Effect of Hesperidin with and without a Calcium (Calcilock®) Supplement on Bone Health in

Postmenopausal Women

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ABSTRACT

Context: Citrus fruits contain unique flavanones. One of the most abundant of the flavanones, hesperidin, has been shown to prevent bone loss in ovariectomized rats.

Objective: The objective of the study was to measure the effect of hesperidin with or without calcium supplementation on bone calcium retention in postmenopausal women.

Design. The study was a double blind, placebo-controlled, randomized order cross-over design of 500 g hesperidin with or without 500 mg calcium supplement in 12 healthy postmenopausal women. Bone calcium retention was determined from urinary excretion of the rare isotope, ^{41}Ca, from bone.

Results. Calcium plus hesperidin, but not hesperidin alone, improved bone calcium retention by 5.5% (p < 0.04).

Conclusion. Calcium supplementation (Calcilock®), in combination with hesperidin, is effective at preserving bone in postmenopausal women.

Both environment and genes influence bone mass and determine the risk of developing

osteoporosis (1, 2), Women are at high risk for bone fracture after menopause due to an increased loss of

bone in response to decreased estrogen secretion.(1)   It is well established that nutrition has an

important role in bone health. Research has shown that calcium, vitamin D, and several micronutrients

are essential for maintaining optimum bone mineral density and strength. (3) Fruits and vegetables

containing various polyphenolic compounds, especially flavonoids, have been associated with improved

bone mineral density in part because of an effect on cell signaling pathways that regulate the cells

responsible for bone formation and resorption (4-6)

Citrus fruits contain hesperidin (hesperetin-7-O-rutinoside), a flavanone that is especially

abundant in oranges (31-43.2 mg/100 g).   Preclinical studies in rats and mice have shown positive effects

of hesperidin on various bone parameters (7).   Dietary supplementation hesperidin (0.5%, w/w) of 3

month old rats resulted in increased bone mass and the same diet in 6 month old rats prevented

ovariectomy-induced bone loss (8). When the diet of twenty month old male rats was supplemented with

0.5% hesperidin for three months, both femoral bone density and trabecular bone volume increased (9) .

Because there is a paucity of studies of the effect of hesperidin on bone in humans, we studied

the effect of hesperidin and its interaction with dietary calcium on reducing bone loss in twelve healthy

post-menopausal women using the isotopic tracer, ^{41}Ca, method (10).

Study Subjects

 Healthy women who were at least 4 years postmenopausal as a result of natural or surgical menopause

established by questionnaire and validated by serum FSH , were enrolled in the study. Women who were

on estrogen replacement therapy, treatment for osteoporosis, or taking dietary calcium supplements were

not eligible for the study. All participants gave written informed consent and the study was approved by

the Purdue University and Indiana University Purdue University Institutional Review Boards.

Study Design and Methods

The study design was randomized order, double blind cross-over with one baseline and three

treatment phases. (Fig. 1)   Interventions consisted of 4 biscuits daily that contained either, 1) 500 g

hesperidin, 2) 500 g hesperidin + 500 mg calcium as CALCILOCK® or 3) placebo (Nestle Research and Development, Maipu, Chile). Study interventions were assigned to each subject in random order. With three interventions (A, B, and C) there were six possible orders (ABC, ACB, BAC, BCA, CAB). A file with the six interventions was randomly sorted to give the orders for the first six subjects. The process was repeated for the six additional subjects. Biscuits were provided at the beginning of each intervention phase. Subjects were instructed to consume 1 serving (two biscuits) in the morning and 1 serving in the evening for each day of the 50 day intervention phase. Each intervention phase was followed by a 50 day wash out period. An initial 50 day baseline preceded the first treatment period (Fig 1). During each 50 day period subjects collected 24 h urine every 10 days (5 collections per phase).   Subjects submitted 4 day diet records including 3 week days and 1 weekend day at the end of each intervention phase. Records were analyzed using the Nutrition Data System for Research (NDSR, version 2011, Minneapolis, MN, USA).

At the end of each baseline, intervention and washout phase there was a clinical visit. Subjects returned any uneaten products. Fasting (>8h) serum and urine samples were collected to evaluate mineral and safety biochemistry and biochemical markers of bone turnover including serum C- telopeptide (Crosslaps® ELISA Immunodiagnostic Systems, Fountain Hills AZ, USA, CV = 4.2%), parathyroid hormone (two-site immunoassay, Nichols Institute Diagnostics, San Juan, Capistrano, CA, USA(CV=7.1%), total 25(OH) D (LC-MS/MS, Novilytic LLC, North Webster IN, USA,), urine deoxypyridinoline (Microvue EIA, Quidel Corp., San Diego, CA, USA, CV=6.9%). Serum and urine creatinine, calcium and inorganic phosphorus were measured by routine chemistry. Anthropometric measures were taken using standard weight scales, stadiometer and calipers. Total body, dual hip and lumbar spine bone mineral densities were measured at the baseline visit (iDXA, software version 4.3e, GE Lunar Corp., Madison WI, USA)

All urine samples were prepared for ^{41}Ca analysis as previously described [11]. Briefly, calcium was precipitated by adjusting the pH to at least 10 using ammonium hydroxide and then adding ammonium oxalate to form calcium oxalate (CaC_sO_4). The supernatant was decanted and the precipitate

was collected on filter paper and dried. The ^{41}Ca:Ca ratios were determined in the filtrate by Accelerator Mass Spectrometry (PRIME Lab, Purdue University, W. Lafayette, IN USA).

Statistical Analysis

  The power of the protocol was based on the ability to detect changes in ^{41}Ca excretion in urine. With at least 10 subjects there was 80% power to detect a 10% improvement in net calcium retention. Daily urinary ^{41}Ca during an intervention was compared with the change predicted from baseline and non-intervention periods. Daily urinary ^{41}Ca:Ca ratios were transformed using the natural logarithm. For each subject a linear regression using non-intervention data was used to predict ratios for intervention periods. For each subject, deviations of the observed intervention value from the corresponding predicted value were averaged to determine the intervention effects. The values were averaged for all 12 subjects and 95% confidence intervals were calculated. The estimates and confidence intervals were expressed as relative change in bone calcium retention (%) as described previously (12). Statistical significance (p<0.05) indicates significant deviation from the predicted change. The bootstrap method was used to verify the accuracy of the asymptotic approximations used. Biomarkers were secondary outcomes and were analyzed using ANOVA methods based on the crossover design.

Results

        Fourteen women were screened for the study. Twelve women age 66.3 y, post-menopausal 16.8 y, and BMI of 25.7 kg/m^2 were enrolled in the study. (Table 1) All 12 women completed all baseline, 3 intervention, and 3 washout phases.   Mean compliance with the consumption of the biscuits was 92.5% .

        A 5.5% improvement in net calcium bone retention was found for hesperidin with the calcium supplement (p=0.04, Fig 2). For hesperidin alone and for placebo, the changes were not statistically significant (p=0.34, P=0.39).   The 95% confidence interval for the combination treatment did not contain zero and thus can be considered an effective treatment for increasing net bone calcium retention.   No significant intervention effects were found for the biomarkers or mineral biochemistry (Table 2) other than PTH was less with the combination treatment than with hesperidin alone or placebo.

There was no indication that dietary intake varied from treatment to treatment when macro and micronutrients from the diet records plus supplements were analyzed other than calcium that was greater in the hesperidin plus Calcilock® phase (Table 3).

Discussion

In this randomized order, placebo-controlled, double-blind clinical trial in postmenopausal women, hesperidin plus a nutritional supplement (Calcilock®) formulated into biscuits improved net bone calcium retention by 5.5%, but hesperidin alone had no benefit. This study employed the novel and sensitive approach of measuring urinary appearance of the rare isotope, ^{41}Ca, from bone to determine the effect of interventions on net bone calcium retention (or loss) relative to predicted values from non-intervention periods and compared with a placebo period. All three intervention periods and recovery periods were tested in each of the 12 participants in 350 days. The more traditional approach of a parallel arm study measuring changes in bone mineral density would have involved an intervention period of 2 or more years and 60 or more participants per arm to have similar power to our study. Thus, both methods give an estimate of changes in bone calcium due to intervention, but the power of the cross-over design and the sensitivity of ^{41}Ca analysis greatly reduce the time and number of participants required. However, bone densitometry could give site specific changes in bone, whereas, calcium tracers reflect whole body metabolism. The longer intervention periods in bone density trials span several bone remodeling cycles which may be more reflective of the long term impact of an intervention. The 50 day intervention periods that we used in this study span a little more than one remodeling cycle, and therefore, may reflect a remodeling transient which could mean a one time benefit that could disappear upon cessation (13). Nevertheless, this is a useful screening approach to identify effective interventions to pursue in the longer, more expensive trials monitoring bone mineral density.

The benefit of hesperidin plus Calcilock® intervention on bone calcium retention of 5.5% theoretically is equivalent to a benefit in net calcium balance or total bone mineral content (BMC) as

calcium is a constant fraction of BMC. This provides a practical and substantial countermeasure to women stable to menopause who on average lose 0.5 to 2.0% BMC annually, depending on the bone site (14). The lack of benefit of the hesperidin alone intervention suggests the benefit is due to calcium rather than the hesperidin, though this was not directly tested. Consistent with our observed lack of effect of 500 mg hesperidin, an unpublished 2 y randomized, controlled trial described in a conference proceeding (15) found no benefit to BMD at any site including total hip, femoral neck, lumbar spine, or whole body. A benefit of calcium of this magnitude in women with average intake of approximately 1 g/d may be surprising given the plateau intake for calcium retention in this age group is approximately 1.2 g/d (16). Yet calcium supplementation has benefits to calcium retention and bone parameters in similar populations (17,18).

The dose and form of hesperidin as well as the inflammatory status of the subjects may influence effects on bone. The same dose of hesperidin as used in this study was effective in reducing two inflammatory markers, C-reactive protein and serum amyloid A protein in patients with metabolic syndrome (19). Hesperitin-7-glucoside, a metabolite of hesperidin, may have been more effective than hesperidin because it is more bioavailable (20, 21). The current study provides little mechanistic insight as the purported effects of flavonoids on osteoblast and osteoclast differentiation were not studied. For example, Kim et al, (22) showed hesperitin suppressed NF-kappaB activation. We powered our study for the main outcome which is a more sensitive measure than for biochemical markers of bone turnover or regulators of calcium metabolism. The bone resorption marker, deoxypyridinoline, was suppressed by hesperidin in a male senescent rat model (9). Serum PTH ($p < 0.05$) was much less in the active intervention, a classic response to increased dietary calcium (17).

In summary, in postmenopausal women stable to menopause, Calcilock®, a supplement including 500 mg calcium with hesperidin, but not hesperidin alone, was effective in improving bone calcium retention. The benefit may be exclusively due to calcium, but a limitation of the study was lack of a calcium alone intervention so an interaction cannot be ruled out. The novel use of a rare calcium isotope, [41]Ca, allowed multiple interventions to be directly compared in a cross-over design in the same subjects

with substantially reduced intervention duration and sample size than a traditional trial of change in bone mineral density.

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