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Combined Effects of Soy Isoflavones and Docosahexaenoic Acid on Osteoclast Formation

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Abstract Soy isoflavones and docosahexaenoic acid (DHA) are effective for maintaining bone health. This study investigated the combined effects of soy isoflavones and DHA on osteoclast formation. Mouse bone marrow cells were pre-cultured with macrophage colony-stimulating factor (M-CSF) for 3 days and then cultured with M-CSF and receptor activator of nuclear factor κB ligand (RANKL) for 6 days. RAW 264.7 cells were cultured with RANKL for 5 days. In mouse bone marrow cells, daidzein, genistein, and DHA significantly decreased the number of tartrate-resistant acid phosphatase-positive multinucleated cells (TRAP(+)MNCs), and the combination of soy isoflavones and DHA further decreased the number of TRAP(+)MNCs. Nuclear factor of activated T-cells c1 (NFATc1) mRNA expression tended to be decreased by daidzein, and was significantly decreased by genistein and DHA. Furthermore, the combination of daidzein and DHA caused significant reduction in NFATc1 mRNA expression compared to the control. In RAW 264.7 cells, daidzein tended to decrease and genistein significantly decreased the number of TRAP(+)MNCs, however, the combination of daidzein and DHA significantly decreased the number of TRAP(+)MNCs compared to the control. These results demonstrated that the combination of soy isoflavones and DHA decreased in osteoclast formation significantly, possibly by modulating the expression of specific genes.

Keywords: *soy isoflavones, docosahexaenoic acid, osteoclast formation, mouse bone marrow cells, RAW 264.7 cells*

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1. Introduction

Osteoporosis is one of the life style-related diseases, and estrogen deficiency increases the risk of bone fracture in postmenopausal women. Therefore, many drugs have been developed to prevent osteoporosis, and hormone replacement therapy is the most effective approach for preventing postmenopausal osteoporosis. However, this therapy increases the risk of breast and uterine cancer [1]. Thus, many researchers have examined its prevention by various functional foods, and some functional food components have to be considered as potentially effective ingredients for bone health.

Soy isoflavones, such as daidzein and genistein, have a structure similar to that of estrogen, and have a weak estrogenic activity. Many studies using rats and mice have showed that soy isoflavones have a preventive effect on osteoporosis [2,3,4]. Furthermore, it has been reported that soy isoflavones inhibit osteoclast formation in cultured cells [5,6,7]. Therefore, soy isoflavones are considered as useful dietary factors for prevention of osteoporosis.

Fish oil is also known to have a preventive effect on bone loss. Previous studies demonstrated that dietary fish oil inhibited bone loss in ovariectomized (OVX) mice [8] and suppressed an increase in osteoclast number in OVX rats [9]. Furthermore, fish oil maintained bone mineral density in aging female mice by lower osteoclast generation [10]. These observations suggested that fish oil inhibited osteoclastic bone resorption and prevented bone loss. Fish oil is abundant in n-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA). It was reported that DHA ameliorated OVX-induced bone mineral loss in rats [11]. Furthermore, there are some reports that DHA inhibits osteoclast formation *in vitro* [12,13,14]. Therefore, it seems that n-3 PUFAs, especially DHA, has an inhibitory effect on bone resorption.

Previous research examined the combined effects of soy isoflavones and fish oil on bone loss in OVX animals [15,16,17]. These results showed that combined intakes of soy isoflavones and fish oil attenuated bone loss in OVX animals. However, there have been no studies on the combined effects of soy isoflavones and DHA. The purpose of this study was to evaluate the combined effects

of soy isoflavones and DHA on osteoclast formation and related molecular markers using mouse bone marrow cell culture. Furthermore, we also tested the combined effects of soy isoflavones and DHA on osteoclast formation using RAW 264.7 cell culture.

2. Materials and Methods

2.1. Bone Marrow Cell Culture

This study was approved by the Massey University Animal Ethics Committee (approval number 13/30). Mice bone marrow cell culture was isolated from the femurs and tibias of 7- to 9-week-old male Balb/c mice, as described previously [18]. Femurs and tibias were cleansed of all soft tissues. The bone marrow cavity was flushed out using culture medium expelled from a syringe through a 25.5-gauge needle. Cells were seeded in a 48-well plate (Corning Incorporated, Corning, NY, USA) at a density of 2.0×10^5 cells/well and pre-cultured for 3 days in phenol red-free α-MEM (Life Technologies, Carlsbad, CA, USA) containing 10% heat inactivated fetal bovine serum (FBS; Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin-streptomycin (Life Technologies), and 30 ng/ml recombinant mouse macrophage colony-stimulating factor (M-CSF; R&D systems, Minneapolis, MN, USA). After pre-culture, the cells were further cultured in the medium containing 30 ng/ml recombinant mouse receptor activator of nuclear factor κB ligand (RANKL; R&D systems) for osteoclast differentiation, and then soy isoflavones and/or DHA were supplemented into the medium. Daidzein (LC Laboratories, Woburn, MA, USA), genistein (LC Laboratories), and DHA (Sigma-Aldrich, St. Louis, MO, USA) were prepared in ethanol and solutions were stored in the dark at −80°C until required. The final ethanol concentration in the culture medium did not exceed 0.5%. The cell cultures were maintained at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

2.2. Tartrate-Resistant Acid Phosphatase (TRAP) Stain and Measurement of TRAP Activity

After 6 days of culture, cells were stained for TRAP and TRAP activity in the medium was measured using a commercial kit (Sigma-Aldrich). Briefly, cells were fixed for 30 s in a fixative solution (25 ml citrate solution, 65 ml acetone and 8 ml of 37% formaldehyde) and washed 3 times with Milli-Q water. Then, cells were stained for 1 h at 37 °C using the TRAP solution (mixture of provided reagents) according to the manufacturer's instruction, followed by counterstaining with hematoxylin solution. TRAP-positive multinucleated cells (TRAP(+)MNCs) were counted under a light microscope, and the number of TRAP(+)MNCs was expressed as percent of the control. For measurement of TRAP activity in the medium, 30 µl cultured medium and 170 µl TRAP solution were mixed in a 96-well plates. The plates were incubated for 3 h at 37 °C in the darkness. The absorbance was measured at 550 nm with ELx808 Ultra Microplate Reader, and the TRAP activity was expressed as percent of TRAP activity of the control.

2.3. Isolation of Total RNA and Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

After 6 days of culture, cells were dissolved using TRIzol reagent (Life Technologies) by repetitive pipetting. Total RNA was isolated from the lysate by using Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instruction. The cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies) and was subjected to quantitative real-time RT-PCR using SYBR Select Master Mix (Life Technologies). Specific primers [19,20] were used for the analysis of nuclear factor of activated T-cells c1 (NFATc1) (forward, 5'-CCGTGCTTCCAGAAAATAACA-3'; reverse, 5'-TGTG GGATGTGAACTCGGAA-3'), TRAP (forward, 5'-CTA CCTGTGTGGACATGACCA-3'; reverse, 5'-GCACATA GCCCACACCGTTC-3'), and β-actin (forward, 5'-TGAC AGGATGCAGAAGGAGA-3'; reverse, 5'-CGCTCAGG AGGAGCAATG-3'). Real-time RT-PCR was performed on a LightCycler 480 System (Roche, Mannheim, Germany). The mRNA expression was normalized to β-actin mRNA as a housekeeping gene.

2.4. Cell Culture for RAW 264.7 Cells

RAW 264.7 cells were maintained in phenol red free DMEM (Sigma-Aldrich) containing 10% heat inactivated FBS and 1% Antibiotic-Antimycotic (Life Technologies). RAW 264.7 cells were stimulated to differentiate into osteoclasts with RANKL as described previously [21]. Briefly, cells were seeded in a 24-well plate (Corning Incorporated) at a density of 2.0×10^4 cells/well and cultured in the medium containing 15 ng/ml recombinant mouse RANKL (R&D systems) for osteoclast differentiation, and then soy isoflavones and/or DHA were supplemented into the medium. The medium was changed at day 3, and the cells were stained for TRAP at day 5. TRAP staining and TRAP(+)MNCs counting were performed in the same manner as described for bone marrow cells above.

2.5. Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM) of 3 independent experiments with three replicates in each experiment. Data were analyzed by one-way or two-way analysis of variance (ANOVA). Where significant effects were found, post hoc analysis using Tukey's test was performed. Differences were considered to be significant when the p value was less than 0.05. Statistical analysis was performed using IBM SPSS statistics 21 (Chicago, IL, USA).

3. Results

3.1. Effect of Soy Isoflavones on Osteoclast Formation in Mouse Bone Marrow Cell Culture

We performed the dose-response effects of daidzein and genistein on the number of TRAP(+)MNCs and TRAP activity (Figure 1). Compared to the control, 1 µM and 10 µM of daidzein significantly decreased the number

of TRAP(+)MNCs. Likewise, 1 μM and 10 μM of genistein significantly decreased the number of TRAP(+)MNCs compared to the control. TRAP activity was significantly lower in the daidzein- and genistein-treated groups at all concentrations compared to the control, however, dose-dependent effect of soy isoflavones was not observed in TRAP activity.

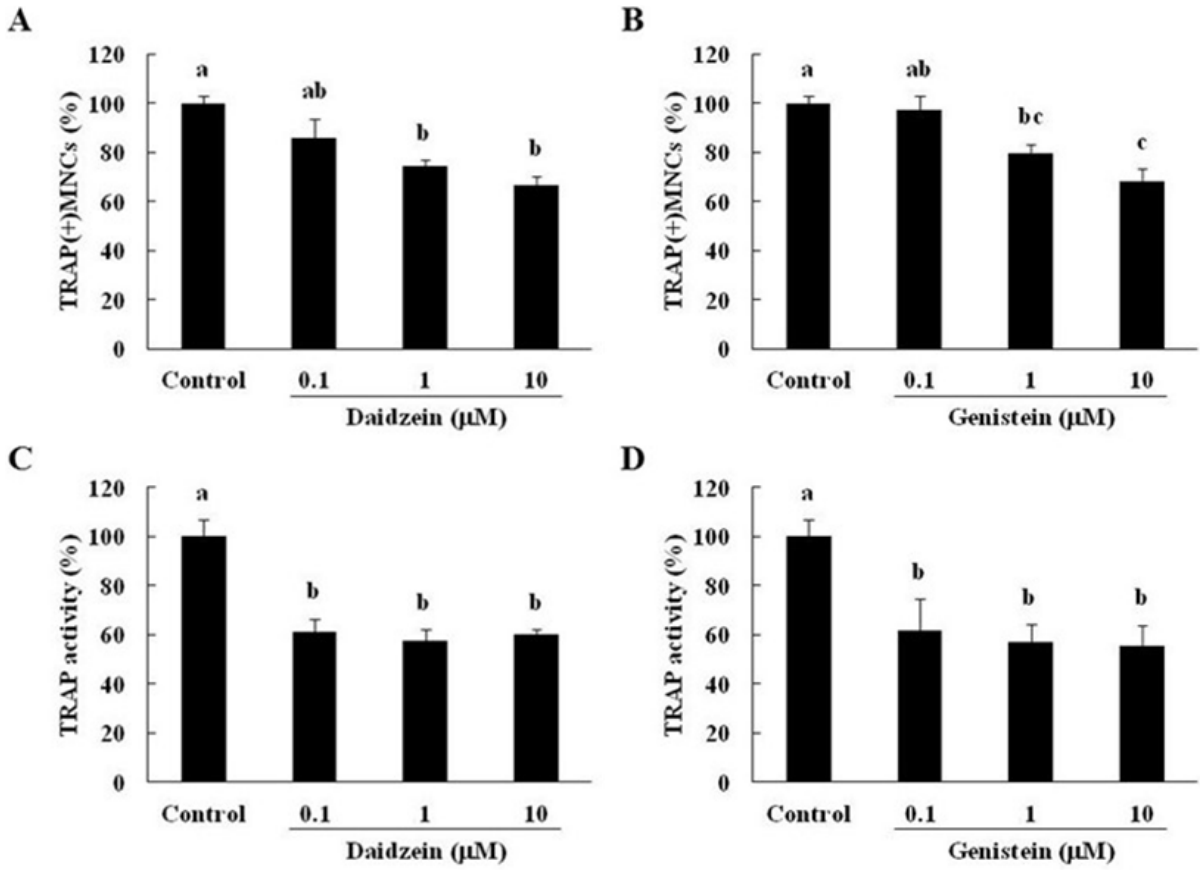


Figure 1. Effect of soy isoflavones on osteoclast formation in mouse bone marrow cell culture. Bone marrow cells were pre-cultured with M-CSF (30 ng/ml) for 3 days and then cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the presence or absence of different concentrations of daidzein or genistein for 6 days. (A) TRAP(+)MNCs for daidzein treatment; (B) TRAP(+)MNCs for genistein treatment; (C) TRAP activity for daidzein treatment; (D) TRAP activity for genistein treatment. Data are expressed as mean ± SEM of 3 independent experiments. [a,b,c]Means sharing the same letters are not significantly different, $p < 0.05$

3.2. Effect of DHA on Osteoclast Formation in Mouse Bone Marrow Cell Culture

We next tested the dose-response effects of DHA on the number of TRAP(+)MNCs and TRAP activity (Figure 2). DHA caused a significant decrease in the number of TRAP(+)MNCs in a dose-dependent manner. TRAP activity was also significantly lower in the DHA-treated groups at all concentrations compared to the control, and 30 μM DHA significantly decreased TRAP activity compared to 10 μM DHA.

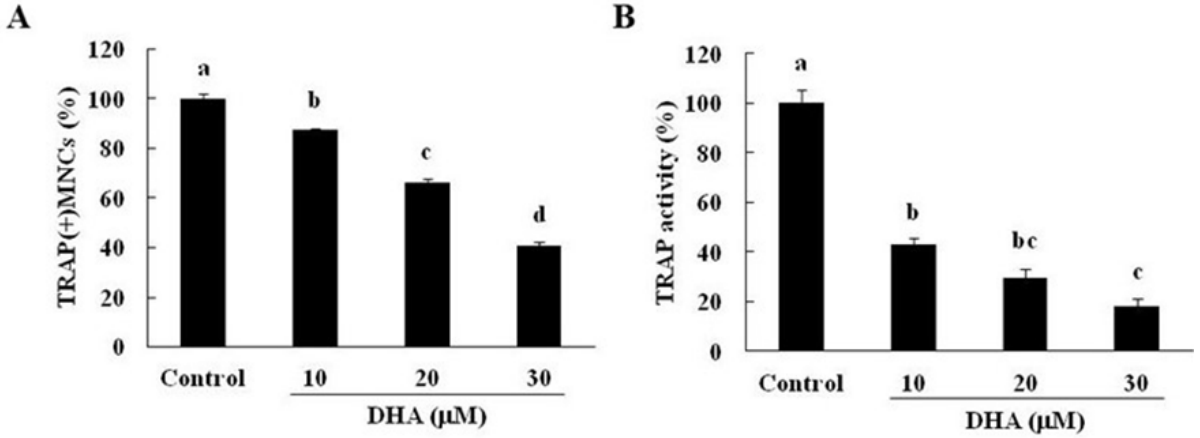
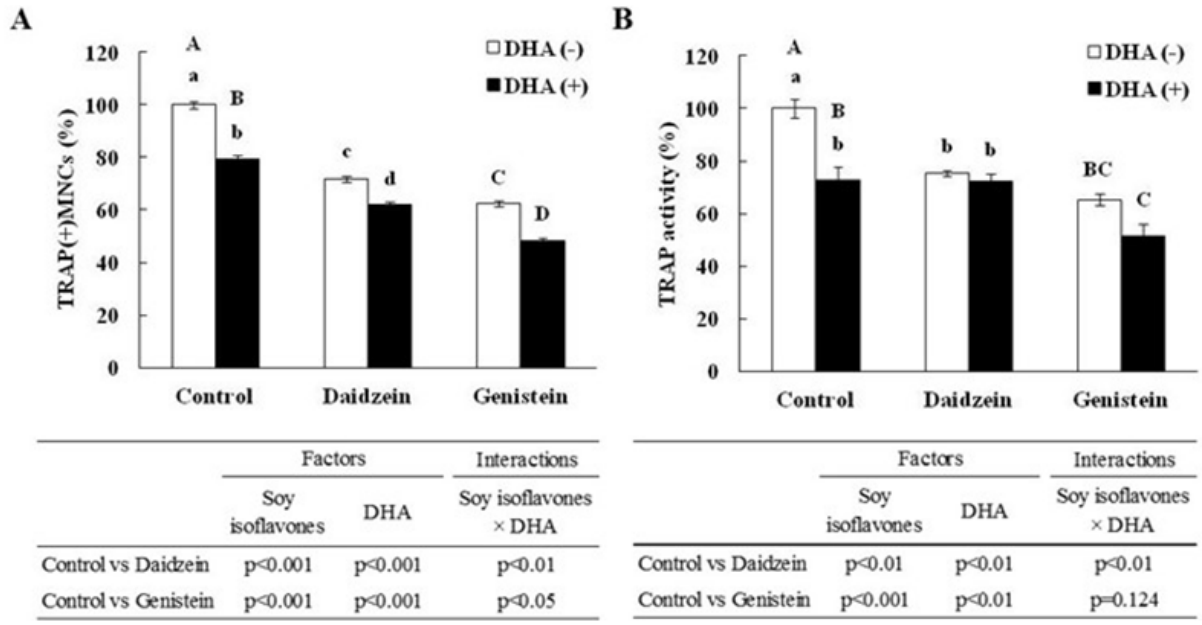


Figure 2. Effect of DHA on osteoclast formation in mouse bone marrow cell culture. Bone marrow cells were pre-cultured with M-CSF (30 ng/ml) for 3 days and then cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the presence or absence of different concentrations of DHA for 6 days. (A) TRAP(+)MNCs for DHA treatment; (B) TRAP activity for DHA treatment. Data are expressed as mean ± SEM of 3 independent experiments. [a,b,c,d]Means sharing the same letters are not significantly different, $p < 0.05$

3.3. Combined Effect of Soy Isoflavones and DHA on Osteoclast Formation in Mouse Bone Marrow Cell Culture

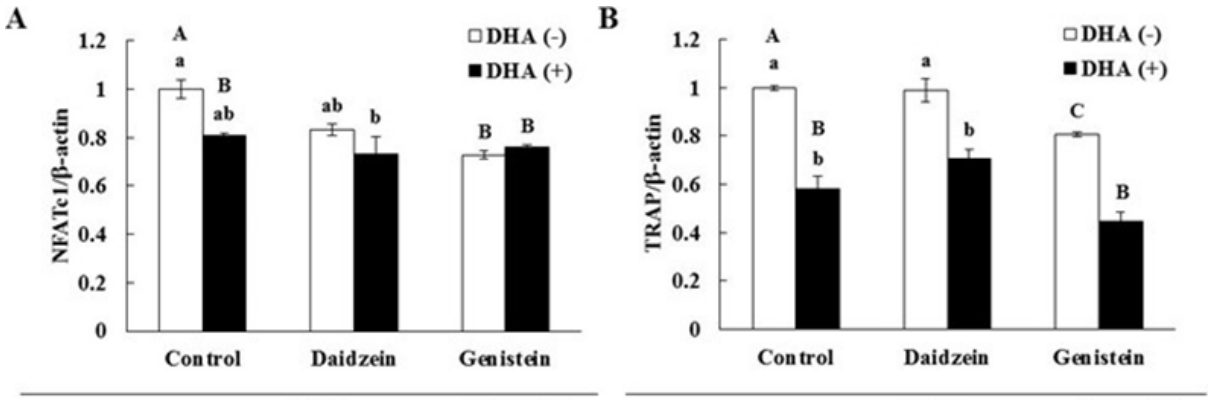
Previous study [18] and this study showed that 10 μM soy isoflavones clearly decreased the number of TRAP(+)MNCs. Furthermore, there was no significant inhibition of cell proliferation by DHA treatment up to 50 μM concentration [12]. We also tested the combined effect of 10 μM soy isoflavones and 20 μM DHA on the number of TRAP(+)MNCs and TRAP activity (Figure 3). Each of daidzein, genistein, and DHA significantly decreased the number of TRAP(+)MNCs and TRAP activity. The combination of soy isoflavones and DHA treatment further decreased the number of TRAP(+)MNCs.



A	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

B	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 3. 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. (A) TRAP(+)MNCs for combination of isoflavones and DHA treatment; (B) TRAP activity for combination of isoflavones and DHA treatment. Data are expressed as mean ± SEM of 3 independent experiments. Means sharing the same letters are not significantly different, $p < 0.05$ (^{a,b,c,d}Small letters: Control vs Daidzein, ^{A,B,C,D}Capital letters: Control vs Genistein)



A	Factors		Interactions
	Soy isoflavones	DHA	Soy isoflavones × DHA
Control vs Daidzein	p<0.05	p<0.05	p=0.294
Control vs Genistein	p<0.001	p<0.01	p<0.01

B	Factors		Interactions
	Soy isoflavones	DHA	Soy isoflavones × DHA
Control vs Daidzein	p=0.209	p<0.001	p=0.132
Control vs Genistein	p<0.01	p<0.001	p=0.399

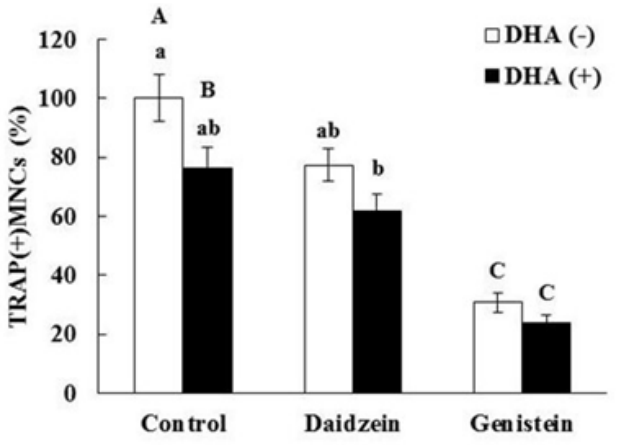
Figure 4. Combined effect of soy isoflavones and DHA on NFATc1 and TRAP mRNA expression in mouse bone marrow cell culture. Bone marrow cells were pre-cultured with M-CSF (30 ng/ml) for 3 days and then cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the presence or absence of 10 μM daidzein or genistein with or without 20 μM DHA for 6 days. (A) NFATc1; (B) TRAP. Data are expressed as mean ± SEM of 3 independent experiments. Means sharing the same letters are not significantly different, $p < 0.05$ (^{a,b}Small letters: Control vs Daidzein, ^{A,B,C}Capital letters: Control vs Genistein)

The combined effect of 10 μM soy isoflavones and 20 μM DHA on mRNA expression of NFATc1 and TRAP are shown in Figure 4. Compared to the control, daidzein tended to decrease (p=0.09) and genistein and DHA significantly decreased NFATc1 mRNA expression. Furthermore, the combination of daidzein and DHA significantly decreased NFATc1 mRNA expression compared to the control. There was no significant difference in NFATc1 mRNA expression between the soy isoflavone-treated groups and the combination of soy

isoflavones and DHA groups. Daidzein treatment has no significant effect on TRAP mRNA expression. DHA treatment significantly decreased TRAP mRNA expression compared to the control. TRAP mRNA expression was significantly lower in the genistein-treated group than in the control, and was significantly lower in the combination of genistein and DHA group than in the genistein-treated group.

3.4. Combined Effect of Soy Isoflavones and DHA on Osteoclast Formation in RAW 264.7 Cell Culture

We further confirmed the combined effect of 10 μM soy isoflavones and 20 μM DHA on the number of TRAP(+)MNCs in RAW 264.7 cells (Figure 5). Compared to the control, the number of TRAP(+)MNCs tended to be lower in the daidzein-treated group (p=0.08), and was significantly lower in the genistein- and DHA-treated groups. Furthermore, the combination of daidzein and DHA significantly decreased the number of TRAP(+)MNCs compared to the control. There was no significant difference in the number of TRAP(+)MNCs between the soy isoflavone-treated groups and the combination of soy isoflavones and DHA groups.



	Factors		Interactions
	Soy isoflavones	DHA	Soy isoflavones × DHA
Control vs Daidzein	p<0.01	p<0.01	p=0.561
Control vs Genistein	p<0.001	p<0.01	p=0.147

Figure 5. Combined effect of soy isoflavones and DHA on osteoclast formation in RAW 264.7 cell culture. RAW 264.7 cells were cultured with RANKL (15 ng/ml) in the presence or absence of 10 μM daidzein or genistein with or without 20 μM DHA for 5 days. Data are expressed as mean ± SEM of 3 independent experiments. Means sharing the same letters are not significantly different, $p < 0.05$ (^{a,b}Small letters: Control vs Daidzein, ^{A,B,C}Capital letters: Control vs Genistein)

4. Discussion

It has been previously reported that genistein inhibited parathyroid hormone-, prostaglandin E_2-, 1,25-dihydroxyvitamin D_3-, or lipopolysaccharide-induced osteoclast-like cell formation in mouse bone marrow cell culture [5]. Other studies showed that daidzein and genistein inhibited TNF-α-induced bone resorption in RAW 264.7 cells [6]. Furthermore, we previously reported that daidzein and genistein suppressed RANKL-induced osteoclast formation in RAW 264 cells and mouse bone marrow cells [18]. Similar to our previous study, daidzein and genistein significantly decreased TRAP(+)MNCs in bone marrow cell culture (Figure 1) and daidzein tended to decrease (p=0.08) and genistein significantly decreased TRAP(+)MNCs in RAW 264.7 cell culture (Figure 5) in the present study. Thus, the data suggest that soy isoflavones have an inhibitory effect on osteoclast formation.

Because soy isoflavones can bind to the estrogen receptors [22], one of the mechanisms by which soy isoflavones could inhibit osteoclast formation is considered to be related to their estrogenic action. NFATc1 mediates osteoclast differentiation as the master transcriptional factor of RANKL-induced osteoclastogenesis [23,24,25]. It was reported that soy isoflavones inhibited TNF-α-induced bone resorption in RAW 264.7 cells by suppressing NFATc1 expression [6]. Our previous study also showed that soy isoflavones suppressed NFATc1 mRNA expression in RAW 264 cells [18]. In the present study, daidzein tended to decrease (p=0.09) and genistein significantly decreased NFATc1 mRNA expression in RANKL-induced mouse bone marrow cell culture (Figure 4). Consequently, a decrease in NFATc1 mRNA expression as a result of soy isoflavones treatment could reflect the result of a decreased the number of TRAP(+)MNCs observed in the present study. A previous report has shown that TRAP is regulated by NFATc1 [23], and TRAP is known to be involved in resorption activity. This study showed that genistein decreased TRAP mRNA expression, which was reflected in the result of NFATc1 mRNA expression in the present study. However, daidzein did not change TRAP mRNA expression, whereas the number of TRAP(+)MNCs, TRAP activity, and NFATc1 mRNA expression were reduced by daidzein treatment. The reasons for this discrepancy are not clear, and detailed examinations are necessary to clarify.

DHA also has a protective effect on bone loss. A previous study using OVX rats showed that DHA ameliorated OVX-induced bone mineral loss [11]. This preventive effect of DHA on bone loss might be explained by the action of DHA on the osteoclasts. Rahman et al. reported that DHA inhibited osteoclast differentiation with a decrease in TNF-α secretion in RAW 264.7 cells [12]. Also, Boeyens *et al.* reported that DHA inhibited RANKL-mediated osteoclast formation in RAW 264.7 cells [14]. In the present study, DHA caused a significant decrease in the number of TRAP(+)MNCs in a dose-dependent manner (Figure 2). Furthermore, NFATc1 and TRAP mRNA expression were significantly lower in the DHA-treated group than in the control (Figure 4). Therefore, these results suggested that DHA inhibited NFATc1 mRNA expression, which caused decreases in osteoclast formation and TRAP mRNA expression in RANKL-induced bone marrow cell culture. Recently, Akiyama *et al.* also reported that NFATc1 gene expression was stimulated by RANKL and downregulated by DHA [26]. However, the mechanism by which DHA inhibits NFATc1 mRNA expression is unclear and further work is required.

The combination of soy isoflavones and DHA treatment further decreased the number of TRAP(+)MNCs in bone marrow cell culture. In RAW 264.7 cells, the combination

of daidzein and DHA significantly decreased the number of TRAP(+)MNCs compared to the control, whereas daidzein tended to decrease the number of TRAP(+)MNCs. These results suggested that the combination of soy isoflavones and DHA enhanced a reduction in osteoclast formation. Compared to the control, daidzein tended to decrease and genistein significantly decreased NFATc1 mRNA expression, although the combination of daidzein and DHA significantly decrease NFATc1 mRNA expression in bone marrow cell culture. Therefore, these results showed that the combination of daidzein and DHA reduced NFATc1 mRNA expression, leading to a reduction in osteoclast formation. However, the combination of genistein and DHA did not further decrease NFATc1 mRNA expression in this study. Furthermore, this study showed that TRAP mRNA expression was decreased by genistein and DHA, whereas a further inhibitory effect by the combination of genistein and DHA was not observed. Thus, it appeared that combination of soy isoflavones and DHA reduced osteoclast formation, in part, by a decrease in NFATc1 mRNA expression.

5. Conclusion

We investigated the combined effects of soy isoflavones and DHA on osteoclast formation in bone marrow cells and RAW 264.7 cells. Daidzein, genistein, and DHA decreased the number of TRAP(+)MNCs, respectively. In addition, the combination of soy isoflavones and DHA further decreased the number of TRAP(+)MNCs possibly via reduction in expression of NFATc1. These results suggest that the combination of soy isoflavones and DHA might reduce osteoclastogenesis and in addition have a bone protective effect.

Acknowledgements

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