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

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Abbreviated Title: Effect of Hesperidin and Calcium on Bone Retention

Key Terms: hesperidin, citrus flavanones, calcium retention, ^{41}Ca, postmenopausal women

Word Count: 1917

Number of figures and tables: 4

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Disclosure Statement: This study was supported by Nestec, Ltd. Vevey CH. M.N.H and E.O.C are research scientists at the Nestle Research Center. C.M.W is a member of the Scientific Advisory Board for Pharmavite® LLC. Other authors have nothing to disclose.

The study was registered in Clinicaltrials.gov (NCT01881204).

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.

Acknowledgements

The authors thank D. Maish for phlebotomy and clinical services. B.R. M., C.M.W., M. H., and G.P.M. designed the research; B.R.M., C.M.W. and M.P. conducted the research; G.S.J. performed the accelerator mass spectrometer measures; G.P.M., L.M., and B.R.M. analyzed the data; B.R.M., C.M.W., M.P., and G.P.M. wrote the paper; B.R.M. and C.M.W. had primary responsibility for the final content. All authors read and approved the final manuscript.

References

1. National Osteoporosis Foundation, Hormones and Healthy Bones, 2009. http://nof.org/files/nof/public/content/file/265/upload/98.pdf , Accessed August 1, 2014.
2. White KE, Koller DL, and Corbin T. Skeletal Genetics. In: Burr DB and Allen MR, eds. Basic and applied bone biology. Boston: Elsevier Inc. 2013: 149-171..
3. Cashman KD. Diet Nutrition, and Bone Health, J Nutr 2007; 137: 2507S-2512S.
4. Weaver CM, Alekel DL, Ward WE, Ronis MJ. Flavonoid intake and bone health, J Nutr Gerontol Geriatr 2012; 31(3): 239-253.
5. Prynne CJ, Mishra GD, O'Connell MA, Muniz G, Laskey MA, Yan L, et al. Fruit and vegetable intakes and bone mineral status: a cross sectional study in 5 age and sex cohorts. Am J Clin Nutr 2006; 83(6): 1420-1428.
6. Chen YM, Ho SC, Woo JL. Greater fruit and vegetable intake is associated with increased bone mass among postmenopausal Chinese women. Br J Nutr 2006; 96(4): 745-751.
7. Sacco SM, Horcajada MN, Offord E. Phytonutrients for bone health during ageing. Br J Clin Pharmacol 2013; 75(3): 697-707.

8. Horcajada MN, Habauzit V, Trzeciakiewicz A, Morand C, Gil-Izquierdo A, Mardon J, et al. Hesperidin inhibits ovariectomized-induced osteopenia and shows differential effects on bone mass and strength in young and adult intact rats. J Appl Physiol 2008; 104(3): 648-654.
9. Habauzit V, Sacco SM, Gil-Izquierdo A, Treciakiewicz A, Morand C, Barron D, et al. Differential effects of two citrus flavanones on bone quality in senescent male rats in relation to their bioavailability and metabolism. Bone 2011; 49(5): 1108-1116.
10. Lee WH, Wastney ME, Jackson GS, Martin BR, Weaver CM. Interpretation of ^{41}Ca data using compartmental modeling in post-menopausal women. Anal Bioanal Chem 2011; 399(4): 1613-1622.
11. Jackson GS, Weaver C, Elmore D. Use of accelerator mass spectrometry for studies in nutrition. Nutr Res Rev 2001; 14(2): 317-334.
12. Weaver CM, Martin BR, Jackson GS, McCabe GP, Nolan JR, McCabe LD, et al. Effects of phytoestrogen supplements compared with estradiol or risedronate in postmenopausal women using Ca-41 methodology. J Clin Endocrinol Metab 2009; 94(10):3798-3806.
13. Heaney RP. The bone remodeling transient: interpreting interventions involving bone-related nutrients. Nutr Rev 2001; 59(10): 327-334.
14. Riggs BL, Melton LJ, Robb RA, Camp JJ, Atkinson EJ, McDaniel L, et al. A population-based assessment of rates of bone loss at multiple skeletal sites: evidence for substantial trabecular bone loss in young adult women and men. J Bone Miner Res 2008; 23(2): 205-214.
15. Horcajada MN and Offord E. Citrus flavanones and bone health. In: Burckhardt P, Dawson-Hughes B., Weaver CM eds. Nutritional influences on bone health: 8th international symposium. London: Springer 2013: 157-169.
16. National Academy of Sciences, Institute of Medicine, Food and Nutrition Board. Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. Washington, DC: National Academy Press, 1997.

17. National Academy of Sciences, Institute of Medicine, Food and Nutrition Board. Dietary reference intakes for calcium and vitamin D. Washington DC: National Academies Press, 2011.
18. Prentice RL, Pettinger MB, Jackson RD, Wactawski-Wende J, Lacroix AZ, Anderson GL, et al. Health risks and benefits from calcium and vitamin D supplementation: women's health initiative clinical trial and cohort study. Osteoporos Int 2013; 24(2):567-580.
19. Rizza S, Muniyappa R, Iantorno M, Kim JA, Chen H, Pullikotil P et al. Citrus polyphenol hesperidin stimulates production of nitric oxide in endothelial cells while improving endothelial function and reducing inflammatory markers in patients with metabolic syndrome. J Clin Endocrinol Metab 2011; 96(5): E782-792.
20. Habauzit V, Nielsen IL, Gil-Izquierdo A, Trzeciakiewicz A, Morand C, Chee W, et al. Increased bioavailability of hesperetin-7-glucoside compared with hesperidin results in more efficient prevention of bone loss in adult ovariectomised rats. Br J Nutr 2009;102:976-984.
21. Nielsen IL, Chee WS, Poulsen L, Offord-Cavin E, Rasmussen SE, Frederiksen H, et al. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. J Nutr 2006; 136(2): 404-408.
22. Kim JY, Jung KJ, Choi JS, Chung HY, Modulation of the age-related nuclear factor-kappaB (NF-kappaB) pathway by hesperetin. Aging Cell 2006; 5(5):401-411.