal gut activity, such as equol from daidzein, may also be found in the circulation as well as in urine[23].

Evidence for the efficacy of isoflavones on bone

Three lines of evidence are used to illustrate the effectiveness of genistein and other isoflavones in bone: animal research, human studies, and *in vitro* cell investigations. Soy extracts or mixtures of isoflavones have typically been used in these studies; enriched soy protein means that additional amounts of soy extracts (isoflavones) have been added to the protein.

Animal research

Ovariectomized (OVX) rats or mice have been commonly used models to examine the effect of isoflavones on bone loss caused by estrogen deficiency. Previous findings from OVX animal studies have been highly consistent in demonstrating that soy isoflavones, either administered alone or with soy protein in the diet, increase BMC or BMD compared to control animals administered vehicle[24]. Table 3 lists the findings in these studies[20,25-33]. A recent study by Picherit et al. (2001) reported that neither BMD nor cancellous bone area was greater in adult OVX rats (7 months) fed with an isoflavone diet than in control OVX rats[34]. These findings suggest that OVX animals at old age may have a lower response to isoflavone treatments than those at young age with higher bone turnover rates. In these studies, no effects on the uterus were found in rats treated with isoflavones, which indicates that at the doses used, no adverse reproductive effects would be likely to occur.

Human investigations

A few human investigations on isoflavone supplement have been reported, but in total they have failed to clear up the uncertain effects of isoflavones on skeletal tissue. The first published report showed that BMD of the lumbar vertebrae in elderly postmenopausal women was increased after six months of treatment with soy protein enriched with isoflavones (96 mg per day)[35]. BMD of the hip was not changed. A corroborative 24-week study of perimenopausal women by Alekel et al. (2000) found very similar results using almost the same dose of mixed isoflavones with soy protein[36].

Data from recent human studies by Gallagher et al. (2000) and Hsu et al. (2001), however, reported that no significant differences were found in BMD after administration of isoflavones (about 50 to 150 mg/day) in postmenopausal women for 6 or 9 months when compared with baseline data. Longer studies are necessary to clarify the bone-sparing effect of isoflavone supplementation because the duration of these studies was too short to observe changes of bone density[37,38].

Epidemiologic studies designed to investigate the relationship between dietary soy intake and bone mineral density in Asian women have shown that high dietary soy

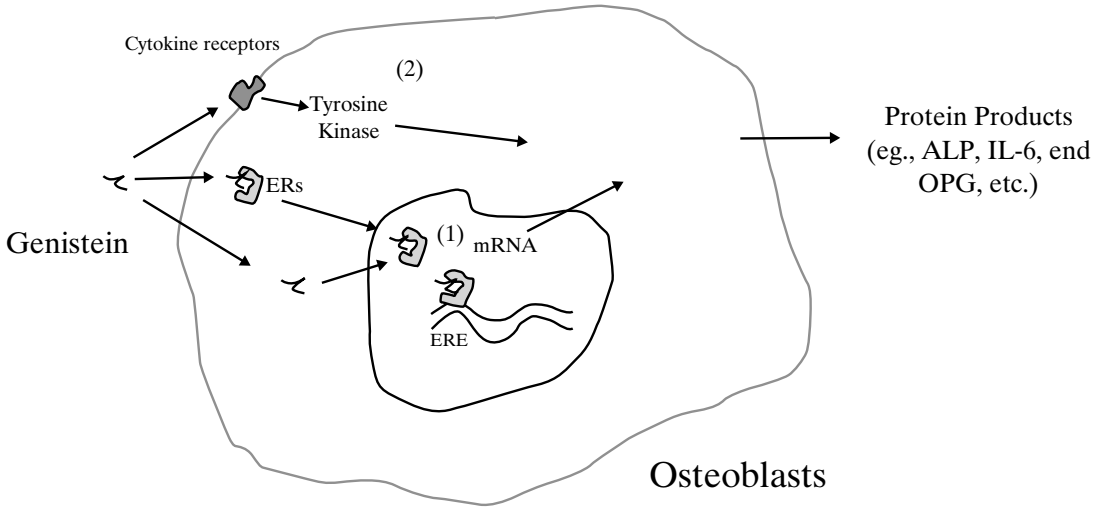

Figure 3. The mechanisms of action of isoflavones on osteoblastic cells using genistein as the model. (1) Estrogen receptor (ER)-dependent pathway: genistein is taken up by passive diffusion across the cell membrane and act as weak agonists via the classical estrogen mechanism. Typical protein products synthesized and secreted by osteoblasts, such as IL-6, are inhibited by the action of genistein. (2) Non-ER-dependent pathway: isoflavones interact with membrane proteins (receptors) and exert an effect that is expressed through second messengers in the cytoplasm (ALP: alkaline phosphatase; IL-6: interleukin-6; OPG: osteoprotegerin).

intake was correlated with a higher bone mineral density in postmenopausal women but not in premenopausal women[39-41]. Further epidemiologic studies are needed to confirm these results in other target populations.

Several bone markers were assessed in the study by Alekel et al. (2000), but only bone-specific alkaline phosphatase (BALP) was found to consistently correlate with the reduction in lumbar BMD of the two groups of bone-losing women that were not given isoflavones[36]. BALP, a marker of bone formation by osteoblasts, remained relatively constant in the women treated with soy protein isolation (with isoflavones), but it fell non-significantly in the other two groups[36]. The higher BALP concentration in the isoflavone-treated women suggests that bone formation is continuing at a higher rate than in control subjects. Other studies of isoflavones using bone markers alone have not been very instructive.

In vitro studies of osteoblast-like cells

Several osteoblast-like cell lines have been used in culture to test the findings on effects of isoflavones on bone remodeling in animal or human studies. Results from *in vitro* studies showed that osteoblast-like cells in culture respond directly to isoflavones. In studies, in which genistein has been used as a tyrosine kinase inhibitor to investigate the signal transduction related to tyrosine phosphorylation during osteoblastic cell proliferation, the concentrations of genistein used were in the pharmacological range (~ 10^{-4} to 10^{-3} M)[42-44]. Investigations on the effects of isoflavones, at dietarily related concentrations, on osteoblastic phenotype development are limited. Sugimoto et al. (2000) reported that genistein had an anabolic effect on osteoblastic MC3T3-E1 cells, and they found that genistein at 10^{-6} and 10^{-5} M caused a significant elevation of alkaline phosphatase activity, and protein and DNA content in the cells[45]. In addition, results from their experiments showed that tamoxifen, an anti-estrogen, clearly abolished the increased protein content and alkaline phosphatase activity induced by genistein. Data from the same laboratory showed that daidzein also had an anabolic effect in osteoblastic MC3T3-E1 cells[46]. In addition, Dang et al. (2000) reported that genistein at concentrations of 10^{-6}M or 10^{-7}M stimulate osteoblastogenesis but inhibit adipogenesis in pre-osteoblastic KS483 cells via estrogen receptor-dependent pathways[47].

At high concentrations (10^{-4} to 10^{-3} M), genistein acts mainly as a tyrosine kinase inhibitor, and thereby inhibits cell growth, arrests cell cycle progression at G2-M, and induces apoptosis[48,49]. At lower concentrations (10^{-10} to 10^{-6} M), which are closer to the dietarily achievable levels, genistein appears to function more as a weak estrogenic agonist via ERs[50,51]. These speculations were also supported by the important finding from our and other laboratories that osteoblast-like cells have a biphasic response to a wide range of concentrations of isoflavones[26,52].

In our laboratory *in vitro* experiments of osteoblast-like cells (MC3T3 cells), treated with genistein at different concentrations, have revealed that genistein, like estradiol, significantly inhibited the production of interleukin-6 (IL-6) by these cells[53]. Genistein presumably acts via ER pathways in the osteoblast-like cells, and then binds to the specific

DNA promoter which down-regulates IL-6 mRNA transcription, and inhibits IL-6 synthesis. IL-6 is considered to be one of the major signals that stimulate osteoclastic bone resorption. This finding implies that genistein, like estradiol, may act on osteoblasts to reduce osteoclastic resorption by first inhibiting IL-6 synthesis, a direct effect, which then leads to decreased amounts of the IL-6 signal leaving osteoblasts to stimulate osteoclastic activity. In addition to IL-6, the osteoblastic synthesis of other osteoclast-stimulating cytokines may also be inhibited.

Furthermore, our studies of bone-forming osteoblast-like cells in culture have shown that an optimal dose range of pure genistein exists for maximal responses, e.g., enzyme synthesis and release, but doses that are too high can produce cell death by the genetically controlled process, apoptosis[26]. Doses that are too low have little effect on the endpoints of these bone-forming cells.

Toxicity and safety

The isoflavones, individually or combined, are considered to be safe up to quite high doses. Human toxicity studies of isoflavones by Busby et al. (2001) (under contract with NCI), currently in publication, suggest that doses ranging from 1 to 16 mg/kg body weight are reasonably safe[54]. At the doses being recommended for prevention of bone loss in postmenopausal women, little concern has been raised about deleterious effects. A few studies of rodents and isolated cells, however, suggest that isoflavones may not be totally safe. Genistein at high doses has been shown *in vitro* to inhibit cell growth and induce apoptosis as mentioned previously[48,49]. In addition, some reproductive disturbances, such as uterotropic effects, have been reported in animals fed a diet rich in isoflavones or other phytoestrogens[55-57]. Therefore, toxicity concerns from studies of rodents and *in vitro* cells must be resolved before isoflavones can be recommended as therapeutic agents.

Questions and uncertainties

For skeletal effects, pure genistein alone (or a combination of genistein with other isoflavones) is effective, but the achievement of significant reductions of total cholesterol and cardiovascular benefits may require soy protein along with the isoflavones. The question of whether soy protein is necessary along with isoflavones for optimal skeletal responses remains unresolved.

The optimal dose for skeletal gains seems to be between 60 and 100 mg per day, perhaps closer to 90 mg a day. In the two studies of post- or perimenopausal women that found benefits in vertebral BMD, approximately 90 mg of mixed isoflavones per day was the dose used. Whether split doses over 24 hours would be more effective has not been tested.

The long-term safety of isoflavones, either mixed with soy protein or as purified supplements, remains to be determined in human subjects. No one questions the safety of soy isoflavones consumed in standard food products, such as tofu and related foods, but the use of supplements alone for long periods is a concern to many investigators.

Summary

The reports reviewed herein have focused primarily on the effects of isoflavones, especially genistein, on bone. Isoflavones need to be consumed by humans at doses approximately twice as great for skeletal improvements as for lipid benefits. Therefore, any skeletal gains in BMD will not likely be observed until doses of 60 to 100 mg of isoflavones are consumed daily for periods of six months or, preferably, longer. The benefit of isoflavones in the diet is that they help maintain a healthy balance between the activities of osteoblasts and osteoclasts that results in better bone conservation and the potential prevention of osteoporosis, and associated fractures.

The positive effects of isoflavone-rich preparations and of soy foods enriched with these phytoestrogens on bone tissue in rodents hold promise for similar benefits in humans. Data from animal experiments suggest that adult humans may receive similar improvements in lumbar BMD, either absolute or relative gains, following isoflavone administration compared to no treatment (placebo). Based on a limited number of human studies, a dose between 60 and 100 mg of isoflavones per day may be needed to show a skeletal benefit. Toxicity studies of human subjects suggest that purified isoflavones are safe at doses at least twice as high as used in reported studies.

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