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Flavonoid Intake and Bone Health

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Abstract

Flavonoids, found in a wide diversity of plant foods from fruits and vegetables, herbs and spices, essential oils, and beverages, have the most potential of dietary components for promotion of bone health beyond calcium and vitamin D. Recent epidemiological studies show flavonoid consumption to have a stronger association with bone than general fruit and vegetable consumption. Bioactive flavonoids are being assessed for properties beyond their chemical anti-oxidant capacity, including anti-inflammatory actions. Some have been reported to enhance bone formation and to inhibit bone resorption through their action on cell signaling pathways that influence osteoblast and osteoclast differentiation. Future research is needed to determine which of the flavonoids and their metabolites are most effective and at what dose, as well as the mechanism of modulating cellular events, in order to set priorities for clinical trials.

Keywords

anti-inflammation; antioxidant; bone; flavonoids; soy isoflavones

Osteoporosis is a debilitating and painful condition of low bone mineral density (BMD) and high fracture risk. There are an estimated 2 million new fractures each year in the USA and 9 million worldwide (1), with an estimated annual cost to our society of ~$100 billion (1). Strategies to prevent osteoporosis include reducing bone loss induced by acute estrogen deficiency post-menopause and more gradual declines in sex steroid levels with age in both men and women. As for other chronic diseases, there is increasing evidence that inflammation is part of the etiology of osteoporosis. Flavonoids as a class of phytochemicals have promise in protecting against bone loss, likely related in part to their anti-inflammatory

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properties. In a large observational study in Scotland, total flavonoid intake was positively associated with BMD and increase in BMD of the spine and hip. Flavonoids in the catechin family had the strongest association with bone (2). The relationship between flavonoid intake and bone health was stronger in general than what has been reported previously for fruits and vegetables (3,4). The most studied of the flavonoids in relation to bone health are the isoflavones, especially those derived from soy beans. Isoflavones, along with flavonoids from other plant foods showing promise for bone health, will be described in this review.

A variety of methods are available to assess bone health. BMD, as determined by dual energy x-ray absorptiometry (DXA), is the most common measure of fracture risk. It can be used to monitor response to intervention in either humans or animal models. The ovariectomized (OVX) rodent is an FDA-approved model for osteoporosis in postmenopausal women. Interventions, such as dietary flavonoids, can be tested for their ability to suppress OVX-induced bone loss if the intervention is started subsequent to OVX or after bone has been stabilized subsequent to OVX; the ability to recover lost bone can then be determined. Because bone strength is determined by microarchitecture and bone geometry, as well as BMD, more recent studies use three-dimensional imaging techniques, such as peripheral quantitative computed tomography (pQCT) in humans or micro-computed tomography (μCT) in animal models. Excised bones of animals can be tested using breaking tests such as 3-point bending for energy to failure. Bone turnover rate is also predictive of fracture. Thus, measures of bone turnover, including biochemical markers of bone turnover or calcium isotope kinetics, are also useful to evaluate interventions for their effect on bone.

Tests such as oxidative radical absorbency capacity (ORAC) have been utilized historically to evaluate the potential of a food or compound to quench free radicals and protect against inflammation-related chronic diseases such as osteoporosis. However, we are beginning to understand, as summarized by Finley et al. (5), that the protective effect of diets high in antioxidant-rich foods goes beyond simply quenching oxygen radicals. Natural compounds act through gene expression and cell signaling pathways to activate enzymes that eliminate oxygen radicals that initiate inflammatory events. Antioxidants can also sequester potential oxidants, such as iron, that regulate oxygen radicals. Also antioxidants can influence redox capacity to regulate oxidation pathways. A cellular antioxidant activity assay using a cell culture model was recently developed to screen foods for biologically relevant antioxidant activity to supersede the chemically based assays to account for these additional functions (6). Cell culture studies can also be used to identify affected pathways. This review briefly summarizes the state of knowledge for the role of flavonoids on bone health and their role in modulating cell signaling pathways.

Soy Isoflavones

The main class of flavonoids that has been studied for their role in bone health is soy isoflavones. Isoflavones are structurally similar to estrogen and bind to estrogen receptors (7). Observational studies suggest that soy consumption contributes to low rates of hip fractures in Asians (8,9). In a large study of approximately 75,000 women participating in the Shanghai Women's Study, hip fracture prevalence was inversely related to soy consumption (10). The relationship is stronger in women who are in early (i.e., <10 y) rather than late menopause, possibly related to greater responsiveness of bone turnover to soy isoflavones during the early phase of estrogen deficiency.

In contrast to observational studies in Asia, the most comprehensive intervention studies using isoflavones have largely been negative. A meta-analysis of randomized control trials (RCTs) showed soy isoflavones (~82 mg aglycones/day) increased spine BMD by 2.4%

(p=0.0001) in the short term (6–12 mo)(11), but randomized controlled trials (RCTs) of 2 or 3 y in duration showed no benefit of soy isoflavones on BMD of fracture-prone bone sites in postmenopausal women (12–14). The longest (3 y) RCT (13) showed no overall treatment effect for lumbar spine, proximal femur, or whole body BMD in either the intent-to-treat or compliant models. However, after adjustment for age, whole body fat mass, and bone resorption, the 120 mg isoflavone (aglycone form) dose compared with placebo was modestly protective (p=0.024) for femoral neck BMD. Further evaluation of volumetric BMD and bone strength at the 1/3 midshaft femur and distal tibia using pQCT in the Alekel trial (15) indicated a protective effect of 120 mg isoflavone/day on cortical volumetric BMD of the femur as time since last menstrual period increased, but no benefit to trabecular bone. There are many differences that may explain the discrepancy between the negative findings of the RCTs and the positive association in the observational studies (16). The RCTs were conducted in western women with heterogeneous genetic backgrounds, as well as low habitual soy food intake. Additionally, because osteoporosis is a long latency disease, the primary outcome measure of the RCTs was BMD rather than fracture. Properties other than BMD may protect against fracture. For example, soy isoflavones may reduce net bone turnover in postmenopausal women (17). All of the RCTs published to date used mixed isoflavones isolated from the soybean, an entirely different form than the whole soy foods consumed in Asia, which contain plant proteins and more than 100 associated phytochemicals in addition to isoflavones (18). Yet, a RCT in Italian women used a purified form of the dominant soy isoflavone, aglycone genistein, demonstrating this isoflavone to be as effective as estrogen therapy in reducing postmenopausal bone loss (19).

Animal studies examining the effect of soy protein or isolated isoflavones on bone health show the strongest benefit during growth rather than in the older OVX rodent model that mimics postmenopausal women (20). Animal models have the advantage of feeding a controlled diet for sufficiently long periods to effect changes in bone properties. Moreover, femurs and vertebra can be excised at the end of long-term feeding trials and direct fracture testing at common sites of fracture (femur neck, vertebra), as well as sites that contain almost exclusively cortical bone (femur midpoint) can be performed. While BMD is the gold standard for assessing fracture risk, other factors such as bone structure, including trabecular thickness and separation, influence bone strength. Thus, combining bone strength measurements with BMD and outcomes of bone structure, obtained by μCT, provide a more comprehensive understanding of how a dietary intervention alters bone at the tissue level. Furthermore, a larger number of comparisons, such as dose response studies or those comparing alternative forms, can be made than is practical in clinical trials. For example, 9 treatments were compared for multiple bone outcomes in an OVX rat model that included a dose response of mixed isoflavones with and without a soy protein background (21). Estrogens, but not soy isoflavones, benefited bone properties and calcium metabolism.

An interesting life-stage that has received recent attention is neonatal exposure to soy isoflavones. Using a mouse model, exposure to purified soy isoflavones (genistein and daidzein) during the first five days of life resulted in higher BMD, improved bone structure, and greater bone strength at adulthood in females but not males (22). Moreover, these benefits to bone development in females protected against ovariectomy-induced bone loss (23). The level of genistein and daidzein used in these mouse studies resulted in similar total serum isoflavone levels in infants consuming soy infant formula (24). Potential differences in metabolism of isoflavones between rodents and human infants and the fact that isolated isoflavones may have different biological effects than when present in the soy matrix suggest that prospective studies of human infants fed soy infant formula are needed to confirm if consumption of soy protein based infant formula benefits bone health at adulthood.

Plant Flavonoids Other Than Soy Isoflavones

Higher intakes of fruits and vegetables have been associated with improved BMD or bone mineral content (3,4), although a systematic review of studies in women aged 45 y and older was inconclusive (25). Benefits to bone have been attributed to several potential constituents, including acid-base balance, potassium or other micronutrients (such as boron or vitamin K), and particular bioactive ingredients. In a comparative evaluation of 53 food items, Mühlbauer et al. (26) used urinary excretion of [^{3}H]-tetracycline following pre-labeling of rat bones to measure bone resorption in response to serial exposures to each food for 10 days. This approach allows several foods to be tested in each rat for direct comparison against a positive reference control of onion. Half of the food items tested showed bone turnover inhibitory capacity. Using this same approach, bone resorption suppression activity was not altered after correcting for the alkaline load of a mixed plant diet with potassium citrate (27), which raises doubt about postulated acid-base mechanisms of action of plant foods and the benefit of extra potassium. In support, a meta-analysis of human studies showed no relation between dietary acid load and osteoporotic bone disease (28), nor did potassium intake influence calcium balance (29). Further, the content of boron or vitamin K in fruits and vegetables is not likely to be the complete explanation for their benefit to bone since boron is typically adequate in human diets and vitamin K has not been shown to attenuate postmenopausal bone loss (30,31).

Instead, Mühlbauer et al. (26) concluded that particular bioactive compounds in some food items have anti-resorptive properties. Total polyphenolic content was not related to efficacy, but specific flavonoids appeared to confer benefits to bone. Onion was used as the positive control because of its strong effect to inhibit bone resorption. Separately, onion powder was shown to dose-dependently prevent OVX-induced bone loss in rats and to increase bone volume and trabecular number (32). Rutin was identified initially as the bioactive compound, but later this group reported that the likely bioactive constituent in onion using bioactivity directed fractionation is Γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide (33). However, this requires confirmation *in vivo*. Other vegetables in the onion family, including garlic and leek, and members of the celeriac family, including cabbage, lettuce, and green beans, were also effective in suppressing bone resorption (26).

The most effective fruit tested in the pre-labeled bone rat model of Mühlbauer et al. (26) described above was plum. Dried plum has been shown to increase vertebral and femoral BMD and to restore trabecular microarchitecture in orchidectomized, male rats (34), but the benefits are even stronger in young male rats (35). Other than soy, plum is the only other flavonoid-rich food that has been tested in a RCT of reasonable duration to study effects on bone properties. In a one-year study of 160 postmenopausal women, loss of BMD of the major fracture sites was prevented by feeding plums (36). Plum contains high concentrations of rutin (3.3 ng/100 g) (35), but the specific bone bioactive compound(s) in plum is uncertain. Rutin is hydrolyzed to its aglycone, quercetin, prior to absorption and can be converted to glucuro- or sulfoconjugates during absorption. Quercetin has antioxidant activity and binds to estrogen receptor-beta (ERβ) (38).

Another fruit with high flavonoid content and high antioxidant capacity that has received recent attention by bone researchers is blueberry. In the OVX rat model, blueberry prevented whole body bone loss, but no change in fracture prone sites (39). No clinical trials with blueberries and bone outcomes have been reported. Hippuric acid, phenylacetic, and hydroxybenzoic acids are likely the bioactive compounds in blueberries, as will be discussed later in the section on mechanisms.

Other fruit products shown by Mühlbauer et al. (26) to be effective in inhibiting bone resorption were orange juice and red wine. Orange juice and pulp prevented bone loss in castrated male rats and increased cortical thickness in a dose-dependent manner (40). The active compound is thought to be hesperidin, the most abundant flavonone in citrus fruits, since similar bone protective effects in OVX rats were reported with this purified compound (41). Resveratrol has been proposed as the bioactive polyphenolic in red wine, but the *in vitro* evidence for an effect on bone formation (42) is at a non-physiological level. Other constituents, such as anthocyanins, should be evaluated for bone health properties.

Antiresorptive food items identified by Mühlbauer et al. (26) also included mushrooms, herbs, and essential oils. A candidate for a bioactive compound in herbs is luteolin, a flavonoid found in celery, green pepper, parsley, perilla leaf and seeds, and chamomile. It has anti-inflammatory properties, inhibits osteoclast differentiation, and protects against OVX-induced bone loss (43). Extracts of king oyster mushroom (Pleuratus ergngii) have been shown to be protective against OVX-induced bone loss (44,45). Bioactive herbs and their oils include parsley, sage, rosemary, thyme, and chili. The effective dose will determine whether inclusion of these herbs have practical bone health benefits.

The studies of soy isoflavones and other plant flavonoids described above collectively suggest positive effects on bone mineral density and content. However, effects on risk of fragility fracture in humans, or overall effect on bone structure and strength properties has been scarcely studied. The magnitude of the effect appears to depend on the flavonoid source; context of exposure (pure compound vs. diet); route of exposure; and age and sex of the target population. Moreover, as discussed below, no single mechanism has been put forward for flavonoid actions on bone.

Potential Mechanisms of Action of Flavonoids on Bone

Recent research has identified molecular targets in cell signaling pathways that affect bone. Some flavonoids appear to have bone anabolic activity, which has exciting implications beyond merely inhibiting bone resorption through suppressing osteoclast activation. Although many gaps remain, we have attempted to develop a unifying model for how flavonoids from various plant sources might affect bone (Figure 1).

The most studied mechanism for benefits of flavonoids to bone has been in the estrogenic actions of phytoestrogens, including soy isoflavones, lignans, and coumesterol. Because of their weak binding to the estrogen receptor ER, and relatively higher affinity for ERβ than ERβ, these phytoestrogens have been largely studied in cell culture for their estrogenic properties (56). Effective concentrations required to produce classical ER activation, nuclear translocation and gene expression cannot be achieved at doses found after eating soy foods. Nevertheless, phytoestrogens ability to bind to ERs may have a positive effect on bone through their antioxidant and anti-inflammatory effects in individuals who have high reactive oxygen species production, such as occurs during aging, menopause, and arthritis, since estrogens also antagonize reactive oxygen species actions in bone cells via non-classical signaling pathways (Figure 1) (48,57). Newer reports show that soy isoflavones may also have roles independent of their estrogenic properties. For example, soy containing diets have been shown to activatesignaling through bone morphogenic proteins (BMPs) (58,59) (Figure 1). Interestingly, the major metabolite of hesperidin in orange juice, hesperetin-7-O-glucuronide, also activates BMP signaling, which increases expression of the major transcription factor Runx2 which drives osteoblast differentiation from multipotent mesenchymal stem cells (MSCs) in bone marrow (60).

The anabolic action on bone of flavonoids from berries is through upstream regulation of osteoblast differentiation through the molecular target, the mitogen activated protein MAP kinase p38. MAP kinases are a complex cascade of enzymes which regulate protein phosphorylation and which are themselves regulated via redox and phosphorylation state. Phosphorylation of p38 results in activation of the Wnt signaling pathway. This pathway involves a series of soluble growth factors (Wnts) which bind to a series of cell surface receptors (LRP5/6-Frizzled proteins) which signal to the cytosolic kinase GSK3β to become phosphorylated. Phosphorylation of GSK3β inactivates it and prevents phosphorylation of a cytosolic master regulator protein β-catenin. As a result of reduced phosphorylation β-catenin is stabilized, translocates to the nucleus and acts in conjunction with transcription factors LEF/TCF to increase transcription of genes involved in bone cell proliferation and differentiation such as Runx2 (61) (Figure 1). After observing increased serum concentrations of hippuric acid, phenylacetic acid, and hydrobenzoic acid after blueberry feeding, Chen et al. (61) tested an artificial mixture of these compounds and found that they stimulated Wnt signaling and differentiation in osteoblast precursors *in vitro* in a similar manner to serum from animals fed whole blueberry. How these phenolic acids activate p38 is not yet understood. Myosin production seems to be involved in the effect of blueberry on osteoblasts, since myosin-related genes are down-regulated following OVX. Feeding blueberry blunts this effect and protects against OVX-induced osteoblast death through a senescence pathway which involves reactive oxygen species (ROS) and the signaling proteins p53 and p21 (Figure 1) (62). In mouse-derived bone marrow stem cells, serum from blueberry fed rats increased osteoblast differentiation and lineage commitment. Moreover, silencing of myosin 2 expression using shRNA inhibited Runx2 mRNA expression in osteoblastic cells and stimulated cell senescence.

Osteoclasts are the primary cells responsible for bone resorption. When activated, these cells release proteolytic enzymes to digest connective tissue proteins and acids that solubilize bone mineral. They attach to bone surfaces and their action forms pits. Osteoblast cells form bone in the pits produced by the osteoclasts. This concerted process is normal and occurs throughout life to model and shape bone and to repair microarchitecture damage. However, estrogen deficiency accelerates bone resorptive activity, which outpaces bone formation. Several signaling proteins produced by osteoblasts including osteoprotegrin (OPG) and the TNFα family member, receptor activator of NFkB ligand (RANKL) are primary regulators of osteoclast activation. ROS and the redox status of bone cells are now thought to have important roles in regulation of bone turnover and survival of osteoblasts, osteoclasts, and osteocytes. ROS produced through the action of NADPH oxidase (Nox) enzymes appears to regulate osteoclastogenesis through control of expression of RANKL in osteoblastic cells and signaling through its receptor RANK on the surface of osteoclast precursors (Figure 1). Chronic bone loss is often accompanied by conditions of oxidative stress and/or inflammation, such as aging, estrogen deficiency, rheumatoid arthritis, inflammatory bowel disease, and alcohol abuse (46). Oxidative stress in bone cells results in production of reactive oxygen species from lipoxygenases and oxidases such as the Nox enzymes. ROS can affect bone cells in many ways, including stimulation of osteoblast apoptosis and senescence (47) and by up-regulation of RANKL, to activate osteoclast differentiation and bone resorption (48). Inflammatory conditions in which there is enhanced production of proinflammatory cytokines, such as TNFα and increased T cell expression of RANKL, are associated with lower BMD (49,50), likely due to up-regulation of osteoclast formation and activation of mature osteoclasts (51–53). Moreover, effects on osteoblast activity include lower production of bone matrix proteins and stimulation of osteoblast apoptosis (54,55).

Dried plum polyphenol extract has been shown to inhibit increases in RANKL expression in response to TNFα, oxidative stress induced by TNFα or by inflammatory response to lipopolysaccharide (that may be mediated in part through modulation of TNFα), and

osteoclast differentiation (63,64). Similarly, resveratrol inhibited reactive oxygen species production and subsequently RANKL signaling and osteoclast activation (65) and up-regulated Runx2 gene expression (66). Green tea polyphenols may offer protection in states of chronic inflammation (67). In rats, administration of green tea polyphenols resulted in higher urinary epigallocatechin and epicatechin, and attenuated the inflammation-induced bone loss that results from administration of lipopolysaccharide in rats (67). Specifically, green tea polyphenols helped preserve femur bone mass and structure, and resulted in lower tartrate resistant acid phosphatase, a marker of bone resorption. These effects were associated with lower mRNA expression of TNFα and cyclooxygenase (COX)-2. It is important to note that the doses used in the plum, resveratrol, and green tea studies were pharmacological and these may not necessarily represent the bioactive metabolites produced upon consuming plum, red wine, or green tea.

Conclusions and Future Studies

Flavonoids in a variety of plant foods hold promise in promoting bone health, both in the primary prevention of bone loss in later life and as a complementary therapy during conditions of high oxidative stress or chronic inflammation. We are beginning to understand their roles in cell signaling, including Wnt-β-catenin and BMP pathways that stimulate bone formation, in addition to their anti-resorptive roles in inhibiting osteoclast activation. This represents an advance in our understanding of flavonoids beyond the classic estrogen-like actions of soy isoflavones and beyond evaluating flavonoids only for their chemical antioxidant properties. The interaction of dietary factors with these signaling pathways is a rich area for future research.

Although we have a growing body of descriptive evidence of various flavonoid-rich foods on bone health in animal models, we have scant clinical data beyond studies on soy isoflavones. To translate these animal data to dietary interventions in humans, we also need comparative data of the various sources of flavonoids. The work by Mühlbauer's group using urinary excretion of bone-seeking tracers from pre-labeled rat bone to screen various fruits, vegetables, and herbs, discussed extensively in this review, has contributed greatly to our understanding of their relative bioactivity. A similar approach has been developed for humans whereby bone is labeled with the rare isotope, ^{41}Ca, and urinary excretion of the isotope measured by Accelerator Mass Spectroscopy is used to compare multiple interventions for their effect on bone balance (17,68). Such an approach will be useful to determine dose response effects and effectiveness of combination drug therapies, as well as to compare various plant flavonoids and their metabolites. Determining the most effective foods/constituents, identifying the bioactive ingredient(s), and their effective doses is prudent prior to investing in large, clinical trials to formulate public health recommendations.

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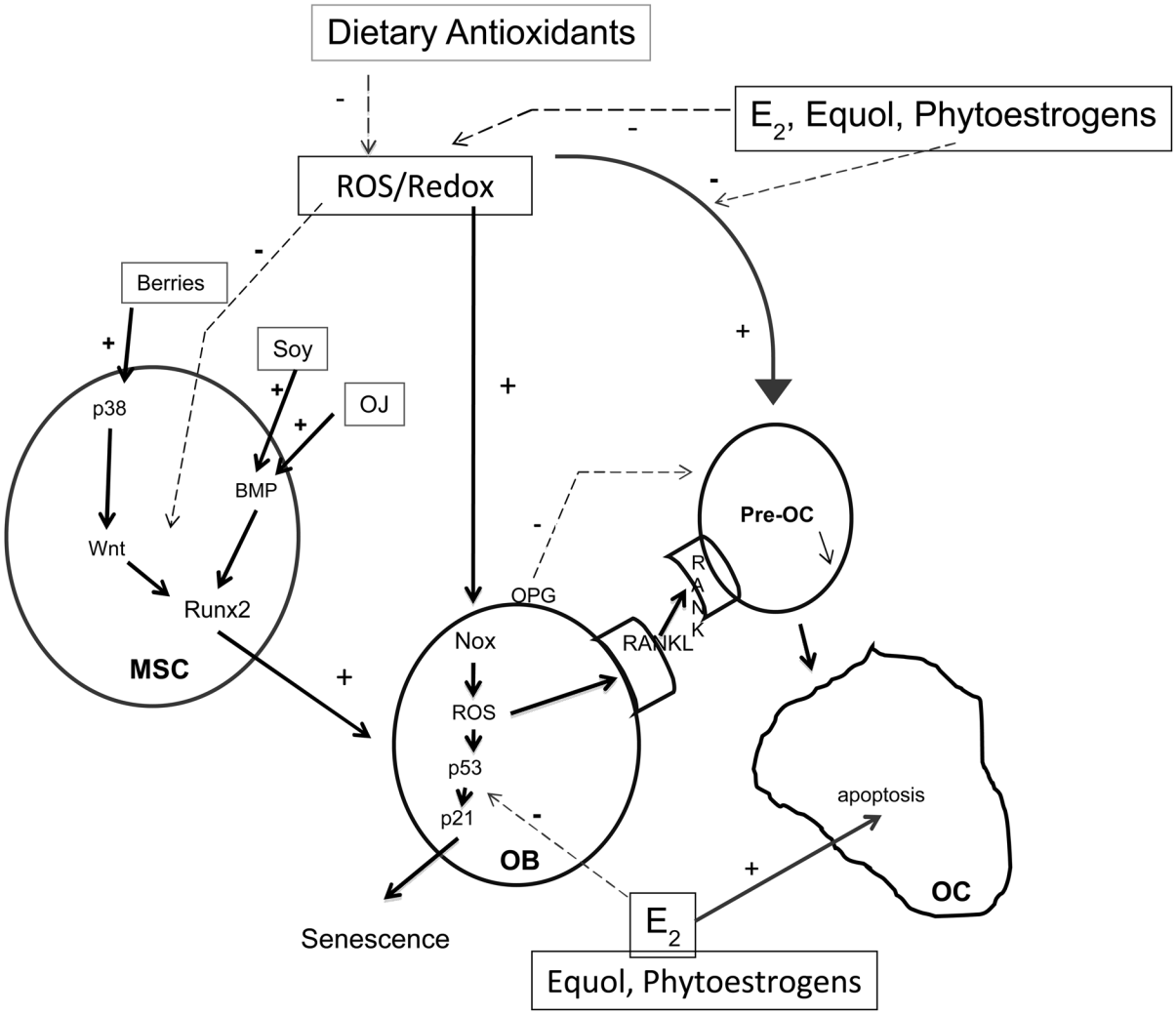

Figure 1.

Dietary intake and factors impact bone turnover and bone cell survival via common signal transduction pathways. BMP, bone morphogenic protein; E_2, 17β-estradiol; MSC, mesenchymal stem cell; NEFA, nonesterified free fatty acids; Nox, NADPH oxidase; OB, osteoblast; OC, osteoclast; OJ, orange juice; OPG, osteoprotegrin; ROS, reactive oxygen species.