Original Article

The effects of green kiwifruit combined with isoflavones on equol production, bone turnover and gut microflora in healthy postmenopausal women

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Background and Objectives: Isoflavone (daidzein and genistein) interventions in postmenopausal women have produced inconsistent skeletal benefits, partly due to population heterogeneity in daidzein metabolism to equol by enteric bacteria. This study assessed changes in microflora and bone turnover in response to isoflavone and kiwifruit supplementation in New Zealand postmenopausal women. **Methods and Study Design:** Healthy women 1-10 years post-menopause were randomly allocated to group A (n=16) or B (n=17) for a 16-week crossover trial. Two consecutive 6-week treatment periods had a 2-week lead-in period at intervention commencement and a 2-week washout period between treatments. Treatments prescribed either (1) daily isoflavone supplementation (50 mg/day aglycone daidzein and genistein) alone, or (2) with two green kiwifruit. At treatment baseline and endpoint (four time points) the serum bone markers C Telopeptide of Type I collagen (CTx), undercarboxylated osteocalcin (unOC), and serum and urinary daidzein and equol, were measured. Changes in gut microflora were monitored in a subgroup of the women. **Results:** Equol producers made up 30% of this study population (equol producers n=10; non-equol producers n=23) with serum equol rising significantly in equol producers. Serum ucOC decreased by 15.5% (p<0.05) after the kiwifruit and isoflavone treatment. There were no changes in serum CTx or in the diversity of the gut microflora. **Conclusions:** 50 mg/day isoflavones did not reduce bone resorption but kiwifruit and isoflavone consumption decreased serum ucOC levels, possibly due to vitamin K1 and/or other bioactive components of green kiwifruit.

Key Words: kiwifruit, bone markers, isoflavones, gut microbiota, postmenopausal women

INTRODUCTION

Osteoporosis is a skeletal disorder typified by low bone mineral density and microarchitectural derangement.[1] Osteoporotic individuals have increased skeletal fragility and fracture incidence.[1] In postmenopausal osteoporosis bone loss is accelerated with an increase in bone turnover due to estrogen deficiency.[2] Postmenopausal osteoporosis is a serious health risk worldwide and generates substantial healthcare costs in developed nations. In New Zealand the estimated annual cost of osteoporotic fracture treatment was $1.38 billion dollars in 2013, with 62% of these fractures experienced by women aged 55+ years.[3] This burden is projected to increase as the aging population expands throughout the developed world.[3] The use of hormone replacement therapy (HRT), which had positive effects in reducing bone loss as well as reducing the symptoms of menopause, has decreased in popularity due to hormone-dependent adverse effects such as an increased risk of developing breast and uterine cancers and cardiovascular disease (CVD).[4] An effective osteoporosis treatment that poses fewer negative health impacts is sought after.

Isoflavones, which are phytoestrogens, have a molecular structure markedly similar to endogenous human estrogen and have been shown in vitro to bind to cellular estrogen receptor β (ERβ) and activate estrogenic gene expression.[5-7] The ERβ-isoflavone interaction in osteogenic cells modulates osteogenic life-cycle and activity,[8,9] and isoflavones have been shown to decrease production of prostaglandin E2 and interleukin-6 in osteoblastic cells.[10] Daidzein and genistein, the isoflavones found in soybean, may therefore reduce the development of postmenopausal osteoporosis and cardiovascular disease

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(CVD) with few or no adverse side effects.[5,11,12] Several long term RCTs failed to show a benefit of soy to BMD in postmenopausal women.[13-15] These studies were conducted with sufficient doses of calcium and vitamin D. A recent RCT however, showed a benefit of isoflavone intervention on bone calcium retention.[16]

Many Asian ethnicities consume soybean in the habitual diet, which exposes these populations to high levels of dietary isoflavones.[17] The osteoporosis burden and hip fracture incidence are lower in Asian postmenopausal women compared to their Western counterparts,[18] where isoflavone intakes are substantially lower[19] which may be partly due to a high intake of isoflavones. Postmenopausal Asian populations also exhibit a lower prevalence of cardiovascular disease (CVD) compared to Caucasian women,[20] which may also be attributable at least in part to isoflavone consumption.[20,21]

Randomised controlled trials (RCT) measuring the effect of isoflavone supplementation on postmenopausal bone density and turnover have produced discrepant outcomes. Disparities amongst RCT may arise from variations in isoflavone dose, isoflavone composition, population characteristics, i.e. the years since onset of menopause, trial duration, and the isoflavone form, glycosidic or aglycone, which affects intestinal absorption.[19,21] However, meta-analyses report that isoflavone supplementation with doses of at least 90 mg/day for 6 months or longer inhibits bone loss at the lumbar spine in postmenopausal women.[22,23] In addition, isoflavone supplementation with doses between 50-90 mg/day for at least 10 weeks significantly reduces urinary levels of the bone resorption marker deoxypyridinoline (DPD) in postmenopausal women.[24,25]

Equol is a daidzein metabolite with greater availability and affinity for ERβ[9,11] and higher antioxidant capacity than its parent daidzein molecule.[26,27] Only 30-50% of the world's healthy population possess an equol-producing gut microbial profile.[28] Inter-individual variation in daidzein metabolism is therefore likely to modulate isoflavone efficacy.[5,28,29] Few isoflavone supplementation trials have accounted for equol metabolism;[5] studies that did prospectively screen postmenopausal participants for equol production reported that bone protective effects were exclusive to equol producers.[30-32] The importance of this factor is further highlighted by the results of a recent intervention study, which showed that supplementation with purified equol significantly inhibited bone resorption in non-equol producers.[32]

The daidzein-metabolising phenotype is dictated by both host genetics[33] and habitual diet:[34-36] only 25-30% of the Western population are equol producers, whereas 50-60% of Asians[33] and vegetarians[35] have this capacity. This phenotype in vegetarians suggests a correlation with dietary fibre intake, and accordingly a dietary analysis of equol producers found a positive correlation to soluble fibre intake.[36,37] Isoflavone and dietary fibre supplementation in an ovariectomised (Ovx) rat model of osteoporosis increased equol production and augmented the bone-sparing effects compared to isoflavones alone.[38] However, additional studies have produced inconsistent results, and the mechanisms by which dietary fibre affects isoflavone metabolism are not yet fully elucidated.

Soluble fibre acts as a prebiotic, which may increase the abundance or activity of daidzein-metabolising microbiota. Kiwifruit have a high soluble fibre content, and thus are capable of modulating enteric microbial composition.[39,40] Combined isoflavone and kiwifruit supplementation had a synergistic effect on bone health in Ovx rodents;[41,42] nonetheless equol production was unaltered in this study.

The current study examined the effects of isoflavones and kiwifruit on bone metabolism, equol production and lipid profile in healthy postmenopausal women. C-telopeptide of type I collagen (CTx), a marker of bone resorption, as well as uncarboxylated osteocalcin (unOC), a marker of bone health and vitamin K status, were measured. Serum lipids were measured as an indirect assessment of CVD risk. We hypothesised that the soy isoflavones would reduce bone turnover and improve the lipid profile in this postmenopausal study population with a greater effect in equol producers. In addition, kiwifruit consumption was expected to enhance equol production and increase the effects on bone and lipid modulation in equol producers.

METHODS

Ethics

This study was reviewed and approved by Massey University Human Ethics Committee: Southern Application 13/15. Participants provided written informed consent and were aware of their right to withdraw from the study. Randomly generated identification codes ensured participant confidentiality. The trial was registered with the Australian and New Zealand Clinical Trials Registry (ANZCTR) http://www.ANZCTR.org.au. Trial Registration: ACTRN 12611000763943).

Participants

Thirty three healthy postmenopausal Caucasian women, 1-10 years since the onset of natural menopause (>1 year since last menstrual period) and >50 years in age, were recruited and screened to fit the exclusion criteria. The postmenopausal status of participants was confirmed retrospectively by demonstrating their serum follicle stimulating hormone levels were >20 IU/L.

Individuals underwent an initial health screening. Exclusion criteria for this study included: frequent constipation (i.e. requisite consumption of fibre products); nicotine and/or excess alcohol consumption (>2 standard drinks per day on average); fracture in the previous six months, a T score less than -2.5 SD at the hip or spine, or the presence of any bone disease or systemic disease affecting bone density; gastrointestinal disease (excluding appendicitis and irritable bowel syndrome) or gastrointestinal infection in the previous month; cholesterol levels ≥6 mmol/L; current kidney and/or liver impairment or history of these impairments, or any endocrine disorders; long-term or recent antibiotic due to the disruption of intestinal microbiota, which lasts for 10-14 days after cessation of antibiotic treatment; use of some medications, such as corticosteroids and heparin, due to their effects on bone and mineral metabolism and lipid metabolism. The log ratio of urinary daizein to equol was used to delineate equol producers and non-producers: a ratio greater than

-1.70 indicated an equol producer.

Intervention design

Research was carried out at the Human Nutrition Research Unit, Massey University, Palmerston North over August-December, 2013. Recruitment was achieved by advertisement in the local newspaper, the Manawatu Standard, and by an email circulated to Massey University staff.

Participants were randomly allocated into one of two groups, group A or B, by a random number generator. These random numbers served as each participant's identification code.

The 16-week study period was segmented: a 2-week lead in period initiating dietary followed by two 6-week interventions, with a 2-week washout in between. Group A had isoflavones alone for the first 6-weeks followed by isoflavones and kiwifruit for the following 6-weeks. Group B had the same intervention sequence in reverse. Isoflavone capsules and kiwifruit were taken in the morning with breakfast.

Soy isoflavone supplement and Zespri Kiwifruit

Participants received 50 mg isoflavones daily from an oral supplement ('Nature Made Soy Isoflavone', Otsuka Pharmaceutical Ltd., Tokyo, Japan) containing daidzein and genistein in an unknown ratio. Of the total isoflavone dose 47.2 mg were aglycone and 2.8 mg were glycosidic. Zespri® International, Mount Maunganui, New Zealand, provided green kiwifruit for the study

Dietary restrictions

Dietary restrictions were implemented beginning at week 0 to reduce confounding factors including: probiotics, soy or soy-based foods, fibre supplements, kiwifruit (outside of the kiwifruit consumed during intervention), omega-3, calcium and vitamin D supplements.

Dietary intakes

Participants recorded three-day food diaries demonstrating their habitual diet (including one weekend day), which were analysed by the computer software program Excel Eiyo-kun, version 7.0 (Kenpaku Co. Ltd., Tokyo, Japan) to determine average macro- and micronutrient intakes. The supplemented kiwifruit were excluded from the analyses.

Measurement of bone mineral density (BMD)

BMD of the total hip, lumbar spine and whole body were measured at baseline using dual energy X-ray absorptiometry (Hologic Discovery A, Madison WA, USA) at the Human Nutrition Research Unit, Massey University. BMD was determined to exclude osteoporotic individuals and to compare between groups.

Blood and urine sample collection

At weeks 2, 8, 10 and 16 fasted blood samples and urine samples, both fasted spot (second void) and 24 h, were collected. The women were instructed to take the isoflavone supplements in the morning.

Blood samples were either clotted at room temperature, for serum samples, or chilled with EDTA as anticoagulant, for plasma samples, until centrifugation for 15 min at 2000g and 4 ℃.

All serum, plasma and urine samples were stored at 80 ℃ until completion of the trial.

Biochemical analyses

Bone markers

Serum CTx was measured by a commercial enzyme-linked immunoassay using the Roche Elecsys (Canterbury Health Ltd, Canterbury, New Zealand). ucOC was measured by a competitive enzyme-linked electrochemiluminescence immunoassay kit (Takara, Tokyo, Japan) with an inter-assay CV of 5.7% and intra-assay CV of 4.6%.

Blood Lipid profiles and hormones

All hormones (Estradiol (E2), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH)) were measured by commercial enzyme-linked immunoassay (Medlab Central Ltd., Palmerston North); lipids (total cholesterol, low-density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c) and triglycerides (TG)) were measured by standard enzymatic colorimetric assay (Medlab Central Ltd., Palmerston North).

Daidzein, equol and vitamin D

Both serum and urinary daidzein and equol were measured by time-resolved fluoroimmunoassay (Labmaster Ltd., Aura, Finland) and vitamin D was measured by isotope-dilution liquid chromatography-tandem mass spectrometry (Canterbury Health Laboratories Ltd., Canterbury). Participants were classified as equol producers if they had a $\log_{10}$ ratio of urinary equol to daidzein $\geq$-1.70 in at least two of the three measured time points: the spot urine sample at week 8 and the 24-hour urine collections at week 8 and 16.

DNA extraction from faeces

Faecal samples were collected at baseline and after 6 weeks of consuming 2 green kiwifruit per day. Samples were collected into a solution containing 100 mM Tris-HCl (pH 9.0) and 40 mM ethylenediaminetetraacetic acid and stored at -80 degrees till analysis. DNA was extracted from the faecal samples according to the method used by Nagashima et al with modifications.[43] The faecal samples were suspended in a solution containing 100 mM Tris-HCl (pH 9.0) and 40 mM EDTA after washing three times with sterile distilled water, and the faeces were then homogenised using a FastPrep FP100A Instrument (MP Biomedicals, Santa Ana, CA, USA). DNA was extracted from the suspension using a GC series genomic DNA whole blood kit and then purified using a Magtration 12GC system (Precision System Science, Chiba, Japan).

PCR conditions and terminal restriction fragment length polymorphism analysis

Amplification of the faecal 16s rDNA, restriction enzyme digestion, size fractionation of terminal restriction fragments, and terminal restriction fragment length polymorphism (T-RFLP) data analysis were performed according to the method used by Nagashima et al with modifications.[44] Briefly, PCR was performed using the total faecal

DNA and the primers of 5'-carboxy-fluorescein-labelled 516f and 1510r. The resulting 16S rDNA amplicons were treated for 3 h at 55℃ with 10 U of Bs/I (5'-CCNNNNN|NNGG-3') (New England Biolabs, Ipswich, MA, USA). The fluorescent-labelled terminal restriction fragments produced by digestion with Bs/I were analysed by electrophoresis on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in GeneScan mode (the injection time was 30 s, and the run time was 40 min).

Clone library analysis

Clone library analysis was carried out according to the method used by Nagashima et al[43,44] with modifications. The length and peak areas of the terminal restriction fragments were determined and a phylogenetic tree was constructed using Gene Maths software (Applied Maths, Sint-Martens-Latem, Belgium). The fingerprint profiles generated by T-RFLP were compared using Pearson's correlation coefficient. The Dice similarity matrix was used to construct a dendrogram of the T-RFLP fingerprint profiles.

Statistical analysis

Data were analysed as a crossover repeated measures design using mixed models approach (Proc mixed, SAS 9.3).[45] The model for raw data included equal producer status, treatment, period, week and all interactions as fixed effects, and subject as random effect. The model for difference from baseline included equal producer status, treatment, period, and all interactions as fixed effects, subject as random effect, and baseline value as covariate. Analysis of variance was followed by post-hoc testing of pairwise differences (Tukey adjustment for multiple comparisons). If necessary, data were transformed to achieve homogeneity of variance. The differences in the faecal intestinal microbiota between equol producers and non-producers were examined by using unpaired Student's t-test. The differences in the faecal intestinal microbiota at baseline and after 6 weeks of intervention were examined by using paired Student's t-test. Results are presented as least-squares means and 95% confidence interval. Significance was declared if $p<0.05$.

RESULTS

Baseline characteristics

The baseline characteristics are presented in Table 1. Anthropometric measurements were not significantly different between groups A and B. Groups A and B had similar BMD, BMC, and T and Z scores for each skeletal site ($p>0.05$). Mean T scores were greater than -1 SD from the reference BMD distribution. The exception was for group B: the T scores for the neck of hip (-1.12±0.215) and the lumbar spine (-0.850±256) were in the osteopenic range (-1 to -2.5 SD) (only when taking into account the SEM of the lumbar spine T score). Serum hormone levels, vitamin D, E2, and FSH, were also comparable between groups A and B ($p>0.05$). The minimum serum FSH was 28.6 and 25.9 IU/L for group A and B respectively; all participants had serum FSH >20 IU/L, this cut-off value used to confirm the postmenopausal status of participants. Baseline serum FSH levels were 68.1±5.0 IU/mL. Menopausal status was confirmed by serum FSH >20 IU/mL.[46] Baseline serum estradiol levels were 41.0±3.2 pmol/L and there was no change in serum estrogen over the treatment period (Table 1). Serum estrogen was monitored to confirm that the isoflavone intake had no effect on endogenous serum estrogen. The current study population consisted of women who were 1-10 years postmenopausal; however, the exact number of years since menopause was not reported. The average serum E2 levels in this study were comparable to values reported by a large prospective study of postmenopausal women that were on average 8-10 years postmenopausal (~12 pg/mL which is equivalent to 44.1 pmol/L),[46] whereas women who were less than 5 years postmenopausal had average serum E2 that was >80.8 pmol/L (converted from 22 pg/mL). Thus it could be estimated that the women in this study were closer to 8-10 years postmenopausal. Participants had sufficient vitamin D status according to the Institute of Medicine (IOM), whose guidelines considers a serum 25(OH)D of 50 nmol/L to be sufficient.[47]

Table 1. Baseline characteristics of groups A and B (mean±SEM)

Baseline characteristics	Group A	Group B	*p* value
Age (years)	56.1±0.8	57.6±0.8	0.203
Height (cm)	164.6±1.2	164.3±1.6	0.877
Weight (kg)	68.6±2.8	66.9±2.3	0.649
BMI (kg/m^2)	25.3±1.0	24.9±1.1	0.795
BMD (g/cm^2)			
Lumbar spine	1.044±0.047	0.995±0.028	0.126
Total hip	0.918±0.030	0.852±0.021	0.087
BMD T score			
Lumbar spine	-0.012±0.428	-0.850±0.256	0.116
Total hip	-0.187±0.247	-0.743±0.168	0.082
BMC (g)			
Lumbar spine	61.3±4.1	53.2±2.4	0.114
Total hip	31.9±1.2	29.2±0.8	0.077
25(OH) D3 (nmol/L)	63.6±4.3	77.2±6.6	0.090
Baseline FSH (IU/L)	66.7±4.6	70.4±5.3	0.595
Baseline E2 (pmol/L)	41.7±4.0	40.1±2.2	0.455

BMI: body mass index; BMD: bone mineral density; BMC: bone mineral content; 25(OH) D3: 25- hydroxyvitamin D3; FSH: follicle stimulating hormone; E2: estradiol.
p values <0.05 represent significantly different means.

Table 2. Nutritional intakes of the 33 women at baseline compared to average intake for some nutrients as assessed using FoodWorks® , and as compared to the NZ Adult Nutritional Survey 2008/2009

Nutrients[†]	Mean	Standard deviation (SD)	Minimum	Maximum	NZ Data base[‡]	NZ women aged 51-70[§]
Energy (kcal)	1964	493	1300	3144	1848	1722
Protein (g)	82.1	18.5	49.1	121.1		71
Fat (g)	74.8	31.7	15.6	168.1		66
Saturated fatty acids (g)	28.5	16.9	8.2	84.6		24.6
Monounsaturated fatty acids (g)	23.3	10.4	6.7	49.3		24.8
Polyunsaturated fatty acids (g)	9.8	6.2	0.8	29.3		10.3
Cholesterol (mg)	218	119	52	480		222
Carbohydrate (g)	236	81	123	443		197
Vitamin B-1 (mg)	1.75	2.08	0.09	12.19		1.2
Vitamin B-2 (mg)	2.68	2.99	0.48	16.21		1.7
Vitamin B-6 (mg)	2.28	1.62	0.11	8.89		1.5
Vitamin B-12 (µg)	5.26	3.95	1.05	19.94		3.5
Vitamin C (mg)	142	95	36	589	142	108
Vitamin D (µg)	2.3	3.4	0.3	16.7		
Vitamin K (µg)	124	85	8	360		
Sodium (mg)	2476	898	845	4494		
Salt equivalent (g)	6.1	2.3	1.8	11.4		
Potassium (mg)	3150	826	1125	5119		2921
Calcium (mg)	848	388	260	1705	931	775
Magnesium (mg)	388	159	107	814	333	
Phosphorus (mg)	1314	404	465	2264	1490	
Iron (mg)	12.0	7.1	5.9	45.1		10.2
Zinc (mg)	10.8	5.1	3.2	29.7		9.1
Copper (mg)	1.30	0.53	0.35	2.68		
Dietary fiber (g)	13.1	6.4	1.1	28.4		18.7

[†]The kiwifruit was removed from the nutritional value calculation.
[‡]Spot checks comparing the intake of some nutrients by the women using the New Zealand Food Data base.
[§]The mean values for women at 50-70-year-old from the New Zealand Adult Nutritional Survey 2008/2009.

Dietary intake

For this analysis all 33 women were included as one group (Table 2). A few nutrients assessed using the New Zealand Food data base are included in table 2 as a comparison with the data from the Eiyo-kun software. In addition, intakes were compared to those reported by the NZ Adult Nutrition Survey (2008/2009) but not all calculated nutrients were reported in the NZ report. In general the intakes for all nutrients in our intervention group were higher than those reported in the survey.

Total energy intake was 1964 kcal (8217 KJ), which was higher than the intake recorded with the nutrition survey for women of this age. The mean calcium intake was 848±388 mg/day and the mean vitamin C intake was 142.2±95 mg/day, higher as compared to the results from the nutrition survey. Mean magnesium intake was 388±259 mg/day; the mean phosphorus intake was 1314.0±404 mg/day. Mean vitamin D intake was 2.3 µg/day and no comparison could be made with the average NZ intake as this was not reported by the survey. The mean vitamin K (K1 (phyloquinone) and K2 (MK-4)) intake was 124±85 µg/day, which is higher than the recommended daily intake of 90 µg/day for women in New Zealand. Addition of two green kiwifruit per day would have raised total vitamin K intake to 192 µg/day and vitamin C intake to about 200 mg/day.

Table 3. The Least Squares mean values (SEM) for the bone markers, lipid parameters, hormones and urinary isoflavones over time relative to treatment

Treatment	Time	Serum ucOC (ng/mL)	Serum CTx (µg/L)	Serum TC (mmol/L)	Serum HDL-c (mmol/L)	Urinary equol (µmol/24 h)	Log ratio of equol to daidzein
Isoflavone	Start	$5.82^{a,x}$	0.531	5.69^{b}	1.70^{b}	0.24^{a}	-0.71
	End	$6.60^{b,y}$	0.514	5.47^{a}	1.63^{a}	2.47^{b}	-1.70
	Difference	0.78	-0.017	-0.22	-0.07	2.23	-0.99
	SEM	0.34	0.028	0.13	0.05	0.69	0.10
Kiwifruit/ Isoflavone	Start	$6.24^{b,y}$	0.508	5.63	1.66	0.24^{a}	-0.82
	End	$5.34^{a,x}$	0.534	5.52	1.63	2.68^{b}	-1.66
	Difference	-0.90	0.026	-0.11	-0.02	2.44	-0.84
	SEM	0.34	0.028	0.13	0.05	0.68	0.10

unOC: undercarboxylated osteocalcin; CTx-1: C-telopeptide of Type I collagen; TC: total cholesterol; HDL-c: high density lipoprotein-cholesterol; SEM: standard error of the means.
Superscripts with different letters indicate significantly different means: a/b compares means within treatment; x/y compares means between treatments at the same time point (SEM).

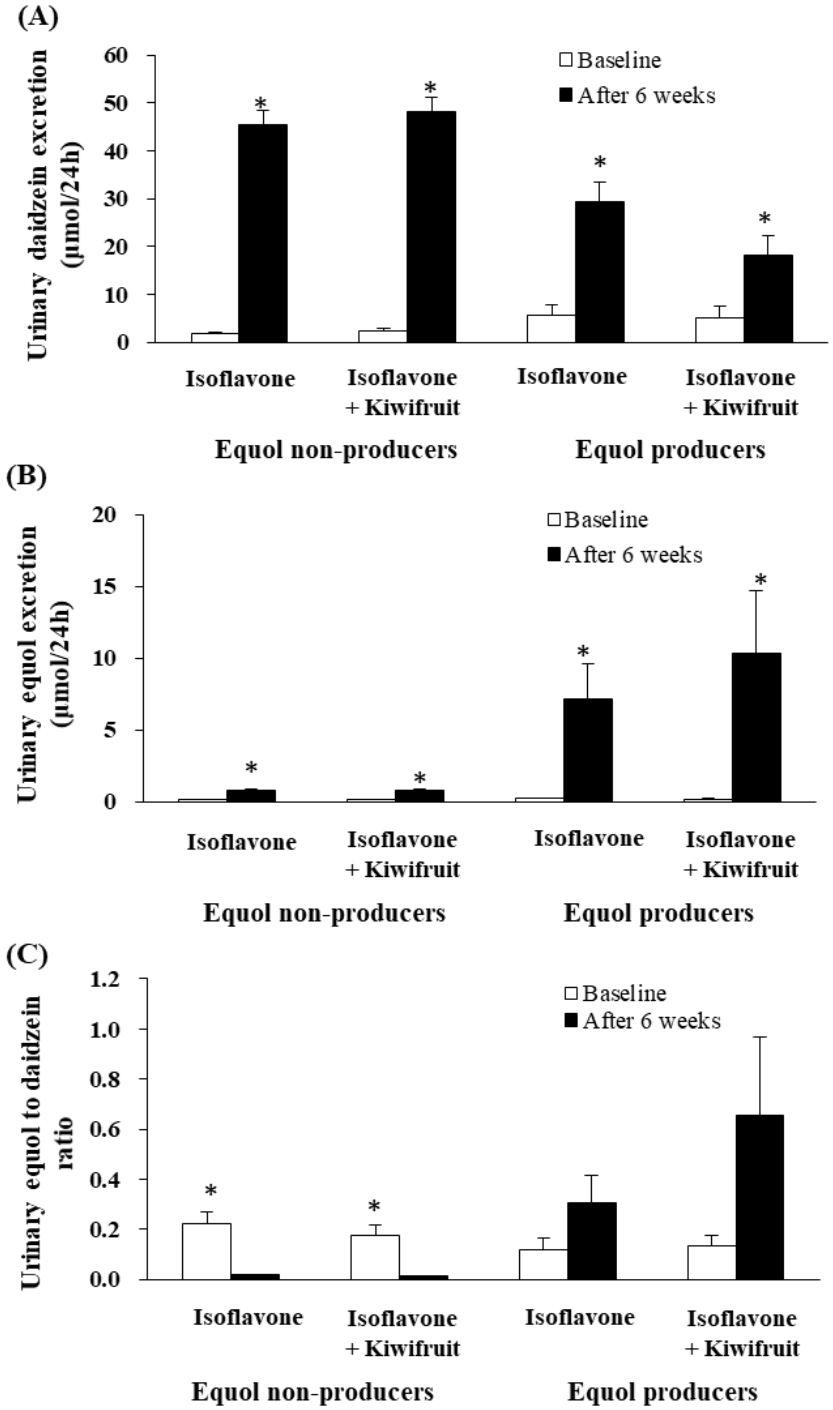

Figure 1. Urinary Daidzein and equol excretion after six weeks of supplementation with either isoflavones alone or isoflavones with two kiwifruit per day. Equol non-producer: Log (urinary equol/daidzein) <−1.70, n=22-3. Equol producer: Log (urinary equol/daidzein) >−1.70, n=10. Error bars represent the SEM; * represents a significant change (p <0.05).

Blood and urine analyses

Table 3 shows the baseline and week six values for the bone markers as well as serum lipids and equol production when the women are analysed together without taking equol production into account. Isoflavone supplementation, with or without kiwifruit increased urinary equol significantly (p<0.05). The data are shown as the response of all 33 women after consuming only the isoflavones supplement or after consuming the isoflavone supplement as well as two green kiwifruit per day. When the women were divided into equol producers (N=10) or non-producers (N=23), urinary daidzein increased both when supplemented only with isoflavones or with isoflavones plus kiwifruit (Figure 1A). The response in equol producers was less for daidzein concentration as the daidzein was then metabolised into equol (Figure 1B). The urinary ratio of equol to daidzein was lower in non-producers versus producers (Figure 1C).

There were no significant differences over time or between treatment groups for serum CTx (Table 3). Serum undercarboxylated osteocalcin (unOC) increased significantly with isoflavone supplementation (p<0.05) while it decreased significantly when isoflavone supplementation was combined with two green kiwifruit per day (Table 3

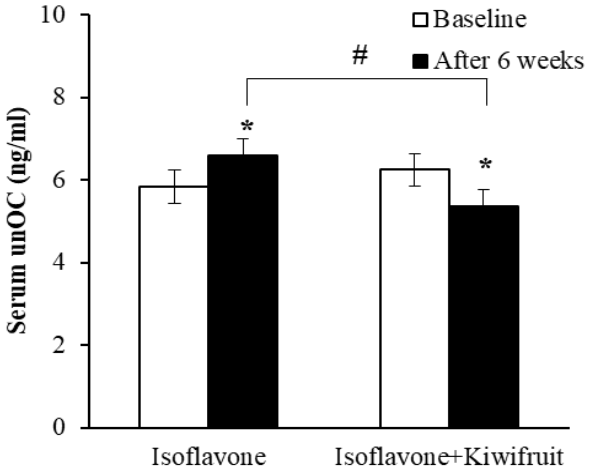

Figure 2. Bar graph showing the mean percentage change in serum ucOC over the treatment periods. Treatment: Iso = isoflavone; Kiwi = Kiwifruit/isoflavone. Error bars represent the SEM; * represents a significant change (p<0.05).

and Figure 2). Isoflavone only treatment, reduced HDL levels significantly but when combined with kiwifruit, this change was not significant.

There was a main effect of equol production on serum CTx independent of treatment. Serum CTx and LDL-c were significantly lower in equol producers compared to non-equol producers: CTx was 0.426 µg/L versus 0.563 µg/L and LDL-c was 3.27 mmol/L versus 3.45 mmol/L in equol producers versus to non-equol producers (data not shown).

Faecal microbiota analysis

Human intestinal microorganisms predominantly consist of the members of approximately nine phylogenetic bacterial groups. No statistically significant differences were observed in the composition of intestinal microbiota between equol producers and equol non-producers at baseline or after 6 weeks of isoflavone + kiwifruit intervention (Figure 3).

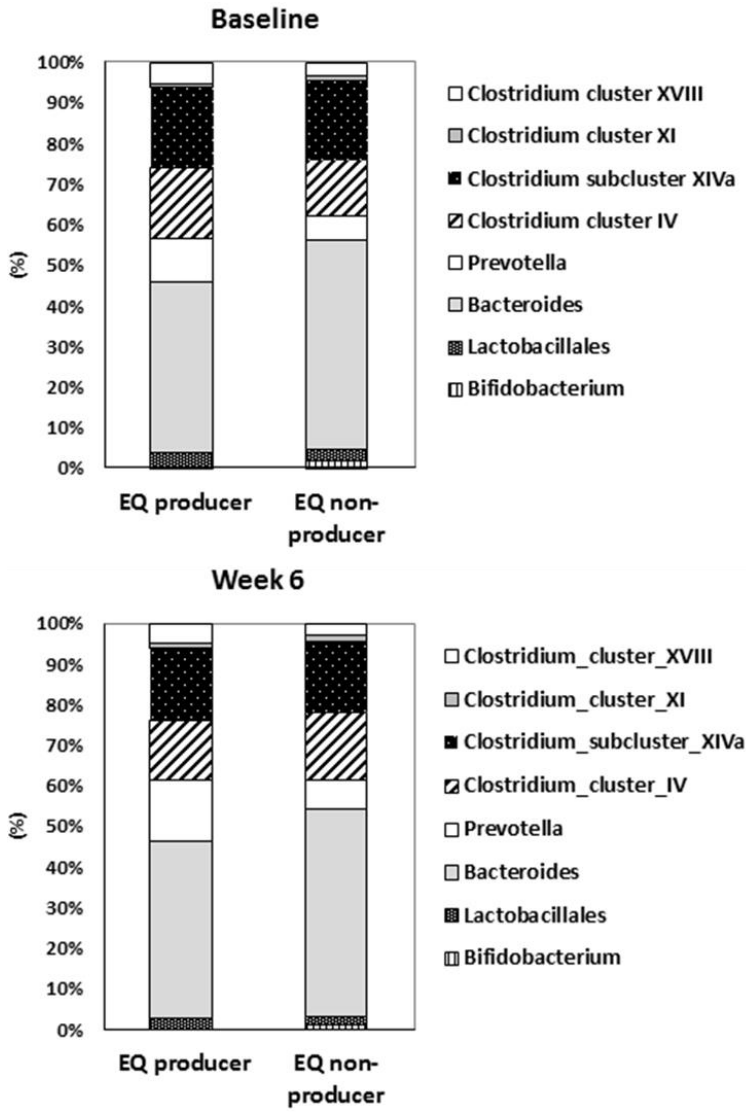

Figure 3. Changes in gut microbiota diversity over six weeks of kiwifruit and isoflavone consumption. Equol (EQ) producers: n=4, EQ non-producers: n=13. Data were analysed by Student t- test (p<0.05). There were no significant differences in the microbiota between EQ producers and EQ non-producers at baseline and week 6. There were no significant differences in the microbiata between baseline and week 6 for EQ producers and EQ non- producers.

DISCUSSION

The aim of this study was to determine the effect of short-term supplementation with aglycone isoflavones, daidzein and genistein, alone or in combination with two green kiwifruit, on biochemical markers of bone turnover, lipid profile and gut microbiota in healthy non-vegetarian postmenopausal women. Postmenopausal women were also assessed for equol-producer status and this was included as a main effect.

Bone resorption was not reduced by isoflavone supplementation, and there was also no significant treatment effect when differentiating between equol producers and non-producers; data not shown. The combined supplementation of kiwifruit and isoflavones decreased serum ucOC significantly, while the isoflavone supplement alone caused a significant elevation in serum ucOC. These changes were significant in both equol producers and non-equol producers when equol production was included as a main effect.

Isoflavones had no effect on the serum cholesterol or LDL-c levels but HDL-c reduced due to isoflavone supplementation. Urinary daidzein increased significantly in both equol and non-equol producers with both treatments, and equol production increased highly significantly in equol producers only with both isoflavone and combined treatment, however there was no additional effect of kiwifruit consumption on equol production (Figure 1).

Vitamin D status

Participants had an average serum vitamin D (25(OH)D) of 70.2±5.7 nmol/L; which is considered sufficient according to the guidelines of the Institute of Medicine (IOM).[47] The Endocrine Society, however, considers a serum level of >75 nmol/L as sufficient, which means most of the women who participated were insufficient.[48] Recent literature, albeit controversial, suggests that this serum vitamin D level is insufficient for the maintenance of bone health and reduced fracture risk;[49,50] insufficiency is defined as serum 25(OH)D between 51-75 nmol/L. Serum vitamin D has significant seasonal variation, due to the fluctuation in UV light levels, with the minimum serum 25(OH)D occurring during winter.[49-51] In this study, the blood samples for 25(OH)D analysis were taken in the second week of the intervention, during August, which is the final month of New Zealand's winter. Thus the vitamin D levels in this group of women would be at their lowest.

Changes in bone markers

This study reports no significant changes in bone resorption marker serum CTx-I (Table 3) in response to dietary intervention. The average levels of the markers were between 0.50 and 0.53 µg/L, which is higher than what was reported in a previous study. Valderas et al[52] reported serum CTx of 0.43±0.15 ng/mL in healthy postmenopausal women (57.5±4.7 years, n=27).

The current study is one of very few interventions that have measured the response of serum CTx to isoflavone supplementation in postmenopausal women. In two other studies, serum CTx did not respond to isoflavone supplementation[53,54] but these studies used pure genistein.

Osteocalcin (OC) is a vitamin K dependent protein produced by the osteoblasts and is the primary non-collagenous protein in bone. The function of OC is to regulate bone mineral maturation and for this function it needs to be gamma-carboxylated by vitamin K. Serum OC carboxylation increased significantly with the kiwifruit and isoflavone treatment: this was shown by the significant decline in serum ucOC (from 6.24 ng/L to 5.34 ng/mL) following the kiwifruit and isoflavone treatment (Figure 2). Conversely, there was a significant increase in ucOC during the isoflavone only treatment. The average baseline serum ucOC level was 6.07±0.226 ng/mL for participants of this study, which is similar to values reported by an isoflavone intervention in postmenopausal Irish women, 6.2±1.3 ng/mL (50-64 years),[55] and postmenopausal Japanese women, 6.50±4.9 ng/mL (mean age 58.9 years).[56]

Serum ucOC is a marker of vitamin K status: serum ucOC levels are inversely correlated to serum vitamin K.[57] Vitamin K1 acts as an essential cofactor for the enzymatic carboxylation of OC's glutamyl side chains. This carboxylation process is required for OC to bind to hydroxyapatite and modulate calcification. Undercarboxylation affects bone health: increased serum ucOC is correlated to increased fracture risk and reduced BMD of the femoral neck.[58,59]

By NZ and Australian standards, the adequate intake (AI) of vitamin K1 is set at 90 µg/day for women aged 51-70 years. Vitamin K1 supplementation has been shown to decrease serum ucOC over both short and long term interventions: a vitamin K1 fortified diet containing 420 µg/day decreased serum ucOC by 41% over five days,[60] whereas supplementing 1000 µg/day of vitamin K1 decreased serum ucOC by 48% over 12 months.[57] Binkley et al[57] suggest that an intake of 1000 µg/day induces maximal carboxylation of serum OC; the 1000 µg/day dose reduced serum ucOC by 74%, which is significantly greater than the alternative doses of 250, 376, and 500 µg/day.

The two green kiwifruit consumed daily during the kiwifruit/isoflavone treatment were equivalent to ~148 g serving (Zespri™ International, n.d.); accordingly the vitamin K1 content of green kiwifruit this serving would provide ~68.4 µg/day, which is 75% of the AI (90 µg/day) for vitamin K1 for this age group. Dietary intakes indicated a mean intake of 124 µg (this includes both K1 and K2) and with the additional vitamin K1 from kiwifruit (68.4 µg) total daily intakes could have been at 192 µg/day. The additional vitamin K content provided during the green kiwifruit treatment would have significantly increased serum vitamin K (not measured), which would have subsequently increased the carboxylation of OC. The significant effect on unOC as shown in Figure 2 could be due to the significantly increased intake of vitamin K1 due to addition of two green kiwifruit per day to the diet.

One potential effect on bone turnover not estimated in this study was the effect of the vitamin C content of kiwifruit on calcium absorption from the diet. Vitamin C has been shown to facilitate calcium absorption and retention in an animal study (Morel & Wolber, unpublished data, as cited in Wolber et al)[61] Given that consumption

of two green kiwifruit daily provided an additional 412-464% of the RDI for vitamin C, the kiwifruit and isoflavone treatment had the potential to increase the fractional absorption of intestinal calcium. However, serum calcium and PTH were not measured, so the effect of kiwifruit on calcium retention is unknown. Nonetheless, this effect seems unlikely as CTx-I did not exhibit a decrease in response to the kiwifruit and isoflavone treatment.

In this study there are several potential reasons for the observed lack of effect of isoflavone intervention on bone resorption markers. Firstly, the intervention duration may have been too short; meta-analysis by Taku et al[22,24,25] deemed that a duration of at least 10 weeks isoflavone supplementation was required to detect a statistically significant change in resorption markers. In addition, the equol producer subgroup (n=10, 30% of participants) was underpowered to detect a change in bone turnover markers. A sample size of 20 participants would be required to detect a statistically significant change in CTx (difference of at least 0.09 nmol/L).

Based on one previous study[32] that measured a significant decrease in urinary DPD in equol producers in response to equol supplementation, the serum equol level in the equol producers of this study may have been too low to elicit a significant change in bone turnover. The average serum equol obtained in the current study was 16.6 nmol/L, which is equivalent to 3.93 ng/mL, whereas in the study by Tousen et al[32] serum equol was 20.7 ng/mL in equol producers. Moreover Tousen et al[32] conducted a longer intervention with a larger sample size (12 months; n=23). Finally, the women in this study ranged from 1-10 years postmenopause, and based on serum E2 as well as FSH levels it is estimated that participants were on average 8-10 years postmenopausal. Bone turnover and bone loss is most rapid in the early postmenopausal phase (1-5 years since the onset of menopause) and isoflavone intervention has been shown to be more effective at preserving bone health in early postmenopausal women.[22] Thus the bone metabolism of the current study population may have been less responsive to an isoflavone intervention.

Equol production and green kiwifruit consumption

Serum equol in non-equol producers did not increase significantly from the baseline average of 3.11 nmol/L over both treatments (data not shown). The equol producers had an average serum equol level of 16.6 nmol/L after both treatments. Although the equol levels were slightly greater after the kiwifruit/isoflavone intervention compared to isoflavone alone, this difference was not significant (serum equol 18.9±2.91 nmol/L versus 13.4±2.91 nmol/L; mean±SEM). From these results it is apparent that kiwifruit had no effect on the equol-producing capacity of daidzein-metabolising bacteria in the participants who were equol producers. Urinary equol excretion by non-producers was not affected by supplementation by isoflavones or with kiwifruit (Figure 2B). In equol producers urinary equol increased significantly after 6 weeks supplementation with isoflavones. Addition of kiwifruit did not significantly affect urine equol. The log ratio of urinary equol to daidzein was used to delineate equol producers and non-producers: a ratio greater than -1.70 indicated an equol producer. This ratio is more accurate than measuring the absolute equol concentration because it accounts for the conversion of a specified dose of aglycone daidzein.[62] In this study, the log ratio of equol to daidzein did not change in the equol producers whereas the ratio declined in non-equol producers (Figure 1C). There was a non-significant trend for the ratio to increase in equol producers following the kiwifruit/isoflavone treatment. In equol producers kiwifruit consumption did not exert an additive effect on serum equol levels compared to the isoflavone only treatment; however, the trend for an increase in the ratio of equol to daidzein during the kiwifruit treatment may indicate that kiwifruit supplementation over a longer period could potentially modulate an increased activity of the equol-producing bacteria. This requires further investigation.

Faecal microbiota analyses

In the sub-group analysis, there were no significant changes in the diversity of the microflora after consumption of green kiwifruit for 6 weeks. The equol producers did not differ from non-producers at baseline or after 6 weeks. Tousen et al[43] reported a significant effect of fresh green kiwifruit on bone density in the ovariectomised (OVX) rat, but cecal microflora were not affected. Katsumata et al[63] reported significant effects by dietary intake of freeze dried kiwifruit on molecular markers of bone resorption in OVX mice, but feeding freeze dried green kiwifruit had no effect on the diversity of cecal microflora. In contrast a study in growing pigs fed with green kiwifruit reported modulation of colonic microbiota.[64] Studies in humans however have indicated that green kiwifruit may have a prebiotic effect by increasing Lactobacilli as well as Bifidobacteria while the fruit are being consumed.[65] The subgroup analyses in our study may have had too few subjects per group to be able to detect significant differences, and the possible prebiotic effect should be further investigated.

The completed study had limitations: Firstly, the duration of the study was not sufficient to test clinically relevant changes of bone metabolism. Secondly, this study was carried out with small populations in each group. Sample size calculation, performed before the start of the study, showed that 25 subjects in each group, a difference of 0.9 ng/mL between the means of serum ucOC concentration could be shown with the a power of 0.80 and two-side type 1 error of 0.05. However, there was significant difference in % change in serum ucOC between the two groups in this study. A second limitation of the study was that Japanese software was used to analyse the diets. Excel Eiyokun is based on standard tables of food composition in Japan. Therefore, nutrients of the food data base are slightly different from those in New Zealand. Software of food composition in New Zealand however contains limited data on the vitamin K content of New Zealand foods, therefore Excel Eiyokun was used in this study. This software however gives information on the combined intake of vitamin K1 (phyloquinone) and K2/MK-4, and consequently the exact intake of phyloquinone could not be calculated.

Conclusion

Long-term intake of isoflavones has been shown to im-

prove bone health, but there is a possibility that the effect is limited to individuals whose gut microbiota enable the metabolism of isoflavones into equol. In this study, post-menopausal women categorised as equol producers based on their urinary diadzein:equol log ratio metabolised more diadzein and produced more urinary equol than their non-producing counterparts, as expected. Supplementation with daidzein-containing isoflavone increased urinary daidzein in all participants, but urine levels of the daidzein metabolite equol increased only in equol producers, again as expected. Interestingly, equol producers had lower levels of serum CTx and LDL-c compared to non-producers at baseline, demonstrating that the capability to metabolise diadzein resulted in measurable health benefits.

In both producers and non-producers of equol, isoflavone supplementation reduced HDL-c but had no effect on levels of LDL-c, total cholesterol, or the bone resorption marker CTx. Kiwifruit supplementation did not change the gut microbiota profile. The absence of an effect on bone resorption and gut microbiota may be due to the interventions being of short duration and/or the participants being late post-menopausal and thus more refractory to intervention. However, isoflavone supplementation alone significantly increased serum ucOC, whereas isoflavone supplementation with kiwifruit had a beneficial effect by significantly decreasing serum ucOC.

We conclude from these findings that kiwifruit can significantly augment the bone health benefits of isoflavones by improving vitamin K status. It will be of interest to assess whether kiwifruit can further improve bone health and alter gut microbiota over a longer period of time.

AUTHOR DISCLOSURES

The authors declare no conflict of interest.

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