Effects of Soy Phytoestrogens and New Zealand Functional Foods on Bone Health

Kruger MC[1], Tousen Y[2], Katsumata S[3], Tadaishi, M[3], Kasonga AE[4], Deepak V[4], Coetzee M[4], Ishimi Y[2].

1) Institute of Food, Nutrition and Human Health, Massey University, New Zealand.

2) Department of Food Function and Labelling, National Institute of Health and Nutrition, Tokyo, Japan

3) Department of Nutritional Science, Faculty of Applied Bioscience, Tokyo University of Agriculture, Japan

4) Department of Physiology, Faculty of Health Sciences, University of Pretoria, South Africa

Corresponding author:

Prof MC Kruger

Institute of Food, Nutrition and Human Health

Massey University

Private Bag 11222

Palmerston north

New Zealand 4442

m.c.kruger@massey.ac.nz

Summary

New Zealand is a rich source of food components that may have bioactivity on

bone. Docosahexaenoic acid (DHA) from fish oil has been shown to maintain

bone in ovariectomised (OVX) rats. Kiwifruit, a source of fibre and carotenoids

may also affect bone via a prebiotic as well as direct cell based mechanisms.

We aimed to 1) ascertain the effects of DHA on two cell models, including interactions with soy isoflavones; 2) and investigate the specific effects of carotenoids from kiwifruit as well as whole kiwifruit in cell based and rodent models as well as in a human study. RAW 264.7 mouse monocytes or mouse bone marrow were used to generate osteoclasts (OC). Cells were exposed to the agents between 5 and 21 days and formation and activity of OC measured, including molecular markers. DHA inhibited OC formation in both cell models, including expression of cathepsin K, NFATc1 as well as actin ring formation. Combination with isoflavones enhanced these effects. In OVX rats and mice fed with kiwifruit for 8 weeks, green kiwifruit reduced rate of bone loss after OVX, and in mice it reduced C-telopeptide of Type 1 collagen (CTX) levels and RANKL expression while in menopausal women, green kiwifruit affected blood lipids and bone markers positively.

Introduction

Osteoporosis is a critical disorder involving bone loss due to estrogen deficiency in postmenopausal women. With the loss of estrogen, bone turnover increases while being uncoupled from the process of formation and the result is a net loss of bone. Hormone replacement therapy is most effective for the prevention of postmenopausal osteoporosis (1), however, hormone replacement therapy can cause adverse effects such as the development of hormone-dependent breast and uterine cancers (2). Soybean isoflavones have similar structures to those of estrogen and have a weak affinity for the estrogen receptors, specifically estrogen receptor β which is predominant in bone, brain, thymus, bladder and prostate (3). Genistein, one of the isoflavones, has been reported to have inhibitory effects on bone resorption *in vitro*, similar to estrogen which is known for suppressing bone resorption activity (4). We also demonstrated that soy

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isoflavones, such as daidzein, genistein, and equol, suppressed osteoclast

formation *in vitro* (5). A recent meta-analysis indicates that isoflavone

interventions significantly attenuate bone loss in postmenopausal women (6). In

addition, the risk for adverse effects of isoflavone treatment appears to be lower

than that of hormone replacement therapy (7). Therefore, intake of soybean

isoflavones can be considered as one of the alternative agents to hormone

replacement therapy and further research on prevention of bone loss or

maintenance of bone health in menopausal women is required. .

Daidzein is metabolized to equol in the intestine by gut microflora and equol

possesses a stronger estrogenic activity than daidzein (8). For example, equol

administration inhibited femoral bone loss in ovariectomized (OVX) mice without

estrogenic activity in the reproductive organs (9). Furthermore, we also

demonstrated that intake of equol-containing soy fermented food inhibited bone

resorption in postmenopausal women not producing equol (10). Therefore, the

clinical effectiveness of isoflavones, especially daidzein, is due the further

metabolism in the gut producing equol. The latter depends on various factors

including the microbiota diversity in the large intestine as well as diet and

ethnicity. In humans, only 30-50% of the population can produce equol (11-13).

Recent animal studies indicate that gut bacteria can be manipulated to modify

the metabolism of isoflavones in the intestine. Fructooligosaccharides (FOS)

increase the bioavailability of isoflavones, leading to cooperative effects in the

prevention of bone loss in OVX mice (14). Also, polydextrose and raffinose

stimulate equol production, and enhance the bone-protective effects of daidzein

in OVX mice (15). A small cross-over study in Japanese women supplemented

women with 37mg isoflavones plus/ minus 5g FOS per day for 2 weeks but

observed no effect of the prebiotic on metabolism of daidzein (16). Lampe et al.

reported that dietary fibre may promote the growth and/or the activity of

bacterial populations responsible for equol production (17). Thus, several

studies have examined the dietary fibre has been associated with ability of equol production.

The purpose of our research programme was to 1) investigate whether kiwifruit or omega 3 fatty acids have specific modulatory effects on bone cells in vitro, and to 2) investigate whether the metabolism of the isoflavones could be affected by selected foods including kiwifruit and omega 3 fatty acids.

Kiwifruit

Kiwifruit contain several components that may have bioactivity in bone. These include amongst others calcium (20-34mg/100g) and magnesium, as well as vitamins such as vitamin C (93-105mg/100g), vitamin K (0.006 – 0.04mg/100g) as well as dietary fibre (18).

A study in Caco-2 cell monolayers where the cells were pretreated with aqueous kiwifruit extracts, showed that gold kiwifruit extract significantly improved calcium uptake (18) with green kiwifruit having no effect. In an animal

feeding study using pigs aged 28 days, the animals were fed fresh kiwifruit at

15g of fruit/kg of body weight/day for 4 weeks. In this study green as well as

gold kiwifruit improved calcium retention significantly in comparison to an

ascorbic acid fed control group (18). This study indicated that vitamin C could

affect mineral uptake. Vitamin C is also essential for the formation of collagen

and the synthesis of hydroxyproline and few studies report a relationship

between vitamin C and bone density (BMD)(19). Other factors present in

kiwifruit may also affect mineral metabolism such as the carotenoids.

Epidemiological reports have shown an inverse relationship between carotenoid

intake and low BMD, risk of fracture and risk for developing osteoporosis (20).

An older in vitro study suggested that carotenoids may inhibit osteoclast

formation and therefore be bone protective. A recent study beta-carotene, lutein

as well as zeaxanthin suppressed osteoclast formation (5).

There is a significant amount of vitamin K in green kiwifruit specifically, up to

60% of the RDI. The requirement of sufficient vitamin K in the diet to support

bone health has been documented well over the past years (21). Osteocalcin is

a vitamin K dependent protein produced by osteoblasts during bone formation

and is the primary noncollageneous protein in bone. Osteocalcin functions as a

regulator of bone mineral maturation (21). The γ-carboxylation of osteocalcin is

the primary mechanism underlying the hypothesized protective influence of

vitamin K on bone. Vitamin K can also modulate certain cytokines involved in

bone turnover, such as osteoprotegerin and interleukin-6 (21), which may be an

additional mechanism by which vitamin K influences bone turnover. Kiwifruit is

also rich source of dietary fibre (22), and has been shown to have a prebiotic

effect of promoting the content of faecal lactobacilli and bifidobacteria in healthy

female adults (23). Furthermore, Han *et al.* reported that inclusion of kiwifruit

fibre in diets modulated the colonic bacterial community in pigs (24). In a rat

study we reported synergistic effects of daidzein and kiwifruit on bone in

ovariectomised (OVX) rats (25). The results showed that the combination of

daidzein with green kiwifruit reduced ovariectomy-induced decline in bone

mineral density (BMD) compared to the OVX control rats, but kiwifruit did not

affect equol production in the rats. In a further study we investigated the effects

of green and gold kiwifruit in the absence of daidzein on bone loss, bone

markers and molecular markers for bone formation/ resorption in OVX mice.

Seven-week old Balb/c-strain mice were either sham operated or OVXed and

then fed freeze dried kiwifruit as 3% of the diet for 8 weeks. The kiwifruit had no

prebiotic effects. Green kiwifruit significantly reduced levels of C-Telopeptide of

Type I collagen (CTX-1) in comparison to the OVX control mice while RANK-L

expression was significantly reduced in the mouse bones by green kiwifruit

(Katsumata et al, unpublished results).

The preliminary data obtained from a rat study (25) and the mouse study as

described above, prompted further investigation of an effect by green kiwifruit in

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menopausal women. In a cross-over design, we supplemented women aged

between 50 and 65 with 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. They were taking the

supplement for two series of six weeks separated by a washout of two weeks,

with our without two fresh green kiwifruit per day. Blood, urine and faecal

samples were collected at various time points and analysed for microflora

diversity, equol production, blood lipid levels and bone turnover markers.

Preliminary data indicate that the green kiwifruit in combination with the

isoflavones were bone protective (Kruger et al, 2014; unpublished data).

Omega 3 fatty acids

There is a substantial body of evidence that has accumulated over the past year

that dietary long chain polyunsaturated fatty acids (LCPUFAs) with a chain

length longer than 18C, are beneficial for bone health. Research over the past 20 years have suggested that PUFAs of the n-3 series, as well as the n-6 fatty acid gamma linolenic acid (GLA), may prove beneficial to bone health when consumed in appropriate amounts (26). In addition, it has been shown that a reduction of the n-6/n-3 PUFA ratio could result in increased bone strength in animals and reduce bone loss in humans (27 - 29).

PUFAs are divided into two classes according to their structure. The longer chain PUFAs can originate from the dietary 18-carbon precursors α-linolenic (ALA,18:3n-3) and linoleic acids (LA,18:2n-6) respectively and these are considered to be essential fatty acids as they cannot be synthesised by human tissue (29). Through a process of desaturation and elongation, the longer chain PUFAs are synthesised from these precursors of which the most important ones, with regards to bone health, are arachidonic acid (AA)(20:4n-6), eicosapentaenoic acid (EPA)(20:5n-3) and docosahexaenoic acid

(DHA)(22:6n-3). During the past 10 years several authors have reviewed

various studies investigating the effects of PUFAs on bone health in humans

and animals (29, 30)

Rahman et al. (31) reported that in RAW 264.7 cells, n-3 PUFAs reduce

NF-kappa-β expression and modulate RANKL signalling whilst n-6 PUFAs such

as AA increase NF-kappa β expression leading to increased

osteoclastogenesis. The observed inhibition correlated with inhibition of several

osteoclast-specific genes such as TRAP, cathepsin K, c-FOS as well as TNF-α.

Pretreatment of these cells with DHA caused reduced activation of NF-kappa-β

and p38MAPK compared to EPA. DHA therefore may be much more effective

than EPA in alleviating RANKL induced proinflammatory cytokine production.

We recently assessed the effects of EPA, DHA, GLA and AA on

osteoclastogenesis using the RAW 264.7 cell model. The cells were treated

with or without 5-20µg/mL of each PUFA in the presence of RANKL to assess

osteoclast differentiation using tartrate-resistant staining (TRAP). All the PUFAs

inhibited RANKL-induced osteoclast differentiation with the strongest effect

observed for DHA and AA (32). Actin ring formation was significantly reduced

by AA and in the presence of DHA actin rings were completely absent.

Investigation of gene expression using RT-PCR and western blot analyses

indicated that both AA and DHA suppress mRNA expression of both cathepsin

K and TRAP but not matrix metalloproteinase with the effect of DHA stronger

that AA (32). These results therefore confirmed that DHA and to a lesser extent,

AA, suppress osteoclast formation, structure and activity by downregulating

expression of genes controlling bone resorption. More recent work also

indicated that DHA at the concentrations of 30μM, is able to significantly

suppress expression of NFATc-1 and TRAP in mouse bone marrow cells

(Katsumata et al, unpublished data). These results are in agreement with those

of Rahman et al (31) as well as those described by Sun et al (33) who reported

an inhibitory effect of both DHA and EPA on osteoclasts using bone marrow culture.

We also investigated the effects of the soy isoflavones, genistein and daizein, on osteoclast formation and the possible interactions with DHA using mouse bone marrow cultures. DHA suppressed the number of TRAP positive mononucleated cells (TRAP(+)MNCs) as well as TRAP activity in a stepwise manner. Individually, daidzein as well as genistein between 0.1 and 10µM, significantly inhibited TRAP activity but both only had a significant inhibitory effect on the number of mononucleated cells at 10µM. When 20µM DHA was combined with either 10µM genistein or daidzein, the inhibitory effects of genistein and daidzein were enhanced by DHA (Figure 1)(Katsumata et al, unpublished data).

| | Factors | | Interactions |
	Soy isoflavones	DHA	Soy isoflavones × DHA
Control vs Daidzein	p<0.001	p<0.001	p<0.01
Control vs Genistein	p<0.001	p<0.001	p<0.05

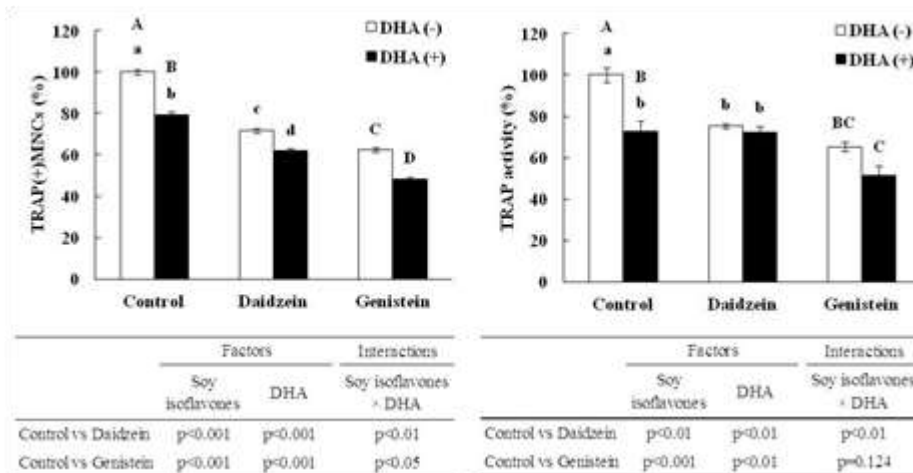| | Factors | | Interactions |
	Soy isoflavones	DHA	Soy isoflavones × DHA
Control vs Daidzein	p<0.01	p<0.01	p<0.01
Control vs Genistein	p<0.001	p<0.01	p=0.124

Figure 1. Combined effect of soy isoflavones and DHA on osteoclast formation in mouse bone marrow cell culture. Bone marrow cells were pre-cultured with M-CSF (30 ng/ml) for 3 days and then cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the presence or absence of 10 µM daidzein or genistein with or without 20 µM DHA for 6 days. Data are expressed as mean ± SEM of 3 independent experiments. Different letters indicate significant differences, p<0.05 (Small letters: Control vs Daidzein, Capital letters: Control vs Genistein).

Conclusion

Long chain fatty acids have significant effects on reducing osteoclastogenesis and disrupting function of the osteoclast and daidzein and genistein enhanced the effects. Green kiwifruit modulate molecular markers of bone resorption and in older women may be bone protective.

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