Soy Isoflavones and Bone Health: A Double-Edged Sword?[⊥]

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Numerous publications and research studies on isoflavones have prompted a nationwide increase in the consumption of soy-based foods and supplements in the United States. Isoflavones are natural endocrine active compounds generally considered to promote health and prevent or slow the onset of certain chronic diseases such as osteoporosis. The beneficial effects of soy isoflavones on bone may, however, be life-stage specific and dependent on the estrogen receptor number and endogenous hormone milieu. Perimenopausal and early menopausal women may therefore be more receptive to the therapeutic effects of isoflavones on bone loss prior to the diminution of estrogen receptors that occurs in the postmenopausal years, whereas laboratory studies in developmental age range animals have demonstrated the potential for adverse effects following exposure to high levels of soy isoflavones. Clinical studies in developing humans that either support or refute findings in animal studies are lacking. The effects of chronic consumption of high levels of soy isoflavones at each life stage to assess risk−benefit ratios should be a high priority of research.

Introduction

By virtue of being structurally similar to 17β-estradiol (E_2), isoflavones are often referred to as phytoestrogens and have gained much public attention based on their apparent potential to elicit influential estrogen-like effects.[1−9] Soybeans (*Glycine max* L.; Fabaceae) and value-added products processed from them generally comprise the most abundant and physiologically relevant source of isoflavones in the human diet.[10] Soybean-derived isoflavones exist either as polar β-glucosides, such as genistin, daidzin, and glycitin, or in the free form, as aglucons that include genistein, daidzein, and glycitein. In nature these soy isoflavones, whether free or attached to a sugar moiety, occur in an approximate 5:4:1 ratio, respectively.[11] Corresponding acetyl and malonyl derivatives of soy isoflavone glucosides also exist,[12] as well as gut microflora-generated metabolites of both genistein and daidzein.[13] From a nutritional standpoint, phytoestrogenic isoflavones are not classified as conventional nutrients; that is, they are not food elements individuals must consume in order to exist, develop, and reproduce.[14] They are instead perceived as nonessential[15] phytochemicals that epidemiological,[16] clinical,[17] and laboratory animal evidence,[18−21] as well as intervention studies[22] and mechanistic data,[23] have indicated contribute to a reduction in chronic disease risk. The general notion is that non-nutrient natural bioactive compounds including isoflavones subtly, but profoundly, influence health over time.[24] However, there is also evidence that suggests the effects of isoflavones on human health may prove to be a double-edged sword.

Current Consumers of Soy Isoflavones

In October 1999, the U.S. Food and Drug Administration (FDA) announced the authorization of a health claim to be used on food labels relating to the association between consumption of soy protein and the reduced risk of coronary heart disease.[25,26] To date, the soy claim does not extend to isolated substances from soy protein such as the isoflavones genistein and daidzein,[27] although the consumption of 25 g/day of soy protein, which is the FDA heart health claim recommendation, is a source of up to ~60 mg of soy phytoestrogens.[28] Currently, the soy dietary products inundating the market place have been more aggressively targeted at females, and health-conscious women across all age groups are opting for isoflavone-containing soy foods, and/or botanical supplements containing them, in anticipation of potential health benefits. Also, a health claim for soy and cancer is under consideration,[29−31] but the relationship of isoflavone consumption to bone health has been less studied and will be the emphasis of this review.

In the year 2000, the U.S. Department of Agriculture (USDA) drafted new legislation that approves unlimited use of soy in school lunch programs (i.e., 100% soy-based foods),[32] a regulatory change that ended long-standing restrictions requiring soy to be used strictly as a food additive in amounts below 30%.[33,34] In a bid to curb childhood obesity[35−37] and maintain the cost-effectiveness of school lunch programs, soy is now routinely included as a major ingredient, possibly exposing American children to unprecedented levels of soy isoflavones. The American Academy of Pediatrics currently recommends isolated soy protein-based formulas as a safe and effective alternative for providing appropriate nutrition for normal growth and development for term infants whose nutritional needs are not being met from maternal breast milk (preferred) or cow's milk-based formulas.[38] This recommendation heavily impacts term infants, because in North America by two months of age most infants are formula-fed,[38] and soy protein-based formulas presently meet the needs of ≥25% of the infant formula market[39] or ~15% of infants.[40] There exist substantial differences between traditional Asian and current American exposures to soyfoods in terms of dosages, form (i.e., fermented versus nonfermented), and the far higher tendency for Asian infants to be breast fed until weaned.[41]

Isoflavone Efficacy: What Should We Expect?

Investigators are still grappling with a number of seemingly baffling contradictions in the data related to the effects and outcomes associated with soy isoflavone intake by humans and animals. Thus far, experimental results pertaining to the potential for soy isoflavones to positively affect health have been somewhat ambiguous, indicating various degrees of efficacy, no discernible significant effect,[42,43] and the capacity for potential harm.[44−46] Due to the inherent nature of gene expression during an organism's development,[47] maturation, and senescence, perhaps we should logically anticipate differing responses and variations in the magnitude of responses at specific life stages, since our physiological and

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biological condition does not remain constant throughout the life cycle. Certain isoflavones may be beneficial at one life stage or under some conditions and alternatively may be ineffectual, or even potentially detrimental, at another.

Isoflavone Mechanisms of Action

Similar to estrogens, the effects induced by isoflavones may be broadly summarized either as long-term genomic actions mediated by intracellular estrogen receptor-induced changes in gene expression or as rapid nongenomic actions that modulate a diverse array of intracellular signal transduction cascades.[48] Evidence pertaining to the health-promoting and estrogen-like effects of dietary isoflavones implicates more extensively their estrogen receptor (ER) binding potential in vivo,[49] since the isoflavone concentrations required to stimulate certain nongenomic activities, such as inhibition of cellular protein tyrosine kinases (i.e., $\sim$>10 μM)[50] and topoisomerase-II (based on in vitro evidence),[51−53] typically exceed the plasma levels that can be attained via a habitual dietary intake of soy-rich foods (i.e., $\sim$2−5 μM). Furthermore, isoflavone aglucons, found predominantly in fermented soy foods[54] and select isoflavone supplements,[55] are by comparison the more bioavailable[54] and the bioactive forms of isoflavones.[56] Unlike their more hydrophilic β-glucosidic counterparts, they easily permeate mucosa and other cell membranes. The estrogen-like activity of agluconic soy isoflavones is still, however, multiple magnitudes lower than that of estradiol.[57,58] In general, on a molar basis, the transcriptional activity of genistein at ERα and ERβ, respectively, only reaches $\sim$0.025% and <0.1% that of E_2,[59,60] and daidzein and glycitein together provide less than 0.0025% or 0.025% of the transcriptional activity of E_2 at ERα and ERβ.[61] Nevertheless, the general abundance of plasma isoflavones following their consumption can manifoldly exceed endogenous estradiol concentrations[62] by an astounding 10 000- to 20 000-fold in adults,[63] by 13 000- to 22 000-fold in infants,[64,65] and by 1000- to 100 000-fold in rodents.[66] This allows naturally high concentrations of isoflavones to compensate somewhat for their relative weakness compared to E_2 by way of plentitude.[67−69]

Isoflavones are also classified as selective estrogen receptor modulators (SERMs) because they selectively modulate ERs and evoke disparate biological responses at the molecular, cellular, and physiologic level. Soy isoflavones have long been promoted as a natural and "safer" alternative than estrogen replacement therapy (ERT), providing postmenopausal women experiencing the adverse effects of a depletion of ovarian estrogen with many of the benefits of estrogen replacement while mitigating some of the disadvantages associated with estrogen-related cancer risk. McNeil has aptly described the perfect SERM as "...a compound that acts as a potent anti-estrogen in the breast and uterus to prevent estrogen-driven cell proliferation and, at the same time, has strong estrogenic effects in bone, the cardiovascular system, and the central nervous system, where hormones can help a variety of postmenopausal conditions."[70] In this respect, isoflavones do not purely mimic estrogens because their dietary-linked tissue-specific effects generally range from estrogenic to antiestrogenic.[71] Activity depends on the spatial and structural differences of isoflavone molecules compared to estrogens, as this is what largely governs the degree of agonism or antagonism exhibited in the amino acid lined binding pockets of hetero- and homodimerized α and β ERs. Factors such as ER number and distribution of ER subtypes in various organs are tissue specific and regulated by the natural life-stage specific changes in gene expression that govern developmental transitions through infancy, prepuberty, adolescence, and pre-, peri-, and postmenopausal periods, and gene expression in this sense is crucial to the potential effects dietary isoflavones may exert.

Bone Health

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased skeletal fragility.[72−74] It is a condition indicative of a chronic pathologic loss of bone, and the associated osteofragility increases an individual's susceptibility to fractures, which represent the most costly[75] and debilitating clinical endpoint.[76] A large body of evidence lends credence to estrogen's ability to contribute significantly to the regulation of skeletal metabolism via constraining and balancing effects on bone remodeling cycles, which keeps bone cell activity adequately balanced so that osteoclastic resorptive activity does not progressively exceed the anabolic activity of osteoblasts. An estrogen insufficiency following surgically induced menopause, or during the natural decline and subsequent cessation of the estrogen-producing capacity of the ovaries in peri- and postmenopausal women, generally results in women being more severely affected by pathologic bone loss than men.

Results thus far gleaned from components of the Women's Health Initiative (WHI) long-term national multicenter health study indicate that postmenopausal estrogen replacement can significantly increase bone density at the hip and reduce the hip fracture rate of women; however a global model for overall risks versus benefits associated with the effects of hormone replacement therapy (HRT) on other disease processes became a cause for concern.[77,78] The WHI estrogen-plus-progestin randomized controlled primary prevention trial investigating the effects of long-term oral HRT was prematurely stopped after a mean 5.2 years of follow up because the increased risk of invasive breast cancer, adverse cardiovascular disease events, and other hazards outweighed the corresponding benefits of a reduction in risk for hip fracture and colorectal cancer.[78] The intervention phase of the estrogen-alone arm of the WHI study was also halted ahead of time when the effect of oral estrogen on heart disease was determined to be neutral and the risk for stroke and thrombosis was observed to increase.[79] Soy isoflavones, as constituents of soy foods and in supplement form, were subsequently more heavily promoted in the market place as a safer alternative or as a complimentary therapy to HRT for the treatment of osteoporosis and other menopause related conditions.

Potentially Important Variables

Types of Estrogen Receptors. The discovery of a second estrogen receptor subtype (ERβ) in 1996[80] accounted for many of the previously inexplicable divergent tissue effects of estrogens. The concept of heterodimeric ERs binding to DNA was born,[81] and the realization that the abundance of ER subtypes in various tissues differentially mediates the effects of estrogen demonstrated the complex nature of estrogen's ER-mediated effects. Before too long, advanced molecular biological techniques were employed to determine the existence of multiple alternative ER splice variants (i.e., structurally altered ER isoforms), many of which are determined to be functionally active.[82] The nuclear receptor superfamily, from which ERs originate, also comprises a number of estrogen receptor-related receptors (ERRα, -β, and -γ),[83] a subfamily of orphan receptors for which there exists no known endogenous ligands.[84,85] Isolated on the basis of their sequence homology and similar domain organization to ERα and ERβ,[86,87] ERRs have been located in numerous tissues, including but not limited to the human ovary, breast, uterus,[82] and bone tissues.[88] In addition to their constitutive activity, they are speculated to play a role in modulating physiological effects when bound by ligands.[89] Despite ERRs not binding estrogens, Suetsugi et al. have shown that genistein and daidzein are exogenous ligands of ERRs and that ERRα activity is as great as ERα and ERβ activity in the presence of the same compounds at similar concentrations.[90] ERRα is reported to be coexpressed with ERs in osteoblastic cell populations derived from human bone, and Bonnelye et al. postulate that there is an osteogenic potential associated with their combined activity that may be down-regulated during postmenopause in the absence of estrogen.[91] The point of mentioning the various types of estrogen receptors is to illustrate that ER-mediated effects are incredibly

Table 1. Summary of Factors that Potentiate Soy Isoflavone Effects

variable	key factors
types of ERs	prevalence of ER-α/α, -β/β, -α/β, various ERRs, and ER splice variants
ER status	ER number and responsivity is dependent on estrogen status, age, and health
estrogen status	age, sexual maturity, reproductive senescence, surgical menopause, and ERT
biphasic effects	dose, high intake may cause ER saturation or PPARγ activation
bioavailability	intestinal microflora profile, health status, medications, glucosides vs aglucons, and age
age-related effects	genetic capacity to express ERs related back to E_2 status and methylation
DNA methylation	increases with age and gradually begins to silence ER expression
isoflavone source	fermented vs nonfermented, purified vs mixtures, combination ratios

diverse and appear to become ever more multifaceted as progress is made in researching them. Currently, we have no clear indication of what the exact biological significance of various soy isoflavones and other estrogen-like ligands encountering the mix of available receptors might be. At present, on this basis alone, the potential for various ER-mediated effects becomes exceedingly difficult to accurately single out of the vast combination of possibilities for interactions and effects on health.

Estrogen Receptor Status. The efficacy of a SERM in relation to bone health is naturally dependent on the presence and relative abundance of ER subtypes in target cells, since the effects of SERMs are proposed to be largely mediated via these transcription factors. Circumstances that may ultimately influence ER expression and possibly ER responsivity include available estrogen and age. These dynamics are of considerable importance to women as they approach and undergo menopause. A reduction in circulating estrogens accompanies natural reproductive aging at the time of menopause and may also lead to the reduced expression of ER in target tissues.[92,93] Batra et al. have shown that in women over 40 years of age ERα and ERβ expression is relatively reduced in osteocytes,[94] the mechanosensory bone cells that play an integral role in skeletal adaptation by way of being connectedly embedded in the mineralized matrix so as to detect mechanical strain or deformation-mediated fluid flow. Hoyland et al. compared bone biopsies from women with normal concentrations of ovarian steroid hormones prior to ovariectomy or post-HRT against biopsies of the same women postovariectomy or pre-HRT for cellular localization of ERα protein or mRNA expression.[95] Hormone-adequate women were characterized by higher levels of bone cells positive for immunodetectable ERα.

Ovariectomized (OVX) rodents have also been shown to display a decrease in ERα and ERβ in heart tissue and bone[96,97] and a decrease in ERRα in bone and uterus tissue,[91] suggesting estrogen status may play a definitive role in influencing ER expression. Conversely, ER mRNA expression in rats has been shown to increase in the uteri[98] and bone[97] during estradiol therapy, and estrogen has been found to directly augment in vitro ERα expression in murine mesenchymal stem cells that can give rise to a variety of cell types including bone cells. Bjarnason et al. found that, despite years since menopause, all HRT regimes tested in postmenopausal women arrested bone loss.[99] Taken together these data suggest that positive regulation of ER expression may be dependent on the extent to which ERs engage their cognate ligands. If soy isoflavones are to function as SERMs, it could be hypothesized that their presence would likely be more efficacious when less opposed by estrogen and before the ER number decreases as a result of chronic hypoestrogenicity. As a result, the optimal response to soy isoflavone supplementation in terms of bone preservation may be more likely to be elicited in the perimenopausal or early postmenopausal periods of a woman's life.

Estrogen Status. A key factor affecting the estrogen-like potential of soy isoflavones relates to the fact that in the first instance they must directly compete with endogenous E_2 in estrogen-responsive target tissues for the opportunity to bind to ERs.[59,100−102] Hormonal status can influence the likelihood of phytoestrogens binding to ERs and may, to some degree, determine whether the magnitude of the effects exerted will be of physiologic significance and/or clinical relevance. If isoflavones are abundant in the body at a time of estrogen sufficiency, a relatively negligible estrogenic effect may be attributable to them given that their affinity to ERs and genetic modulatory potency is less than that of E_2.[42] In fact, such an effect may even be interpreted as antiestrogenic,[103,104] because the existing hormonal potential of E_2 may be dampened and replaced to some extent by isoflavones,[105] which are comparatively less effective ER modulators. Alternatively, during hypoestrogenic states (e.g., postmenopause) an abundance of isoflavones unopposed by endogenous estrogen may be observed to exert an estrogen-like effect[106] in estrogen-responsive tissues, including bone.[107] In the absence of sufficient endogenous estrogen, the theoretical implication is that any agonist activity of phytoestrogens at ERs, even if it is partial agonism, will likely influence overall estrogenicity. The effects elicited by soy isoflavones under these circumstances will depend on not only the level of endogenous estrogen but also such factors as dosage,[108] bioavailability, ratios of component isoflavones if combined, and ER status (e.g., number and subtype distribution) in various target tissues.[68]

There appears to be consensus among researchers in relation to the lack of effect of soy isoflavones on bone mineral density (BMD) in estrogen-replete subjects. An isoflavone-rich soy preparation regularly consumed over a 12-month period in young healthy adult females with normal menses demonstrated no effects on bone mineral content (BMC) or BMD.[42] Arjmandi et al. showed that soy protein, with its constituent isoflavones, more positively influenced bone and calcium homeostasis in postmenopausal women not on HRT.[109] Cai et al. found there was no benefit to bone when isoflavones were added to the diet of mature OVX rats with or without estrogen administration.[110] A study by Nakai et al. showed intact 3-month-old female Fischer 344 rats fed isolated soy protein (ISP) with high or low levels of isoflavones (200 and 100 g/kg, respectively), or a diet containing high- or low-level extracts of ISP (17.2 and 34.4 g/kg, respectively), revealed no significant bone parameter differences between the casein control and treatment groups, except for a lower level of the resorption marker deoxypyridinoline in the high-soy group and a higher lumbar BMD in the low-soy group ($P < 0.05$).[111] A study of similar design by Nakai et al. using intact female Sprague−Dawley rats also showed that femur and lumbar BMD was not significantly different between control and treatment groups and had a potentially negative effect on the uterus.[112] These results and others[113] suggest soy isoflavones have little or no osteogenic effects on premenopausal women and rodents with adequate circulating concentrations of estrogen or on postmenopausal mammals on HRT.

Biphasic Effects. Conflicting results pertaining to the osteoprotective and osteogenic potential of isoflavones may, in part, relate to the combinations of isoflavones present,[114] the administered dose, duration of exposure,[115] and route of administration.[116,117] After investigating the effect of a single purified isoflavone on bone tissue in an OVX, lactating rat model, Anderson et al. proposed that genistein's osteoprotective potential may be governed by a dose-dependent threshold, such that biphasic effects may ultimately be elicited.[118] This study demonstrated that the lowest administered dose of genistein (0.5 mg/day) was significantly more effective than the intermediate and high dose (1.6 and 5.0 mg/day, respectively) in terms of retaining bone mass, including cancellous bone tissue, where the latter was assessed by scanning electron microscopy. Remarkably, the beneficial effect of low-dose genistein on cancel-

lous bone was reported to be approximately equally as effective as estrogen when based on trabecular number and density in the tibia subepiphyseal region. Picherit et al. reported a biphasic effect of soy isoflavones on cortical bones of adult OVX rats, where the lowest administered dose was most effective.[66] In contrast, only the highest doses of isoflavones in this same study elicited a protective effect on the trabecular-rich metaphyseal region.[66] The different dose-related effects elicited by isoflavones on trabecular bone as reported by Picherit et al. and Anderson et al. may be explained by the disparate effects of a mixture versus single purified isoflavones.

Genistein biphasicity may be a phenomenon attributable to an ER saturation effect. At low or "physiological concentrations", genistein, if it is not in competition with E_2 or other compounds with a more concentrated presence and/or a higher affinity for ERs, may occupy available ERs to exert estrogen-like effects. At higher concentrations, genistein in excess of that necessary to populate available receptor sites may exert other non-ER receptor-mediated effects that may not be conducive to osteogenesis nor serve to prevent bone loss. An example of this biphasic propensity was demonstrated by Dang et al. when the effect of genistein on osteogenesis and adipogenesis in the mesenchymal KS48 mouse clonal cell line and in mouse bone marrow cells was investigated.[119] It was concluded that genistein has the potential to activate ERs and PPARγ (i.e., peroxisome proliferator activated receptor gamma, a transcription factor involved in adipogenesis) in a biphasic manner to elicit opposing effects dependent on genistein dose. At low concentration ($<1\ \mu$M), genistein was shown to act like an estrogen in an ER-dependent manner, stimulating osteogenesis and inhibiting adipogenesis. At high concentrations ($>1\ \mu$M) genistein was considered to act as a ligand of PPARγ, resulting in the up-regulation of adipogenesis and down-regulation of osteogenesis. Also, when the potent antiestrogen ICI182780 was used to block ERs in the presence of isoflavones, osteogenesis was inhibited while adipogenesis was stimulated. The biphasic nature of genistein is not a phenomenon unique to bone. Accounts of the biphasic activity of genistein have also been reported in MCF-7 breast cancer cells.[120,121] Low concentrations of genistein in the micromolar range have been shown to exert proliferative effects, whereas high concentrations appear to inhibit growth[121,122] and/or are found to be cytotoxic.[123] Genistein has also been found to elicit biphasic effects on LNCaP prostate cancer cells,[124,125] atrial myocytes,[126] ovaries,[69] intestinal cell proliferation,[40] spermatozoa motility,[127] and steroidogenic enzymes.[128]

Bioavailability. The absorption, bioavailability, metabolism, and elimination of soy isoflavones are important factors governing their potential activity, and the discovery of various interindividual and interspecies differences makes it increasingly challenging to design studies that yield conclusive results. The importance of intrinsic β-glucosidases and microfloral enzymatic activity in the metabolism of isoflavones is emphasized by the fact that glucosides are not generally detected in plasma,[129−132] and the excretion of isoflavone metabolites is greatly diminished following antibiotic treatment of human subjects[133] and in "germ-free rats" [sic].[134] Enzymatic cleavage of isoflavone glucose moieties has recently been shown to begin in the oral cavity of some humans following hydrolysis by buccal bacteria and enzymes in the cytosols of sloughed off epithelial cells.[135] A more than 20-fold variability among subjects was demonstrated, however, for the oral hydrolytic deconjugation of genistin, and this suggests differences between subjects in terms of their natural oral microflora, which may be of some biological importance (e.g., antiproliferative effects on oral squamous carcinoma cells).[136] Furthermore, Walle et al. showed that antibacterial mouthwashes (i.e., Listerine and chlorohexidene) inhibit the potential of subcultured oral bacterial colonies to hydrolyze glucosides. It has also been suggested that in vivo β-glucosidase activity may be stimulated during periods of inflammation.[137]

Interindividual differences in the key metabolic enzymes that emanate from a host's unique intestinal microflora may divergently predispose physiological outcomes linked to soy isoflavone consumption at various periods during a lifetime. Intestinal biotransformations are reported to be hampered by the lack of a fully developed population of microflora in early infancy.[138,139] However, available evidence has shown infants can digest and absorb dietary phytoestrogens in active forms as effectively as adults.[44,140] *S*-Equol, a specific enantomeric isoflavone metabolite derived from daidzin and daidzein precursor molecules via biotransformation by colonic bacteria, is found in only approximately 20 to 35% of human adults,[58] but conversely is predominantly synthesized in rodents.[141,142] A number of reports suggest that equol may be more estrogenically potent at ERβ than genistein.[56,61,143] Unless human producers of equol are distinguished from the nonproducers in human studies investigating the effects of mixtures of soy isoflavones, this aspect may contribute to confounding findings related to clinical effectiveness.[144] Using the results of experiments where rodents are surrogate models for human conditions, and where daidzin or daidzein is the component isoflavone under investigation, will obviously have limitations in relation to the general population. Individuals or animals consuming mixtures of soy isoflavones in soy food, various ratios of particular isoflavones in supplements, or single purified sources of one or more isoflavones may experience different degrees of isoflavone efficacy, and this has been borne out in the evidence that exists thus far.

Age-Related Effects. The genetic and subsequent protein expression of an organism can be affected in many ways. Nutrients,[145,146] phytochemicals,[147] toxins,[148] and various environmental exposures[149] are exogenous factors that can influence genetic expression[149] either directly or indirectly via metabolic and/or signaling pathways.[104] One important endogenous factor contributing to the pattern of gene expression is an organism's biological age[149] and/or stage of development. It appears that age is inextricably linked to endogenous estrogen status and subsequent ER expression, and it is hypothesized that estrogen levels positively regulate ER number and activity in bone.[150] Steroid receptor and hormone levels contribute to regulating adaptive physiological responses in organisms, and with a few exceptions, their abundance is not usually constant throughout life.[151] Furthermore, a decreased responsiveness of various target tissues to steroid hormones, related to aging, has been reported.[152] Batra and colleagues report that the predominance of either ERα or -β in the skeleton is age- and cell type-dependent according to their findings.[94] Other evidence also suggests that specific gene expression patterns are associated with the aging process of individual organs.[153−155]

In the absence of physiological abnormalities, and without pharmacological or surgical intervention, the potential for endogenous estrogen synthesis is largely age-related in both sexes. For example, estrogen synthesis by the ovaries[156] and testes[157] is limited prior to puberty. A women's ability to synthesize ovarian estrogen terminates following depletion of her finite complement of ovarian follicles and typically ends with the onset of menopause around $\sim$51 years of age.[158] Men of a comparable age undergo more subtle hormonal transitional changes,[159,160] due to the fact that they continue to synthesize up to 85% of their circulating estrogen via peripheral aromatization of androgen precursors[161] that are naturally more abundant in males. Without the advent of menopause, the decline in estrogen production that men experience is more gradual and more likely to manifest at an older age coincidental with the slow progressive age-related decrease in circulating androgens.[151] Generally this renders men less susceptible, although by no means invulnerable, to pathologic bone loss as they age. Congenital dysfunctions,[162] disease states, or other disorders that cause androgen[163] and aromatase deficiencies[161,164] not associated with aging have also been documented to severely alter estrogen production in men and are associated with adverse skeletal effects

including osteopenia and osteoporosis. Evidence of a disruptive ER gene mutation resulting in male estrogen resistance has also been shown to perturb bone turnover and considerably diminish BMD.[165] Unfortunately, data related to ER number in men as they age have proven to be elusive, if they exist at all.

Methylation. In mammals, the expression of ERs is inversely correlated with the extent of de novo DNA methylation that occurs on cytosine-guanine dinucleotides located in promoter regions of ER genes.[166] Methyl-rich sequences of these dinucleotides appearing at a high frequency on a stretch of DNA are also referred to as CpG islands,[167] and methylation effectively condenses chromatin structures,[168] rendering them unavailable and transcriptionally repressive. Hypermethylation is the inappropriate addition of covalently bound methyl groups (i.e., 5-methylcytosines 5′ to guanine in CpG dinucleotides) to active DNA promoter regions that effectively prevents their interaction with DNA-binding proteins and silences expression of the genes implicated.[169] An increase in the methylation of the promoter region of various genes,[170,171] including ER genes,[172−175] is an epigenetic modification reported to be a natural phenomenon of aging[169,176−179] and prevalent in the pathogenesis of chronic disease processes such as cardiovascular disease[167,180] and cancer.[168,181−187] Aberrant methylation patterns, such as hypermethylation of tumor suppressor genes[188] or hypomethylation of ER genes,[189] have been documented in cases of tumorigenesis. Recent research suggests that estrogenic compounds[190] and the phytoestrogen genistein can in fact influence DNA methylation in specific cell types, with genistein demonstrating the potential to maintain a protective methylation profile of genes implicated in prostate cancer.[191] Whether or not there is a diminution in bone cell ERs during disease processes such as osteoporosis, or a diminution in association with the phenomenon of ER hypermethylation that occurs during the aging process, remains to be determined in light of the lack of related research in this area.

Animal Data. Numerous experiments using relatively young OVX rats as a model for postmenopausal bone loss have shown that soy isoflavones can contribute to the maintenance of BMD and thus the prevention of bone loss.[143,192−201] The data are less consistent for older rats and in human studies. Picherit et al. have shown a significant dose-dependent cortical and cancellous bone sparing effect of dietary soy isoflavones (genistein 159 mg/g, daidzein 156 mg/g, and glycitin 33 mg/g) after administering 20, 40, and 80 μg isoflavones/g body weight (bwt)/day (or approximately 6.4, 12.8, and 25.6 mg isoflavones/day, respectively, based on final bwt) in 7-month-old OVX rats.[66] In turn, Cai et al. found that enrichment of feed with isoflavones (0.2, 0.3, 0.4, and 0.8 mg/g diet, which approximately converts to 2.8, 4.2, 5.6, and 11.2 mg/day, respectively, based on average food intake) did not prevent trabecular or cortical bone loss in 6-month-old OVX virgin female rats based on femur histomorphometry analysis.[110] Bioavailability of β-glycosidic conjugates compared to aglucons and the ratios or combinations of isoflavone components may be key factors of consequence when comparing and assessing isoflavone efficacy.

Most of the rodent studies that demonstrate a positive effect of isoflavones on bone are designed so that the animals are supplemented with phytoestrogens immediately, or very soon, after OVX, possibly when ER number is less likely to be as adversely affected or down-regulated by the lack of circulating E_2. However, phytoestrogens had no benefit in the Cai et al. study even though treatments were started one week postovariectomy. In many instances, considerable time elapses between the onset of menopause and the initiation of human supplementation with isoflavones when assessing the efficacy of phytoestrogens in clinical trials—with perimenopausal and very early menopausal women being an exception. Avoiding such delays may prove to be pivotal in the quest to demonstrate favorable outcomes in human studies. A less common approach was taken in a rodent experiment in another study by Picherit et al. when assessing the dose-dependent "bone-curative" [sic] effects of daily soybean isoflavone intake on rats.[202] The animals underwent OVX at the age of 7 months and were fed total isoflavones (comprising a mix of genistin 159 mg/g, daidzin 156 mg/g, and glycitin 33 mg/g) at the levels of 0, 20, 40, and 80 mg/(kg bwt/day) for 84 days from day 80 after OVX surgery. Although this postponement of phytoestrogen administration did not prevent a trend for a dose-dependent reduction in bone turnover in response to phytoestrogens, as measured by bone biomarkers, the BMD of isoflavone-treated rats was not significantly different from that of untreated rats. Arjmandi et al. also began treating rats with isoflavones (i.e., genistin, genistein, daidzin, and daidzein at 1462, 25.1, 590, and 11.3 mg/kg, respectively) in a soy-based diet after 5 weeks had elapsed post-OVX and bone loss was evident.[193] The intervention was largely ineffective at restoring or preventing bone loss. These results indicate the effectiveness of isoflavones may be compromised in older animals the further in time from OVX that isoflavone consumption is initiated and the longer a hypoestrogenic state is endured.

Human Data. To date, findings based on clinical trials examining the effects of isoflavones on bone health for a period of one year or less have been inconsistent, but generally suggest that isoflavones can attenuate bone loss in perimenopausal and in younger postmenopausal women.[16] A double-blind randomized clinical trial (RCT) by Alekel et al. revealed that bone loss from the lumbar spine was attenuated in perimenopausal women receiving 80.4 mg/day soy isoflavone components (details of the components were not disclosed) for 24 weeks, but not in women on an isoflavone-poor (4.4 mg/day) or isoflavone-free diet. Furthermore, the time elapsed after the onset of menopause appeared to have a negative effect on the efficacy of isoflavones as a therapeutic intervention to prevent bone loss according to a double-blind RCT reported by Kreijkamp-Kaspers et al.[203] The daily use of soy protein supplements containing mixed isoflavones (i.e., 99 mg of isoflavones/day comprising 52 mg of genistein, 41 mg of daidzein, and 6 mg of glycitein) failed to improve BMD in healthy postmenopausal women when the intervention commenced after the age of 60 years or older. However, a subgroup analysis demonstrated a significant improvement in the intertrochanteric region ($P = 0.4$) and a trend toward a more favorable response at both the hip and lumbar spine in women more recently menopausal following one year of soy, as opposed to placebo, treatment.

The most dramatic investigation to date was a 12-month randomized double-blind placebo-controlled study that was undertaken by Morabito et al. to assess the effect of the isolated phytoestrogen genistein (54 mg/day) on bone metabolism and BMD in 90 confirmed postmenopausal women compared to HRT.[204] The age range across the three treatment groups of $n = 30$ women was placebo (51 ± 4), genistein (52 ± 3), and HRT (52 ± 5 years). Following genistein treatment, BMD was significantly improved at locations including the femoral neck, Ward's triangle, and lumbar spine, compared to the placebo group, which sustained bone loss during the study. The BMD parameters for the genistein group were less than, although not significantly different from, the HRT group, which demonstrated at least a 3% positive change in BMD from baseline. While the average age of subjects appeared to be close to the typical age for menopause (51 years), years since menopause in each of the groups ranged as follows: placebo $= 6 \pm 5$, genistein $= 7 \pm 6$, and HRT $= 7 \pm 3$ years, or possibly anywhere between the range 1−11, 1−13, and 4−10 years postmenopause, respectively. Within these groups there is a potentially wide variation in range for which there is inadequate information related to how skewed or centered the actual distribution may be. In light of the favorable osteogenic outcome pertaining to genistein intake, it would be of enormous interest to know whether more women within each group, or whether women between groups, were in fact closer

to being categorized as newly menopausal (i.e., 1 to 2 years), as opposed to being menopausal for more than 5 or 10 years for example.

Purified Isoflavones. Genistein's virtual estrogen-like potency and bone trophic effect on subjects in Morabito's investigation may be related to it being administered alone. This result is in contrast to the less dramatic effect that is commonly reported in other studies where mixtures of isoflavones were used. Genistein in the presence of daidzein and/or glycitein may ensure these bioactive compounds must compete with one another to bind to ERs and instigate an effect, and since daidzein and glycitein are not as transcriptionally active as genistein, soy isoflavones of lesser bioactive potential may in effect antagonize genistein, which is capable of a higher level of activity when unopposed and given access to ERs. Other studies, albeit in a different contexts, have shown genistein and daidzein compete with one another for stimulatory activity,[205] and equol has even been shown to counter the effects of genistein,[13] demonstrating the combinations of effects that come into play when isoflavones are present in a mixture.

Purified isoflavones administered separately to individual treatment groups of 12-month-old OVX rats (i.e., genistein, 10 μg/g bwt/day; daidzein, 10 μg/g bwt/day) for 3 months revealed that daidzein was more efficient than genistein in preventing ovariectomy-induced bone loss.[143] In this instance, daidzein's effect may be attributable to rodents exhibiting the unconditional capacity to produce equol from daidzein, which has been reported in one investigation to be 10- to 100-fold more estrogenic than daidzein and at least 10-fold more estrogenic than genistein in fish;[57] however, effects may be tissue- and species-dependent. At present, the optimal amounts, ratios, and/or combinations of isoflavones required for estrogen-like activity in bone are not known, or generally agreed upon, and as such require further investigation.

The Double-Edged Sword

The developmental stages of life are particularly susceptible to endocrine disruption.[47,206] Prepubertal estrogen levels in humans of both sexes are comparatively low,[207] sometimes below the detection limit of available assays,[208] providing little binding competition for circulating estrogen-like compounds at available ERs. There is extensive evidence suggesting that an intense period of exposure to relatively high levels of endocrine active compounds[44] in utero, or during neonatal and postnatal development, potentiates immediate and/or long-term developmental effects.[209] Due to the ethical constraints, prohibitive expense, and practical difficulties associated with long-term clinical trials, there exist very limited data on the effects of soy isoflavone exposure on developing humans.[39] The available animal data are, however, rife with examples demonstrating the biopotency of soy isoflavones in very young rodents and the potential for numerous adverse effects. Data supporting the notion that biochemical events, occurring in a discrete period early in life, are capable of exerting long-lasting effects that may potentially delay or prevent chronic diseases that normally occur later in life[41] are generally given more weight and acceptance compared to data that suggest the potential for adverse effects following soy isoflavone exposure in the young.

Some of the more immediate effects linked to early isoflavone exposure and detected in developing rodents have included permanent changes in morphogenesis,[69] such as altered anogenital distance,[210] increased thymus mass,[211] abnormal cellular maturation in the vagina,[212] and premature vaginal opening.[213,214] Differentiation patterns of estrogen-sensitive tissues can also be rapidly altered during development, a prime example being enhanced mammary gland differentiation in response to prepubertal genistein exposure.[215] Latent effects pertaining to endocrine disruption may be other than structural[209] and far more subtle. In terms of early soy isoflavone exposures in rodents, latent effects have included altered steroidogenic enzyme expression,[216] dysfunctional reproductive behaviors,[209,210] altered dispositional behavioral patterns,[217] and long-lasting effects on immune systems in adulthood.[211] There is also evidence to suggest that early soy isoflavone exposures, at levels comparable to the ranges of human exposure, can cause changes that alter the responsivity of estrogen-sensitive target tissues to endogenous hormonal stimuli in mature rodents, representing a deferred outcome with ramifications pertaining to long-term reproductive health.[218]

Delayed effects may be imperceptible at the time of exposure, such that they become apparent, or of consequence, only as temporal endocrinologic changes occur during puberty, adulthood,[209] or pregnancy.[218] To demonstrate this phenomenon, Naciff et al. used a microarray technique to analyze 8740 genes derived from the uterus and ovary of gestational rats that were transplacentally exposed to genistein and revealed that a treatment effect was immediately evident in 344 genes.[47] Histological examination of these same organs, as is the protocol in many short-term studies attempting to identify irregularities following isoflavone exposure, showed no apparent changes or gross micromorphology abnormalities. Less discrete sequelae to these immediate genetic alterations are considered likely to surface as latent developmental effects.[47] For example, a 35% increase in the incidence of uterine adenocarcinomas at 18 months of age was reported following exposure of neonatal mice to genistein (50 mg/kg/day) on days 1−5 after birth.[219] While dosage and route of administration will always be important and affect the magnitude of effects elicited by soy isoflavones, latent potentials should provide the impetus for more long-term human investigations.

For those infants that are nourished on soy-based formulas the daily exposures are in the vicinity of 8 mg/kg bwt,[65] or 6- to 11-fold higher on a bwt basis than is the dosage for adult humans that consume soy foods.[64] Furthermore, infants fed cereals containing isoflavones can increase their phytoestrogen intake by as much as 25% depending on the brand selected,[44] and school lunch programs may now provide a source of food that is rich in soy isoflavones to growing and developing children. Isoflavones are currently recognized for their physiological modulating capacity in human adults at relatively lower exposure levels than are encountered by soy-formula-fed infants. This factor alone should be triggering more concern than is evident at present in light of the extensive animal data that suggest the timing of exposure to phytoestrogens is crucial[220−222] and, in many instances, may be linked to aberrant effects in estrogen-sensitive tissues in developing rodents,[213,217,223−226] many of which may be long-term effects that we have yet to recognize or link to the levels of early soy consumption.

Heavy isoflavone consumption patterns among young developing humans has been implicated in the increased incidence of reproductive abnormalities in males that have reportedly occurred over the last half century[227] and the trend for a decrease in the age of onset of pubertal development in Western countries during the past century.[208,205] Relying almost entirely on epidemiological evidence pertaining to Asian nations, where consumption of fermented soy foods has been an enduring tradition, and then classifying the bioactive compounds of soy in any form, in any quantity, and at any life stage as unconditionally "healthy" and "relatively harmless" is overlooking many of the other environmental, lifestyle, and usage differences that undoubtedly contribute to observed health effects. This may be as unlikely a healthful action as singling out wine consumption as the reason for the French Paradox without factoring in lifestyle behaviors, and expecting the same positive health-related effects in a different population, or assuming Asian cultures can increase dietary consumption of dairy products to promote bone health, as seen in Western cultures, without considering their genetic predisposition to lactose intolerance. These analogies highlight the potential pitfalls in assuming Asian patterns of soy consumption are completely safe, or effective, in Western populations without sufficient research.

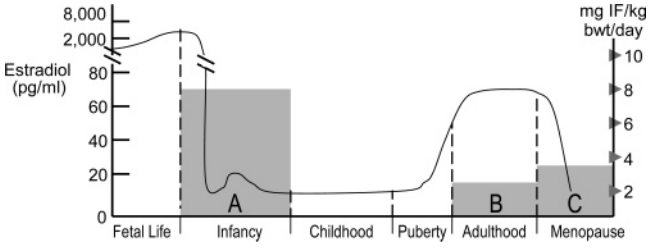

Figure 1. Female hormone levels[229] and isoflavone intake[64,65] during various life stages. Abbreviations: IF = isoflavones, bwt = body weight. The line represents estradiol levels (left axis values) and the shaded area represents the approximate range of IF consumption (right axis values) for (A) infants fed soy-based formula, (B) adults consuming a soy-rich diet, and (C) menopausal women consuming a soy-rich diet in addition to soy supplements. No comparable data available for fetal life, childhood, and puberty.

Whereas soy formula is available off the supermarket shelf in the United States, as a precautionary measure it is only available by prescription in Europe.[228] Here in the U.S., however, phytoestrogen use in nutritional and pharmaceutical applications for infants and children is increasing.[219] Natural estrogen levels for females throughout life are depicted in Figure 1. The rationale for consumption of soy isoflavones during the onset of menopause is to compensate for lowered estrogen levels in a bid to derive estrogen-like health effects for the mature skeleton. With these expectations in mind, we need to ask ourselves what benefits will be derived and what possible risks are involved in stimulating estrogen-like effects during the critical developmental years of infancy and childhood? Both menopausal and prepubescent life stages are characterized by naturally low estrogen levels and preceded by life stages where estrogen levels are high and ERs abundant as a result of the presence of estrogen. It is hypothesized that these periods are potential windows of opportunity for the optimal soy isoflavone effect, because at these times isoflavones are less opposed by estrogens, enabling them to exert their maximal ER-mediated effects.

Menopausal women are also being encouraged to consume potent isoflavone-containing supplements and to substitute soy-based foods in their regular diet. Various reports have estimated isoflavone intakes for Japanese, Chinese, and Korean women consuming traditional diets at between 15 and 50 mg/day,[230−234] or in the vicinity of 1 mg/kg bwt/day,[235] amounts that are frequently exceeded in Western clinical intervention studies.[60,236] As a general precaution, in July 2002 the Italian Ministry of Health recommended that isoflavone supplements not exceed 80 mg/day dosage.[104] It would seem feasible that efficacy[50] and risk assessment be considered relative to the development phase or life stage of the individual at the time of exposure[237] as the previously mentioned variable factors come into play to govern the potential for isoflavones to exert healthful effects.

Concluding Comments

Thus far, data in rodents, and to a lesser extent in humans, appear to suggest a potential for delivery of bone health benefits as a result of soy isoflavone consumption in the early postmenopausal years. However, the magnitude and clinical relevance of those estrogen-like benefits are yet to be adequately determined. Soy isoflavones are bioactive compounds that exert estrogen-like effects in mammals via estrogen receptors and via other nongenomic signaling pathways. An extended period of subtle estrogen-like effects is considered desirable at the end of a woman's reproductive years; however, we need to determine whether isoflavone-induced estrogen-like effects of any magnitude are advantageous during the formative years prior to advocating the inclusion of soy in the diet of prepubescents without caveat. This perspective is not intended to be alarmist, because adverse effects in animal models may not predict the human situation in infants and children at all. However, if we are willing to infer that data indicating the potential for soy isoflavone health benefits in adult rodents may to some extent be applicable to adult humans, should we discount the possibility that health risks in the very same animal models at a young age may be indicative of effects that have some relevance to developing humans? On all levels, more research examining the effects of soy isoflavones on humans must be carried out, particularly on developing humans, to ensure the physiological effects elicited by these compounds are of the categorical benefit we currently ascribe to them.

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