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Postprandial effects of almond consumption on human osteoclast precursors—an ex vivo study

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Abstract

Consumption of almonds has been associated with increased bone mineral density, but the direct effects of almonds on bone cells are not known. We determined whether serum obtained following the consumption of a meal containing 60 g of almonds affects human osteoclast formation, function, and gene expression in vitro. Human osteoclast precursors were cultured in medium containing 10% serum obtained from 14 healthy subjects at baseline and 4 hours following the consumption of 3 test meals containing almonds, potatoes, and rice and balanced for macronutrient composition. Osteoclast formation was determined by the number of tartrate-resistant acid phosphatase (TRAP)$^+$ multinucleated cells, and osteoclast function was assessed by measuring TRAP activity in the culture medium and calcium released from OsteoAssay (Lonza Walkersville, Walkersville, MD, USA) plates. The expression of cathepsin K, receptor activator of nuclear factor kB, and matrix metalloproteinase–9 genes was measured by real-time reverse transcriptase–polymerase chain reaction. Compared with serum obtained at baseline, serum obtained 4 hours following the consumption of the almond meal reduced osteoclast formation by approximately 20%, TRAP activity by approximately 15%, calcium release by approximately 65%, and the expression of cathepsin K, receptor activator of nuclear factor kB, and matrix metalloproteinase–9 by 13% to 23%. No effects were observed with serum obtained from the other test meals. Serum obtained 4 hours following the consumption of an almond meal inhibits osteoclast formation, function, and gene expression in cultured human osteoclast precursors, and provides evidence for a positive effect of almonds on bone health.

1. Introduction

Growing evidence indicates that the consumption of almonds has many beneficial health effects including reducing the risk of cardiovascular disease [1], improving blood lipid profiles [1-3], and reducing oxidative damage [3]. Recent evidence suggests that regular consumption of almonds (>4 servings per week) may also protect against osteoporosis in postmenopausal women [4]. Almonds contain several nutrients that are involved in the maintenance and development of bone such as calcium [5], protein [6], fatty acids [6], and various antioxidants [7]. As such, the possible beneficial effects of almonds on bone might be due to multiple components. Moreover, intermediates produced from the metabolism of bioactives

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Author contributions: IP designed and completed the ex vivo studies, conducted the statistical analysis, and prepared the initial manuscript. AJ conducted the clinical study and statistical analysis. CK, DJ, and AE obtained funding and provided supervision. All authors contributed to the data interpretation and critically revised the manuscript for important intellectual content.

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found in almonds could also affect bone physiology. Bone cells, such as the bone-forming osteoblasts and bone-resorbing osteoclasts, can be studied in vitro and can be used to test the direct effects of various dietary factors on their formation and function. One drawback of such experiments, however, is that they do not enable the direct testing of a whole food that requires digestion or the many metabolites produced during metabolism. To overcome this limitation, cultured cells can be treated with serum obtained from donors fed whole foods to directly test the effects of these foods and their metabolites at the cellular level. Previous studies have examined the effects of serum obtained from humans following an 11- to 14-day diet and exercise intervention on prostate cancer cells to determine the likely effect of such interventions on cancer progression [8-11]. However, no studies have examined the effects of serum obtained following an acute (postprandial) dietary intervention or a dietary intervention alone on cultured human cells. The purpose of this study was to determine if postprandial sera obtained from healthy donors 4 hours following the consumption of an almond meal affects cultured bone cells by examining the direct effects of baseline and postprandial sera on human osteoclast formation, function, and gene expression in vitro.

2. Materials and Methods

2.1. Subjects

Serum samples were obtained from a study in which the effects of 3 test meals containing almonds, potatoes, and rice, and with an identical macronutrient composition, were examined on postprandial glycemia, insulinemia, and oxidative damage in healthy individuals [3]. Serum samples were obtained from 5 individuals (7 men and 8 women) with an average (mean ± SD) age of 26.3 ± 8.6 years (range, 19-52 years) and a mean body mass index of 23.4 ± 3.4 kg/m^2 (range, 17.4-29.5 kg/m^2). Healthy subjects were recruited from staff and students at the University of Toronto and from subjects taking part in studies at Glycemic Index Laboratories (Toronto, Canada). Exclusion criteria were smoking, current use of vitamin or mineral supplements, impaired fasting blood glucose, diabetes, liver or kidney disease, or disorders of the gastrointestinal tract. Because the original study examined the effects of the test meals on oxidative damage to proteins, subjects were asked to avoid consuming any foods containing the antioxidant lycopene for 1 week and to avoid any water-soluble antioxidant-rich foods for 24 hours before study commencement and for the duration of the study. Subjects were provided with a list of specific foods and beverages high in antioxidants that they were to avoid, which included tomatoes, tomato products, watermelon, papaya, apricots, berries, tea (black, green, and herbal), coffee, chocolate, melon, kiwi, citrus fruit, and fruit juices. Before each study session, subjects were asked to complete a 3-day food record, outlining in detail all foods and beverages consumed. Compliance was assessed from the food record. Informed consent was obtained from the subjects. This study was approved by the Research Ethics Boards of the University of Toronto and St Michael's Hospital.

2.2. Study protocol and meals

Details of the original study have been published previously [3]. Briefly, 15 subjects attended the clinic between 8:00 AM and 9:00 AM after a 12-hour overnight fast once a week for 4 weeks. Subjects were instructed to eat the same meal the evening before each study day and to maintain the same level of physical activity the day before and the morning of the study. Venous blood samples were drawn from a forearm catheter kept patent with saline. Following the baseline sample, a test meal consisting of 60 g of almonds and 97 g of white bread (Wonder Bread, Interstate Bakeries Corp, Kansas City, MO, USA) was provided, which subjects were asked to consume within 10 minutes. In addition to the almond meal, 2 additional test meals, one containing parboiled rice and the other instant mashed potatoes, were consumed on separate visits to the clinic. Cheddar cheese and butter were added to these meals to balance the quantity of available carbohydrates, fat, protein, and total energy with the almond meal, as described in the Table 1. Subjects consumed the 3 test meals in a random order.

2.3. Analyses

Capillary blood samples were collected in Sarstedt (Sarstedt Inc, Montreal, QC, Canada) tubes at 0, 15, 30, 45, 60, 90, 120, and 240 minutes and were immediately placed in a −20°C freezer until analyzed for glucose. Venous blood was also collected at 0, 30, 45, 60, 90, 120, and 240 minutes. All venous blood samples were collected in serum red-top BD (Oakville, Canada) Vacutainer blood tubes with no additives. Upon collection, blood tubes were wrapped in tin foil to minimize light penetration and remained at room temperature for 1 hour to clot before being spun in a

Table 1
Macronutrient and energy content of the 3 test meals

Test meal	Energy	Available carbohydrate	Protein	Fat
	kJ		g	
Almond meal	2499	51.8	21.1	33.7
60 g almonds				
97 g bread				
Parboiled rice meal	2473	49.2	21.1	34.2
68 g cheese				
14 g butter				
60 g parboiled rice				
Mashed potato meal	2499	51.3	21.3	33.8
62 g cheese				
16 g butter				
68 g mashed potatoes				

Beckman GPR (Beckman Coulter Inc, Mississauga, ON, Canada) centrifuge at 1008*g* for 15 minutes. After centrifugation, serum was aliquoted into amber Eppendorf tubes and stored at −70°C. Serum insulin was analyzed at the St Michael's Hospital Core Laboratory using a chemiluminescent ultrasensitive insulin immunoassay (coefficient of variation [CV], 3.1%-5.6%) (Access Immunoassay System-Beckman Coulter Inc; Mississauga, ON, Canada). Venous blood collected at 0, 90, 120, and 240 minutes was analyzed for total antioxidant capacity (TAC) and protein thiols. Total antioxidant capacity was determined by the ferric-reducing antioxidant power assay [12,13]. The CV of samples analyzed in triplicate was 3.8%. Protein oxidation was measured using the 5,5'-dithio-*bis*(2-nitrobenzoic acid) assay [14] to assess the loss of reduced thiol (−SH) groups as a measure of protein oxidation. Reduced glutathione standards from 100 to 1000 μmol/L were used. The CV of serum samples analyzed in triplicate was 2.2%.

2.4. Preparation of serum

Serum obtained at baseline and 4 hours following the consumption of the test meals was used in the cell culture experiments. This time point (4 hours) was chosen because serum insulin, which is known to affect osteoblast and osteoclast formation and function [15,16], did not differ from baseline concentrations following the almond meal (Fig. 1). In addition, serum protein thiol concentration, which is a measurement of TAC, did not differ between baseline and 4-hour postprandial serum samples for any test meal. Before initiation of cell culture experiments, the sera were filter sterilized using radiosterilized 0.22-μm Millex-GP Syringe Filters (Millipore, Etobicoke, ON, Canada).

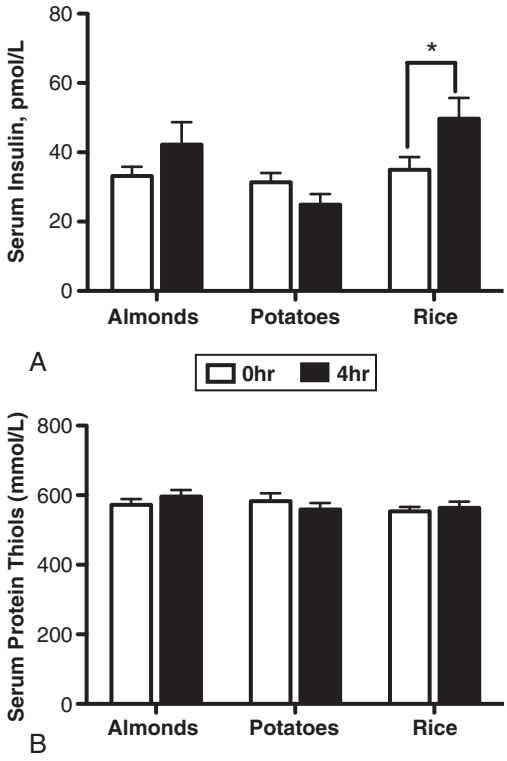

Fig. 1. Postprandial capillary serum insulin (A) and protein thiol concentrations (B) in healthy subjects at baseline and 4 hours after consumption of an almond, potato, or rice meal. Values are means ± SEM. n = 15.

2.5. Cell culture protocol

Primary human osteoclast precursor (OCP) cells and the Osteoclast Precursor Growth Medium (OPGM) BulletKit were obtained from Lonza Walkersville (Walkersville, MD, USA). Primary human OCPs were isolated by density centrifugation, immunoaffinity purification, and selective culturing techniques. The OCPs were seeded in 96-well (regular or OsteoAssay (Lonza Walkersville)) plates at a density of 10^4 cells per well in OPGM. The next day, the OPGM was removed and replaced with OPGM containing 10% donor serum rather than the 10% serum supplied in the BulletKit. Baseline and postprandial serum from one subject was used to test the feasibility of treating the OCPs with human serum. As a result, 84 preparations of OPGM were tested in the cell culture experiments corresponding to 14 subjects (serum from the 15th subject was used in the development of the assays), 3 test meals, and 2 time points. Sera from subjects fed the rice and potato meals were examined as controls to determine whether any differences in osteoclast formation between baseline and postprandial serum samples were the result of a feeding effect. The media were changed twice a week for 2 weeks.

2.6. Quantification of osteoclast formation

Osteoclasts were identified as tartrate-resistant acid phosphatase $(TRAP)^+$ cells containing 3 or more nuclei. The wells were viewed field by field under a phase-contrast microscope at 100× magnification, and the total numbers of osteoclasts per well were quantified as the sum for each field. For TRAP staining, the cells were fixed with 2.5% glutaraldehyde for 5 minutes, washed 2 times with phosphate-buffered saline preheated to 37°C, and treated with TRAP stain for 20 minutes at 37°C. Tartrate-resistant acid phosphatase staining was carried out using the protocol described in BD Biosciences Technical Bulletin #445. The TRAP staining solution consisted of 50 mmol/L acetate buffer, 30 mmol/L sodium tartrate, 0.1 mg/mL Naphthol AS-MX phosphate, 0.1% Triton X-100, and 0.3 mg/mL Fast Red Violet LB (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada).

2.7. Determination of TRAP activity

The TRAP activity was determined in cultured media using an adapted Sigma protocol as described [17]. Briefly, cultured medium was added to enzyme-linked immunosorbent assay plates containing phosphatase substrate

(*p*-nitrophenyl phosphate) and 40 mmol/L tartrate acid buffer and incubated at 37°C for 30 minutes. The reaction was stopped with the addition of 2 N NaOH, and absorbance was measured at 405 nm. Tartrate-resistant acid phosphatase catalyzes the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol, which has a maximal absorbance at 405 nm and represents TRAP activity in the sample. The TRAP activity was calculated from a standard curve obtained from *p*-nitrophenol standards.

2.8. Quantification of calcium released from the OsteoAssay plates

Following 2 weeks of treatment with the donor sera, the calcium concentration of the culture media was determined using the TECO Diagnostics (Anaheim, CA, USA) colorimetric Calcium Reagent Set, according to the manufacturer's instructions.

2.9. RNA isolation and one-step real-time reverse transcriptase–polymerase chain reaction

After 7 days of treatment with the donor sera, the cells were washed twice with phosphate-buffered saline and lysed in 0.5 mL of nucleic acid purification solution (Applied Biosystems, Streetsville, ON, Canada). Total RNA was isolated using an ABI 6100 Nucleic Acid PrepStation (Applied Biosystems). A one-step reaction was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) to reverse transcribe the messenger RNA (mRNA) into complementary DNA, which was subsequently amplified using the QuantiTect Multiplex RT-PCR Kit (Qiagen; Mississauga, ON, Canada) and TaqMan Gene Expression Assays (Applied Biosystems). All reactions were performed in 96-well plates with a final volume of 25 mL per well. Cycling conditions were 20 minutes at 50°C followed by 15 minutes at 95°C to activate the HotStarTaq DNA Polymerase, and 50 cycles of 45 seconds at 94°C and 45 seconds at 60°C. The TaqMan Gene Expression Assays used for cathepsin K (CSK), receptor activator of nuclear factor–kB (RANK), and matrix metalloproteinase (MMP)–9 were Hs01080388_m1, Hs00187189_m1, and Hs00957555_m1, respectively. The target genes CSK, RANK, and MMP-9 were each coamplified with VIC-labeled b-2-microglobulin (Applied Biosystems, #4326319E), which served as an internal control. Data were obtained as threshold cycle (C_T) values, which represent the cycle at which the first significant increase in fluorescence is detected, which corresponded to the amount of starting template in the sample. The difference in C_T (DC_T) values between the internal control (VIC-labeled b-2-microglobulin) and target gene of interest (FAM-labeled) was calculated to determine the relative change in C_T (DC_T) values between samples. The DC_T of each baseline sample was subtracted from the DC_T of its corresponding 4-hour sample to derive a DDC_T value, which represents the change in mRNA expression between the baseline and 4-hour treatments. Relative mRNA levels corresponding to the 4-hour samples were calculated as 2^{-DDCT} and expressed as relative arbitrary units compared with its corresponding baseline sample that produces a 2^{-DDCT} of 1. The mean 2^{-DDCT} from the 14 samples at 4 hours was compared with the baseline value of 1.

2.10. Statistical analyses

Results are expressed as mean ± SEM from the different subjects. Differences were analyzed using a paired *t* test. *P* values < .05 were considered significant. All data were analyzed using GraphPad Prism Software, Version 5.0 (La Jolla, CA, USA).

3. Results

3.1. Postprandial capillary blood insulin concentrations from healthy donors obtained at baseline and at 4 hours following the test meals

Fig. 1A presents the mean serum insulin concentrations over the 4-hour testing period for the 3 study meals. As demonstrated in this figure, mean serum insulin concentrations did not differ at baseline and 4 hours following the almond and potato meals, but differed at baseline (34.9 pmol/L) and 4 hours (49.7 pmol/L) following the rice meal ($P < .05$).

3.2. Postprandial capillary blood TAC of sera from healthy donors obtained at baseline and at 4 hours following the test meals

Fig. 1B shows the mean serum protein thiol concentrations of the 15 healthy donors over the 4-hour testing period for the 3 test meals. Serum protein thiol concentrations, a marker of protein oxidative damage, and serum postprandial TAC did not differ between baseline serum samples and those obtained 4 hours following any of the test meals.

3.3. $TRAP^+$ multinucleated cell formation from human following treatment with sera from healthy donors obtained at baseline and at 4 hours following the consumption of the test meals

Multinucleated cells were first observed under a phase-contrast microscope (100× magnification) after 14 days of treatment with human sera. As demonstrated in Fig. 2A, sera obtained from 14 healthy subjects 4 hours following the consumption of 60 g of almonds decreased the mean number of osteoclasts formed by approximately 20% compared with sera obtained from the same subjects at baseline (0 hour) ($P = .01$). Postprandial sera from both the potato and rice test meals had no effect on osteoclast formation compared with sera obtained at baseline (data not shown). Thus, the remainder of the experiments focused

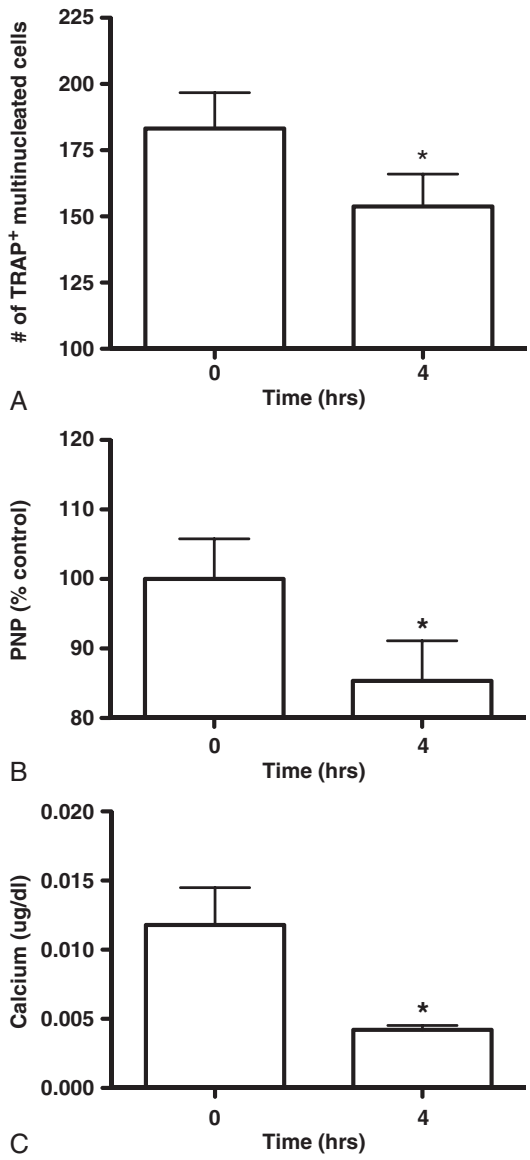

Fig. 2. Effects of serum obtained at baseline and 4 hours after consumption of an almond meal on osteoclast formation (A), as determined by the number of $TRAP^+$ multinucleated cells, and on functional osteoclast activity, as measured by TRAP activity in cultured media (B) and by the amount of calcium released from human bone fragments in an OsteoAssay plate (C). Values are means ± SEM. n = 14.

solely on the serum obtained at baseline and at 4 hours following the almond meal.

3.4. TRAP activity in cultured medium following treatment with sera from healthy subjects obtained at baseline and at 4 hours following the consumption of an almond meal

The TRAP activity was measured in the total culture media collected at each media change. Osteoclast precursors were treated with sera obtained at baseline and 4 hours following the consumption of the almond meal. As shown in Fig. 2B, postprandial sera reduced TRAP activity in the cultured media by approximately 15% compared with baseline sera ($P = .03$).

3.5. Calcium released from OsteoAssay plates following treatment with sera from healthy subjects obtained at baseline and at 4 hours following the consumption of an almond meal

Calcium was measured in the cultured media following 14 days of treatment with human sera. Compared with cells treated with sera from baseline samples, cells treated with sera obtained 4 hours following the consumption of the almond meal released 65% less calcium from the OsteoAssay plates (Fig. 2C).

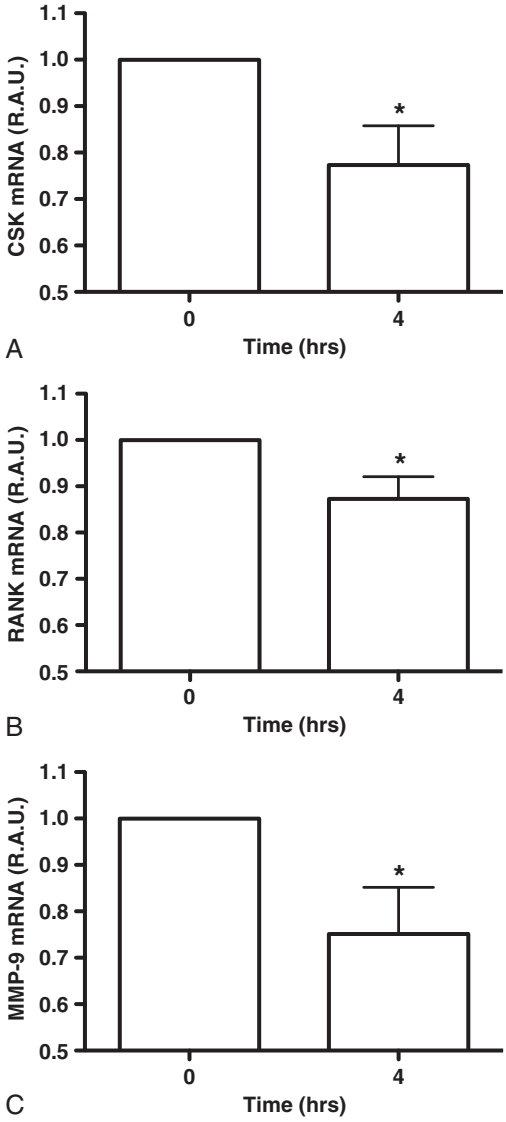

Fig. 3. Osteoclast gene expression (A, CSK; B, RANK; C, MMP-9) following treatment with serum obtained at baseline and 4 hours after consumption of an almond meal. Values are mean ± SEM. n = 14.

3.6. CSK, RANK, and MMP-9 expression in OCPs following treatment with sera from healthy subjects obtained at baseline and at 4 hours following the consumption of an almond meal

Gene expression in OCPs was measured following 7 days of treatment with sera obtained from healthy subjects at baseline and 4 hours following the consumption of the almond meal. As seen in Fig. 3, gene expression of the osteoclast markers CSK, RANK, and MMP-9 in cells treated with sera obtained 4 hours postprandially was lower than in cells treated with sera obtained at baseline. Compared with cells treated with sera from baseline, cells treated with postprandial sera showed a 23% reduction in CSK expression (Fig. 3A, $P = .02$), a 13% reduction in RANK expression (Fig. 3B, $P = .01$), and a 21% reduction in MMP-9 expression (Fig. 3C, $P = .04$).

4. Discussion

The present study demonstrates that serum obtained following the consumption of an almond meal inhibits human osteoclast formation, function, and gene expression in vitro. These data provide direct evidence to support the association between regular almond consumption and a reduced risk of developing osteoporosis [4]. Moreover, the present study established that serum obtained from an acute dietary intervention could be used to treat cultured human cells to determine the direct effects of whole foods or mixed meals on cellular differentiation and function. The ability to test the direct effects of metabolized whole foods overcomes the limitations of examining individual bioactive compounds because the actual effects of the individual compounds are likely modified by other bioactive compounds. As such, the ability to test the direct effects of metabolized whole foods likely provides more clinically relevant information than testing the effects of isolated compounds. The methods outlined in this study could be widely applied to test the direct effects of other foods, specialized diets, and designer foods on any type of cultured cell.

The beneficial effects of serum obtained following the consumption of an almond meal on osteoclast formation were not observed with the potato or rice meals, suggesting that the antiosteoclastogenic effects of almonds are not caused by a general effect of being in a postprandial state. To rule out any potential effects of serum insulin on osteoclast formation, activity, and gene expression, we tested the effects of serum samples obtained 4 hours following the consumption of the test meals because serum insulin concentrations at this time point did not differ from serum concentrations at baseline in both the almond and potato meals.

Osteoclast formation is stimulated by reactive oxygen species [18-20], and almonds contain various antioxidants [7] that reduce oxidative damage [3]. However, the inhibitory effect of almonds on osteoclast formation and activity is likely not due to a reduction in oxidative stress because serum obtained at baseline and 4 hours following the consumption of any test meal did not differ in their TAC.

Although the test meals did not differ in their macronutrient or energy composition, the fatty acid composition of the almond meal was markedly different than that of the potato or rice meals. Almonds are high in oleic and linoleic acids [21,22], whereas dairy fats from the cheese and butter that were added to the potato and rice meals are high in myristic, palmitic, stearic, and *trans*-vaccenic acids [23,24]. Thus, the inhibitory effects of serum obtained postprandially from the almond meal, but not the potato and rice meals, may be due to differences in the fatty acid compositions of the meals. We examined the effects of purified linoleic acid (25-100 mmol/L) on osteoclast formation and function and determined that this fatty acid does not affect osteoclast formation or activity (data not shown). Therefore, it is unlikely that the high linoleic acid content of almonds is responsible for the observed effects on osteoclasts. Despite the potential for other fatty acids and their metabolites to affect osteoclastogenesis, it is possible that more than one fatty acid, metabolite, or component of the almond is responsible for the inhibitory effect on osteoclast formation and function.

Almonds contain many bioactives that may be responsible for the observed effects on osteoclasts. Many nutrient-nutrient interactions may occur following the consumption of almonds, and other known and unknown metabolites produced during the digestion of almonds could also affect osteoclast formation and function. As such, the combined effects of the various components of almonds are likely more relevant than the effects of each individual component.

In summary, our findings demonstrate a direct inhibitory effect of almonds on osteoclast formation and activity, and support the inverse association observed between regular almond consumption and a reduced risk of osteoporosis [4]. To our knowledge, this is the first study to demonstrate the feasibility of testing the postprandial effects of a meal on cultured bone cells in vitro.

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