Various Selected Vegetables, Fruits, Mushrooms and Red Wine Residue Inhibit Bone Resorption in Rats

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ABSTRACT To make a broad survey of the effect of components of the human diet on bone resorption, a few items from the following categories were added to rat diets: vegetables, fruits, beans, nuts and seeds, mushrooms, carbohydrate sources and beverages. The effect on bone resorption was measured by the urinary excretion of tritium released from bones of 9-wk-old rats prelabeled with tritiated tetracycline from weeks 1 to 6. The number of rats per experiment was 26 — 6, 5, 5 and 5 in the untreated control group fed the plain semipurified diet, the positive control group fed onions and three groups fed one of the newly investigated items, respectively. New experiments were added until 10 rats were fed each item in each of two separate experiments. The results for each item were compared to those for the untreated control group (n = 12) investigated simultaneously. We found that feeding rats 1 g/d of dry fennel, celeriac, oranges, prunes, French beans and farmed and wild mushrooms (Agaricus hortensis and Boletus edulis) as well as the freeze-dried residue from red wine significantly (P < 0.05 or lower) inhibited bone resorption. Eighteen items had no significant effect. To date we have found 25/53 items that exhibit inhibitory activity. Activity appears to be restricted to the following categories: vegetables, salads, herbs, mushrooms, fruits and red wine residue (25/36 items effective). Furthermore, as assessed in a similar experimental design with various doses of a mixture of active items, we determined the minimum effective dose of the dry items to be 170 mg/d. These results open the possibility for targeted interventions in humans.

KEY WORDS: bone resorption • vegetables • urinary excretion • acid • osteoporosis

Bone mass in adult humans decreases with age, leading to an increased risk of fractures (1). Osteoporotic fractures, besides causing suffering to the patient, are a major burden to health care resources; the direct annual expenditure for osteoporosis and associated fractures in the United States is ~$17 billion (2). From a medical and economic viewpoint, it would therefore be desirable to prevent loss of bone mass. A nutritional approach would be an inexpensive means of achieving this goal. However, the effects of the nutritional strategies recommended today are rather modest. Indeed, even the beneficial effect of calcium in milk on the relative risk of hip fracture seems to be restricted to the 10% of the female population with the lowest intake of calcium (3). Thus, research into novel nutritional strategies for preventing bone loss is needed.

Osteoporosis occurs most frequently in postmenopausal women following the decrease in estrogen levels. Hormone-replacement therapy (HRT) (3) is effective in preventing bone loss. However, compliance with HRT therapy is low because of side effects. This has stimulated research into alternatives to the classical HRT therapy, i.e., the use of phytoestrogens to prevent bone loss.

It has been suggested that the high consumption of soy products in the traditional Japanese diet, providing 30 to 60 mg/d of the estrogenic isoflavones genistein and daidzein, may contribute to the low prevalence of postmenopausal osteoporosis in Japan (4). Indeed, treatment with soy protein containing isoflavones inhibits bone loss in an animal model for postmenopausal osteoporosis, ovariectomized rats (5). Treatment of premenopausal and postmenopausal women with 40 g/d of a soy protein isolate providing 80 or 90 mg/d of isoflavones, respectively, attenuated the loss of bone mineral density (BMD) in the spine but not at other sites; lower doses were ineffective (6, 7). A randomized double-blind placebo-controlled study recently found that a dose of 54 mg/d of genistein was as effective as HRT in preventing bone loss in the spine and in the femoral neck in early postmenopausal women (8). Thus, genistein appears to be a promising pharmacologically active agent. However, the doses required for this effect are beyond the possibilities of nutritional supplementation. Indeed, achieving an intake of 54 mg/d of genistein from soy products, for example, would require the consumption of ~ 200 g/d of tofu (9). Such a strategy would imply a fundamental modification of Western nutritional habits, which is hardly feasible.

We have, therefore, chosen a different approach—we have


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3 Abbreviations used: BMD, bone mineral density; BRIFI, bone resorption inhibitory food items; HRT, hormone replacement therapy; [3H]-Tc, tritium-labeled tetracycline.
investigated whether components of the Western diet display bone-modulating activities. We have previously shown that 14 common vegetables, salads and herbs that are part of the normal Western diet—arugula, broccoli, cucumbers, Chinese cabbage, red cabbage, dill, garlic, wild garlic, leeks, lettuce, onions, Italian parsley, common parsley and tomatoes—significantly inhibit bone resorption in rats when administered at a dose of 1 g/d (10). Others have shown that the consumption of fruit and vegetables is associated with greater bone mineral density in humans (11–13), an effect that is claimed to be caused by the base excess of fruits and vegetables buffering noncarbonic metabolic acids that would otherwise be buffered by bone mineral, leading to bone dissolution (11,12). We demonstrated, however, that at least in rats the effect on bone resorption of the 14 foodstuffs outlined above is not mediated by their base excess but possibly by pharmacologically active compounds (14). Because some vegetables, such as potatoes, carrots and soy, consumed at the same dosage, did not significantly inhibit bone resorption (10), it cannot be generally stated that the consumption of vegetables is beneficial to bone density. This prompted us to perform the present survey.

Here we demonstrate that individual foods shown to inhibit bone resorption are widely distributed among vegetable components of the human diet.

MATERIALS AND METHODS

Animals and experimental design. Male Wistar Hannlbm rats were kept in standard animal facilities that comply with the Swiss and U.S. National Institutes of Health guidelines for care and use of experimental animals. The experiments performed were approved by the State Committee for the Control of Animal Experimentation. At completion of the experiment all rats were killed with carbon dioxide.

Feeding and diets. From the time the rats were placed in the metabolic cages, they were provided with demineralized water to drink and the diets were presented in a stainless steel crucible as wet food to minimize spillage in the cage; deionized water was added to batches of the food powder to give a dough-like consistency easily formed into food balls. During the 10-d acclimatization period in the metabolic cages and the diet described below. During the acclimatization period the rats were trained to consume 23 g/d of wet food (13.1 g/d of dry matter); rats that repeatedly did not eat the whole daily amount were eliminated during this period.

During the 10-d treatment the rats were fed the semipurified diet 2160 (Kliba-Mühlen, Kaiseraugst, Switzerland) with high Ca and P concentrations (11 g Ca/kg and 12 g P/kg) similar to those in the semipurified diet described below. During the acclimatization period the rats were trained to consume 23 g/d of wet food (13.1 g/d of dry matter); rats that repeatedly did not eat the whole daily amount were eliminated during this period.

For the treatment the dry additives were mixed with the semipurified diet (Table 1). Appropriate amounts of the items to be investigated were added to batches of wet food sufficient for 10 d of dietary intervention in 5 rats. These diets were then divided into daily portions and kept frozen at −20°C until used.

The calcium and phosphate concentrations of the diets were verified with triplicate ashed samples dissolved in 1 mol/L HCl. Calcium was determined by atomic absorption spectrophotometry and phosphate by photometry (16,17). The values given by the manufacturer were confirmed.

Processing of foodstuffs and beverages for the various additions to the diets. Processing of foodstuffs was similar to that previously described (18). Briefly, fennel, celeriac and red pepper were purchased locally, carefully washed with tap water, minced, air-dried at about 50°C and ground to a fine powder. Ready-to-use frozen spinach was freeze-dried before grinding. Plums, oranges and bananas and apples were carefully washed, stoned (plums) and peeled (oranges and bananas), cut and freeze-dried before grinding. Dried commercially available farmed shiitake and field agaric mushrooms (Lentinus edodes and Agaricus hortensis, respectively) and locally harvested yellow boletus wild mushrooms (Boletus edulis) were air-dried at about 50°C and stocked dry, then dried further by adsorption over silica gel before normal diet (15). In order to make the rats insensitive to Ca and P in the food items to be tested, a semipurified diet with high concentrations of Ca and P (11 and 12 g/kg, respectively) was used (Table 1).

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### TABLE 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration g/kg diet</th>
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<tbody>
<tr>
<td>Sodium caseinate</td>
<td>200.0</td>
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<tr>
<td>Corn starch</td>
<td>521.0</td>
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<tr>
<td>Dextrose</td>
<td>100.0</td>
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<tr>
<td>Pork fat</td>
<td>30.0</td>
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<td>Cellulose</td>
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<tr>
<td>Potassium carbonite</td>
<td>5.6</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1.1</td>
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<tr>
<td>Vitamin mix2</td>
<td>1.0</td>
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<tr>
<td>Trace element mix3</td>
<td>35.0</td>
</tr>
<tr>
<td>Choline chloride (50%)</td>
<td>2.3</td>
</tr>
<tr>
<td>dl-methionine (25%)</td>
<td>3.0</td>
</tr>
</tbody>
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1 Powdered onion or spinach, fennel, celeriac, red pepper, prunes, oranges, bananas, apples, Brazil nuts, peanuts, French beans, kidney beans, flax seed, Chinese mushrooms, wild and farmed mushrooms, rice, dark bread, sugars, beer, red wine, cola and cocoa were added to the plain control diet at a dosage per rat of 1 g/d, corresponding to 76.8 g/kg diet. Green and black tea were administered at a dosage per rat of 0.25 g/d, corresponding to 19.2 g/kg of the plain control diet. Instant coffee was administered at a dosage per rat of 0.1 g/d, corresponding to 7.7 g/kg of the plain control diet. Mix 14 consisted of equal parts of dry arugula, broccoli, cucumber, Chinese cabbage, red cabbage, dill, garlic, wild garlic, leek, lettuce, onion, Italian parsley, common parsley and tomato. Dosages per rat of 0, 50, 75, 100, 250, 500 and 1000 mg/d, corresponding to 3.84, 5.76, 7.68, 19.2, 38.4 and 76.8 g/kg of the mix 14 diet, were added to the plain control diet. 2 Providing the following addition of vitamins per kg of diet: retinyl acetate, 1.34 mg; cholecalciferol, 0.025 mg; vitamin E, 100 mg; menadione, 4 mg; thiamin, 6 mg; riboflavin, 6 mg; nicotinic acid, 30 mg; panthenolic acid, 16 mg; folic acid, 2 mg; pyridoxine, 7 mg; cobalamin, 0.05 mg; biotin, 0.2 mg and choline 1000 mg. 3 Providing the following additions of trace elements per kg of diet: copper, 8 mg; zinc, 38 mg; iron, 80 mg; iodine, 0.8 mg; manganese, 11 mg and selenium, 0.18 mg.
grinding. Roasted peanuts were shelled before grinding. Flax seed and kernels of Brazil nuts were purchased from a local retailer and ground without prior treatment. French beans and dry kidney beans were cooked in water, mashed in a blender together with the water and freeze-dried. Parboiled steamed rice was freeze-dried, and dark bread without crust, made from wheat flour type 1050, was air-dried at about 50°C before grinding. A mixture of 15.1 parts glucose, 12.9 parts fructose and 17.2 parts sucrose ("sugars"), similar to the proportions contained in dried onion (19), was prepared from analytical grade sugars.

Regular instant coffee and soluble cocoa powder were used as purchased, whereas black and green tea were ground prior to use. Normal cola was freeze-dried because a pilot experiment revealed that rats drinking cola in liquid form had a much higher liquid intake after a few days than control rats drinking demineralized water (fluid intake increased from 28 ± 5 mL/d in control rats to 88 ± 3 mL/d in rats drinking cola). Because the rats drinking cola also consumed all the food, this led to a gain in body weight of 43 ± 7 g compared with 7 ± 1 g in the control rats during the same time period. To overcome these difficulties the dry residue from cola was added to the diet. To avoid a similar problem with beer and red wine, the alcohol was removed under reduced pressure at 60°C (Rotavapor R110, Büchi, Flawil, Switzerland) and the remnant was freeze-dried. All these items were packed in polyethylene bags from which the air was evacuated before sealing, and they were stored at 4°C until used.

**Monitoring of bone resorption.** The urinary excretion of tritium-labeled tetracycline ([3H]-Tc) from chronically prelabeled rats, an extensively validated method, was used to monitor bone resorption (22). For each experiment three Wistar Hanlbm dams with 12 3-d-old male pups each were purchased (RCC Ltd., Fullinsdorf, Switzerland). From the first week of life the 36 pups were injected twice a week for 6 wk with increasing amounts of [3H]-Tc (20). The [3H]-Tc is deposited into bone and is released when bone is resorbed (20). After discontinuation of labeling, the rats were transferred to metabolic cages. After 10 d of acclimatization baseline bone resorption was monitored by measuring the daily urinary [3H] excretion. After 10 d of baseline measurement the 10-d dietary intervention was begun in rats that were homogeneously assigned to the groups; that is, the baseline [3H] urinary excretion of all rats was ranked and one animal with a similar rank was assigned to each treatment group until the complete number of animals per group was assigned (n = 6 per control group; n = 5 per treatment group). Using this protocol the mean [3H] excretion was similar for all groups at the start of the dietary intervention.

Concentration of [3H] in urine was determined by liquid scintillation counting. Aliquots of 1 mL of urine were counted in 10 mL of Irga-Safe Plus scintillator (Packard International, Zurich, Switzerland) and the result (Bq) was multiplied by the 24-h urine volume.

**Statistical methods.** Where appropriate, the 95% CI of the pertinent control groups was calculated by multiplying the SEM by 1.96 (shaded box in Fig. 1). Means of groups outside the 95% CI are significantly different from those of the control group (P < 0.05) (23). The correlation between the dose of vegetables and inhibition of bone resorption was analyzed by linear regression, and the significance of the slope was tested by ANOVA using GraphPad InStat version 3.05 statistical software (GraphPad Software, San Diego, CA).

**RESULTS**

The inhibition of bone resorption in the positive controls fed 1 g/d of onion powder was significant in 20 out of 22 groups (Fig. 1). In this model, when 1 g of a food item to be tested is added to the semipurified diet, some components of the diet might be “diluted” by 8% because the additions are not compensated for by a reduction of the cornstarch component by 8%. However, as shown with 1 g of sugars, where we expected no effect, because these sugars are devoid of secondary plant products, 8% dilution per se did not affect bone resorption. Thus, the model we used is reliable.

Using this animal model, we found that vegetables such as fennel and celeriac but not spinach and red pepper significantly inhibited bone resorption. Fruits with a significant effect were prunes and oranges, but not banana and apple. From the beans, nuts and seeds group, only French beans displayed significant activity, but not Brazil nuts, peanuts, kidney beans and flax seed. Farmed and wild mushrooms (yellow boletus and field agaric) displayed a significant activity, but Chinese mushrooms (shiitake) were devoid of activity. The three carbohydrate sources, rice, dark bread and sugars, also showed no effect on bone resorption. Finally, of the beverages tested, only the residue from red wine significantly inhibited bone resorption, but not beer, green tea, instant coffee, cola or cocoa. A pilot experiment showed that green tea administered at a dosage of 1 g/d was not well tolerated; it reduced food intake and body weight. Therefore, green and black tea were administered at a dosage of 0.25 g/d. As green tea showed no effect in both experiments, a second indepen-

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**FIGURE 1** Bone resorption in rats fed the control diet or diets containing onion (O; positive control) or various items of the following categories: vegetables, fruits, nuts, beans and seeds, mushrooms, carbohydrate sources or beverages. The 95% CI of the respective untreated control groups investigated simultaneously is shown as a shaded box. The item fed to the rats at a dose of 1 g/d, if not stated differently, is given by full name. Values are means ± SEM (n = 12 for control; n = 10 for treatment). Mean values outside the 95% CI are significantly different from those of the control (P < 0.05) (23).
dent experiment for black tea was not done. Bone resorption in rats fed 250 mg/d of black tea in the single experiment, expressed as treated/control, was 1.02 ± 0.03 (n = 5), and the 95% CI of the untreated control was ±7.7% (n = 6). Thus, black tea was also devoid of bone resorption inhibitory activity. In order to make sure that freeze-dried cola corresponds to native cola in terms of acid concentration, we compared the two items. For this, freeze-dried cola was reconstituted to its original volume with demineralized water, and the pH and the titratable acid concentration was measured as done previously (14). The pH of reconstituted cola was 2.55, compared with 2.52 for the control cola. The consumption of 0.01 mol/L NaOH to titrate 1 mL of reconstituted cola to pH 7.4 was 0.88 ± 0.1 mL, whereas the control cola required 1.10 ± 0.1 mL. Thus, reconstituted cola appears to have a 20% lower titratable acid concentration, compared with native cola. Nevertheless, rats fed 1 g/d of cola powder still received a conspicuous load of noncarboxylic acid. Despite this, bone resorption was not significantly different from that of the control group.

Of the 25 newly investigated nutritional items, 8 significantly inhibited bone resorption in rats when tested at a dosage of 1 g/d (Fig. 1). Seven items were clearly negative (mean ± SEM within the 95% CI of the controls), whereas 10 items were located at the lower limit of the 95% CI of the controls. No conclusions could be drawn for red pepper because the discriminative performance was poor in this set of experiments. For the other items, the question of whether a significant effect could be obtained with larger doses remains open. The daily dose of 4 g dry foodstuff/kg body weight corresponded to 8% of the daily intake of dry matter and was, therefore, already a high dose of an individual food item. The daily doses of coffee (400 mg of coffee extract/kg body weight) and of green tea (1 g of tea leaves/kg body weight) correspond to about 1 L/d if extrapolated to a 70 kg human; similarly, 1 g of cola powder corresponds to 2.2 L for a 70-kg human if the 20% loss of titratable acid is taken into account.

Twenty-four food items and the red wine residue were shown to significantly inhibit bone resorption when administered at a dosage of 1 g dry weight in this study and in the previous work (10,24) (Fig. 2). To allow a comparison of the activity of various food items as they are consumed in real life, the results for the inhibitory activity of bone resorption when administered at a dosage of 1 g/d of dry matter in the present and previous work (10,24) were converted to fresh weight using their recorded loss of weight (water and ethanol) during drying. The values are means ± SEM and are ranked according to potency. Copyright Roman C. Mühlbauer, 2003. Reproduced with permission.

Dose-response value for mix 14 lies outside the 95% CI of the control (dose 0 mg) calculated separately (Fig. 3b). Using this procedure we found 170 mg/d of mix 14 to be the minimum effective dose per rat.

**DISCUSSION**

As a result of this survey, seven new foodstuffs and the residue from red wine can be classified as bone resorption inhibitory food items (BRIFI), components of the human diet with significant inhibitory activity on bone resorption in our animal model, when tested at a dosage corresponding to 8% of the daily intake of dry matter. The activity cannot be associated with a single category of food items, but is rather distributed among various categories. Of the 50 food items of vegetable origin studied previously (10) and in the present work, 25 were BRIFI. Of the 36 items studied in the categories of vegetables, salads, herbs, mushrooms, and fruits, 25 were BRIFI, whereas 13 of 14 items studied in the categories of nuts, beans (dried kernels) and seeds, carbohydrate sources and beverages were devoid of activity.

We found that bread made of dark wheat flour containing 5.2% dietary fiber had no inhibitory effect (19). Thus, it appears likely that no effect on bone resorption is to be expected from flours with a lower fiber concentration commonly used to make white bread and many kinds of pasta. Other processed carbohydrate sources, parboiled rice and sugars, were devoid of effect, and because potato also displayed no significant inhibitory effect (10), the major carbohydrate sources in the human diet are not BRIFI. Foodstuffs of animal origin were also devoid of inhibitory effect in this animal model (10). Therefore, BRIFI seem to be restricted to the categories of vegetables, salads, herbs, mushrooms, fruits and red wine residue.

Our results in rats can possibly confirm and explain the effects of fruits and vegetables on bone mineral density observed in human studies (11–13) and expand BRIFI to the
decreasing full line is the same regression line as depicted in (a) (14) and graphical extrapolation of the minimum effective dose of mix 14 on bone resorption in rats (b). (a) The 65 individual data points and the mean value for onion stem from least two different classes of active molecules.

There is only scant information concerning the nature of the compounds in BRIFI that cause their inhibitory activity. To date we have identified nine monoterpenes contained in the essential oils of sage, rosemary, thyme and other plants as inhibitors of bone resorption (24). In our previous work, we also showed that the active component in onion is extractable with water or with ethanol/water, inhibits resorption in vivo and in vitro and prevents bone loss in an osteoporosis model.

Others have shown that rutin, a flavonoid abundant in onion, inhibits bone resorption in rats. Unfortunately, however, a single pharmacological dose was used (25) which was much higher than that contained in the 1 g/d dose of vegetables we used (10). It is therefore open whether rutin can partly explain the activity of BRIFI (26,27). Likewise, hesperidin, a citrus flavonoid, has been shown to inhibit bone loss in mice at a pharmacological dosage (28).

Beer could possibly be expected to have a significant effect on bone, considering the carryover of the estrogenic humulone from the hops used in brewing (29). Because intact male rats were used, the lack of effect might be due not only to an insufficient dosage, but possibly also to the fact that the model we used is not tailored to be exquisitely sensitive to phytoestrogens. Accordingly, our previous studies also found no significant effect with soy at a dosage of 1 g/d in rats (10); for an effect larger dosages of 2.5 g/d were necessary (unpublished observation). We chose this model, which is sensitive to inhibitors of bone resorption in general (30), because bone loss occurs in old age in both sexes.

Prunes have been shown to inhibit bone loss in rats at a very high dosage (25% of daily intake), the rationale for the study with prunes being their abundant polyphenol content, thought to protect bone by scavenging free radicals (31). We found prunes to have a significant effect when fed at a dosage corresponding to 8% of the daily intake. If polyphenols in general were responsible for the effect observed, other foodstuffs containing large amounts of polyphenols could also be inhibitory. However, among the beverages tested, only red wine inhibited bone resorption, despite the fact that some beverages have a large concentration of polyphenols. The polyphenol concentration provided per rat with the additions we used, calculated from published data (32), was within a range of 2.4 to 3.9 mg/d for beer, 40 to 161 mg/d for red wine, 55 to 82 mg/d for black tea, 50 to 87 mg/d for green tea and 120 to 180 mg/d for cocoa. Thus, it appears unlikely that polyphenols in general can explain BRIFI.

Coffee, tea, cola and cocoa are rich in caffeine. Caffeine intake is a risk factor for bone loss in humans because caffeine increases the urinary excretion of calcium. However, it appears that the risk can be offset by moderate milk consumption (33). We used a high calcium diet throughout these studies to make the rats insensitive to the calcium concentration of the food items tested, which makes it plausible that any stimulation of bone resorption by caffeine-rich food items might have been masked.

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A mechanistic understanding of how foods influence bone loss and bone formation is important for bone health. We recently reviewed the evidence that a diet rich in soybean foods has a protective effect on bone loss (2). Here we discuss plant foods that have been identified as potential sources of phytoestrogens, such as soybean (Glycine max (L.) Merr.) and flaxseed (Linum usitatissimum L.) (3,4) and provide an overview of their effects on bone density in postmenopausal women and in animal models of bone loss.

Phytoestrogens: A Brief Review

Phytoestrogens are plant-derived substances that have a structure similar to that of estrogen and can bind to estrogen receptors (5). They are found in a variety of plant foods and beverages, such as soybeans, flaxseed, and other legumes, and their effects on bone health have been studied extensively. The main phytoestrogens found in soybeans are genistein and daidzein, while those found in flaxseed are enterolactone and enterolignans. These compounds have been shown to have an estrogen-like effect on bone metabolism, but their mechanisms of action are not fully understood.

The Effect of Soybean Foods on Bone Health

Soybeans are a rich source of isoflavones, and studies have shown that soy consumption can decrease bone loss in postmenopausal women. In the Shanghai Women’s Health Study, a large prospective study of Chinese women, the consumption of soy products was associated with a lower risk of hip fracture (6). Another study found that the consumption of soy products was associated with a 20% lower risk of fractures in postmenopausal women (7). These findings are supported by data from the Nurses’ Health Study, which showed that the consumption of soy products was associated with a reduced risk of hip fractures (8).

In animal studies, soy consumption has been shown to decrease bone loss and increase bone density. In a study using ovariectomized rats, a diet rich in soy protein was found to decrease bone loss and increase bone density (9). Similarly, a study using ovariectomized mice found that soy consumption decreased bone loss and increased bone mineral density (10).

Soy products also contain other compounds that may contribute to their bone protective effects. For example, soy is a rich source of vitamin D, which is essential for bone health. Soy foods also contain other nutrients that are important for bone health, such as calcium, magnesium, and potassium.

In summary, the evidence suggests that soy consumption is associated with a lower risk of bone loss and fractures in both men and women. However, more research is needed to fully understand the mechanisms by which soy products affect bone health.

References