

Effect of High-Calcium Diet on Coronary Artery Disease in Ossabaw Miniature Swine With Metabolic Syndrome

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Background—Calcium is a shortfall essential nutrient that has been a mainstay of osteoporosis management. Recent and limited findings have prompted concern about the contribution of calcium supplementation to cardiovascular risk. A proposed mechanism is through the acceleration of coronary artery calcification. Determining causality between calcium intake and coronary artery calcification has been hindered by a lack of sensitive methodology to monitor early vascular calcium accumulation. The primary study aim was to assess the impact of high calcium intake on coronary artery calcification using innovative calcium tracer kinetic modeling in Ossabaw swine with diet-induced metabolic syndrome. Secondary end points (*in vitro* wire myography, histopathology, intravascular ultrasound) assessed coronary disease.

Methods and Results—Pigs (n=24; aged ≈15 months) were fed an atherogenic diet with adequate calcium (0.33% by weight) or high calcium (1.90% from calcium carbonate or dairy) for 6 months. Following 5 months of feeding, all pigs were dosed intravenously with ^{41}Ca , a rare isotope that can be measured in serum and tissues at a sensitivity of 10^{-18} mol/L by accelerator mass spectrometry. Kinetic modeling evaluated early coronary artery calcification using ^{41}Ca values measured in serial blood samples (collected over 27 days) and coronary artery samples obtained at sacrifice. Serum disappearance of ^{41}Ca and total coronary artery ^{41}Ca accumulation did not differ among groups. Secondary end points demonstrated no treatment differences in coronary artery disease or function.

Conclusion—There was no detectable effect of high calcium diets (from dairy or calcium carbonate) on coronary artery calcium deposition in metabolic syndrome swine. (*J Am Heart Assoc.* 2015;4:e001620 doi: 10.1161/JAHA.114.001620)

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In recent years, the potential contribution of dietary and supplemental calcium to elevating cardiovascular risk has been widely debated.^{1,2} Adequate intakes of calcium, along with vitamin D, have been encouraged due to the important

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physiological and structural roles of these nutrients, particularly in reducing bone fracture risk. Moreover, supplemental calcium has been advocated³ because the amount of calcium obtained from foods consistently falls short of current dietary recommendations.⁴ Although traditionally regarded as safe, recent findings have led to concerns regarding a putative association between calcium supplementation and myocardial infarction and cardiovascular-related mortality,^{5–8} prompted by a randomized controlled trial that demonstrated elevated cardiovascular event risk with calcium supplementation versus placebo among postmenopausal women.⁹ Evidence from observational studies and exploratory analysis of a randomized controlled trial, however, has failed to demonstrate associations between calcium intake and coronary artery calcium scores in adults.^{10–12} Furthermore, a recent meta-analysis did not demonstrate an increased risk for heart disease or all-cause mortality risk in elderly women taking calcium supplementation with or without vitamin D versus those assigned to placebo.¹³ Currently, the available literature is sparse and inconclusive. Because both cardiovascular disease and osteoporosis contribute significantly to mortality

and morbidity,^{14,15} further assessment of the causal relationship between calcium intake and cardiovascular risk is of importance.

A suggested mechanism by which supplemental calcium may elevate cardiovascular risk is through the acceleration of vascular calcification induced by acute elevations in circulating calcium.¹⁶ Vascular calcification is a clinical indicator of atherosclerosis and may predict cardiovascular-related mortality.¹⁷ The investigation of a causal role of dietary or supplemental calcium in cardiovascular-related outcomes has been hampered by a lack of sensitive experimental methodology for assessing early vascular calcification and by the extensive intervention period necessary to monitor coronary artery disease (CAD) progression in human populations.

In the present study, we used a relevant animal model for the study of CAD and both innovative and traditional methods for detecting soft tissue calcium, cardiovascular function, and CAD burden. The Ossabaw miniature swine model has translational relevance to humans because it demonstrates the full spectrum of human disease progression from metabolic syndrome to CAD with calcified atherosclerotic plaques when fed an atherogenic diet.^{18,19} Furthermore, their size makes the Ossabaw pig an ideal model for using imaging techniques and kinetic studies that necessitate ample blood and tissue sampling. Our group has demonstrated previously that the Ossabaw pig is an adequate model for assessing coronary artery calcification (CAC) through the use of novel calcium tracer kinetic modeling.²⁰ The isotope ⁴¹Ca is a long-lived ($t_{1/2} > 10^5$ years) calcium tracer that can be measured in low concentrations (10^{-18} mol/L) by accelerator mass spectrometry. Previously, our group has used ⁴¹Ca tracer kinetics to monitor bone turnover and bone calcium retention.²¹ We recently verified that compartmental kinetic modeling of ⁴¹Ca can be used to assess early CAD-associated calcium deposition in the coronary arteries.²⁰

The primary aim of this study was to investigate the impact of high calcium intake from a supplemental source (calcium carbonate [CaCO₃]) or dairy on coronary artery calcium deposition in the Ossabaw miniature swine fed an atherogenic diet, using novel ⁴¹Ca tracer kinetics to characterize early CAC. Secondary objectives included the investigation of the effect of high calcium intake on cardiovascular function and CAD progression, using more traditional techniques including *in vitro* wire myography, histopathology, and intravascular ultrasound.

Materials and Methods

Animals and Diets

This protocol was approved by the animal care and use committees of Purdue University and the Indiana University

School of Medicine. Female Ossabaw miniature swine (n=24) aged 14 to 16 months (sexually mature) were randomized to 1 of 3 dietary calcium conditions (n=8 per group) including a control condition (adequate calcium; 0.33% Ca by weight) and elevated calcium conditions from CaCO₃ (1.90% Ca) or from dairy (1.90% Ca). The compositions of the experimental diets are listed in Table 1. The calcium composition of the dairy diet was achieved through the addition of 7.50% nonfat dry milk and 6.85% TruCal D50 (Glanbia Nutritionals), a milk mineral complex. The TruCal D50 is a dairy ingredient that is derived from milk and that provides milk minerals in a profile similar to that of milk (per 100 g: 24 800 mg Ca, 620 mg sodium, 700 mg potassium, 1400 mg magnesium, 12 500 mg phosphorous) without providing additional lactose that has the potential to cause gastrointestinal side effects in the pigs when fed in large quantities. The 2 high-calcium diets (CaCO₃ and dairy) were designed with the intention of reflecting human calcium intakes at the tolerable upper limit recommended by the Institute of Medicine (≈ 2000 to 2500 mg/day). All diets were supplemented with phosphorous to prevent deficiency due to calcium binding, with the dairy diet containing the most phosphorous (1.85% by weight versus 0.90%) to reflect the mineral composition of a human diet that incorporates high amounts of dairy. All pigs were placed on a high-fat, high-cholesterol atherogenic

Table 1. Composition of Experimental Diets

	Control	Dairy	CaCO ₃
	0.33% Ca	1.90% Ca	1.90% Ca
	0.90% P	1.85% P	0.90% P
Chow diet	53.98	53.98	53.98
Hydrogenated soybean oil	8.40	8.40	8.40
Cholesterol	2.00	2.00	2.00
Cholate	0.70	0.70	0.70
High-fructose corn syrup	5.00	5.00	5.00
Hydrogenated coconut oil	4.70	4.70	4.70
Nonfat dry milk	—	7.50	—
Milk mineral complex*	—	6.85	—
Monosodium phosphate	2.28	2.28	2.28
Calcium carbonate	0.55	—	4.49
Soy hulls	17.66	6.77	14.55
Soy protein concentrate†	3.90	1.49	3.21
Fructose†	0.84	0.32	0.69

Data presented as percentage of diet (wt/wt). Macronutrient composition of the 3 diets is as follows: 40% kilocalories from fat, 23% kilocalories from protein, and 37% kilocalories from carbohydrate.

P indicate phosphorous.

*The milk mineral complex used was TruCal D50.

†Soy protein concentrate and fructose were added to balance the energy and protein contents of the diet.

diet to induce early development of CAD. The atherogenic diet consisted of standard chow supplemented with cholesterol (2.0% wt/wt), hydrogenated coconut oil (4.70%), hydrogenated soybean oil (8.40%), cholate (0.70%), and high-fructose corn syrup (5.0%). Pigs were meal fed 1500 g of their assigned diet daily for 1.5 hours to encourage gorging behavior. If applicable, leftover food was weighed and recorded to calculate true total food and calcium intakes.

The pigs were housed and fed in individual pens with a 12-hour light-dark cycle. Water was provided ad libitum. One pig died early in the intervention period due to apparent kidney disease, and 1 pig died during catheter insertion surgery, accounting for the final sample size of 22 for some of the study end points.

Body weights were measured weekly. Pigs were acclimated to restraint in a specialized sling²² regularly throughout the intervention period. Conscious systolic and diastolic blood pressures were measured weekly using a noninvasive blood pressure cuff on the left forelimb while the pig was elevated and resting in the restraint sling.

Intravenous Catheter Insertion and Blood Collection

After 5 months of dietary intervention, all pigs underwent placement of a central venous line catheter into the left or right jugular vein. Anesthesia was maintained by mask with 3% to 5% isoflurane in 100% oxygen as a carrier gas. Body temperature, heart rate, blood pressure, and respiratory rate were continuously monitored throughout the procedure.

Following a 24-hour recovery period, baseline venous blood samples (10 mL) were obtained by jugular catheter after an overnight fast. An iStat Clinical analyzer (Abbott Point of Care Inc) was used to immediately analyze ionized calcium, glucose, and creatinine on fresh whole blood. Subsequently, all pigs received an intravenous infusion of 50 nCi ⁴¹Ca as ⁴¹CaCl₂ in 3 mL of sterile saline over 2 minutes by jugular catheter, and blood (10 mL) was drawn through the same catheter at 5, 15, and 30 minutes; at 1, 1.5, 2, 2.5, 3, 4, 5, 7, 10, 12, and 24 hours; and then at 2, 3, 5, 7, 9, 11, 16, 18, 23, and 27 days following the intravenous delivery of the ⁴¹Ca dose. Blood specimens were centrifuged, and the serum fraction was separated and stored at -40°C for future analysis.

Pharmacokinetics

To assess serum calcium response to a test meal, half-rations of the typical daily portions of test diet (750 g) were given concurrently with the ⁴¹Ca dose to ensure that all pigs could complete the serving within 60 minutes. Serum total calcium

was measured in the samples drawn over the first 12 hours (as listed earlier) using a COBAS Integra 400 analyzer (Roche Diagnostics). Baseline-adjusted calcium concentration versus time curves were constructed, and area under the concentration-time curve (AUC_{0-t}) was estimated using SAS 9.2 (SAS Institute). The peak change in serum calcium over the 12-hour span was defined as the C_{max} (mmol/L), and the time to reach the C_{max} was defined as the T_{max} (minutes).

Serum Assays

Fasted serum samples were analyzed for calcium, phosphorous, triglyceride, total cholesterol, and high-density lipoprotein cholesterol (HDL-C) concentrations using a COBAS Integra 400 analyzer. Low-density lipoprotein cholesterol (LDL-C) content was calculated with the Friedewald equation.²³

Serum C-reactive protein, a marker of chronic inflammation that may predict atherosclerosis risk,²⁴ was assessed using a porcine C-reactive protein enzyme-linked immunoassay (Alpco Diagnostics).

In Vivo Analysis

Anesthesia

A central venous line was used to administer anesthesia solution for in vivo imaging and sacrifice. Pigs were anesthetized with 10 mL of telazol solution (tiletamine and zolazepam; 10 mg/mL) and xylazine (10 mg/mL), which was given in a slow intravenous push through the venous catheter. The pigs were placed on inhalational anesthesia by mask and given 5% isoflurane carried on 100% oxygen for 5 minutes. This was interrupted briefly as the animals were intubated to ensure that a proper airway was maintained. Inhalational anesthesia was continued using isoflurane ranging from 0.5% to 3.5% with 100% oxygen as carrier gas. Pigs were continuously monitored for blood oxygen saturation, electrocardiogram, heart rate, blood pressure, capillary refill time, mucosal membrane color, and respiratory rate.

Dual-phase Electrocardiogram-gated Dynamic Contrast-enhanced Computed Tomography

Dynamic contrast-enhanced computed tomography (CT) provided high-resolution temporal and spatial CT images suitable for quantification of cardiac function (Figure 1). Left ventricular volumes were segmented across the cardiac cycle, and end-diastolic and -systolic phases were used to estimate ventricular stroke volume and ejection fractions. Calcified lesions were evaluated, as described previously, in published work, and CAC scores were assigned by trained personnel using a previously published scoring system based on number and area of calcified lesions detected.²⁵

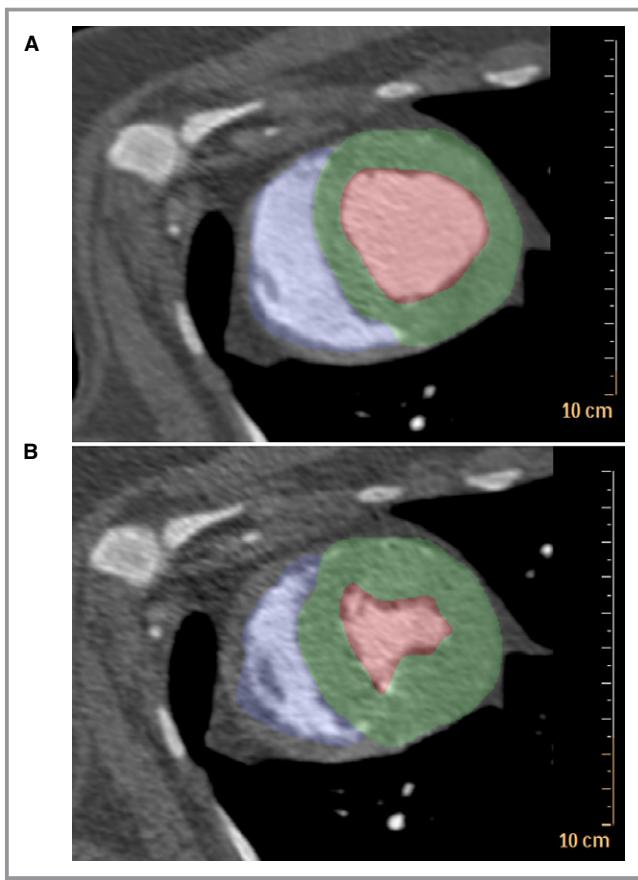


Figure 1. ECG-gated dynamic contrast-enhanced computed tomography allowed for quantification of end-diastolic volume and end-systolic volume to assess cardiac function in Ossabaw swine with metabolic syndrome. A, Diastole. B, Systole. Blue indicates the right ventricular cavity. Green indicates the left ventricle; Red indicates the left ventricular cavity.

Intravascular Ultrasound

After induction of anesthesia, intravascular ultrasound was performed in the left anterior descending (LAD) coronary artery, as described previously.¹⁸ Intravascular ultrasound images were analyzed using ImageJ software (National Institutes of Health) for every 1 mm in the proximal 15 mm of the LAD coronary artery to pair with the arterial segment used for in vitro wire myography studies described below. An initial area was obtained by tracing the tunica media–tunica intima boundary; this represented an “initial” lumen. A secondary measurement was obtained by tracing the actual lumen (Figure 2). Plaque burden was determined by calculating $(IL - AL)/IL \times 100$, in which IL is the initial lumen and AL is the actual lumen.

Sacrifice and Tissue Collection

Under anesthesia, hearts were removed, weighed, and perfused with ice-cold Krebs bicarbonate buffer containing

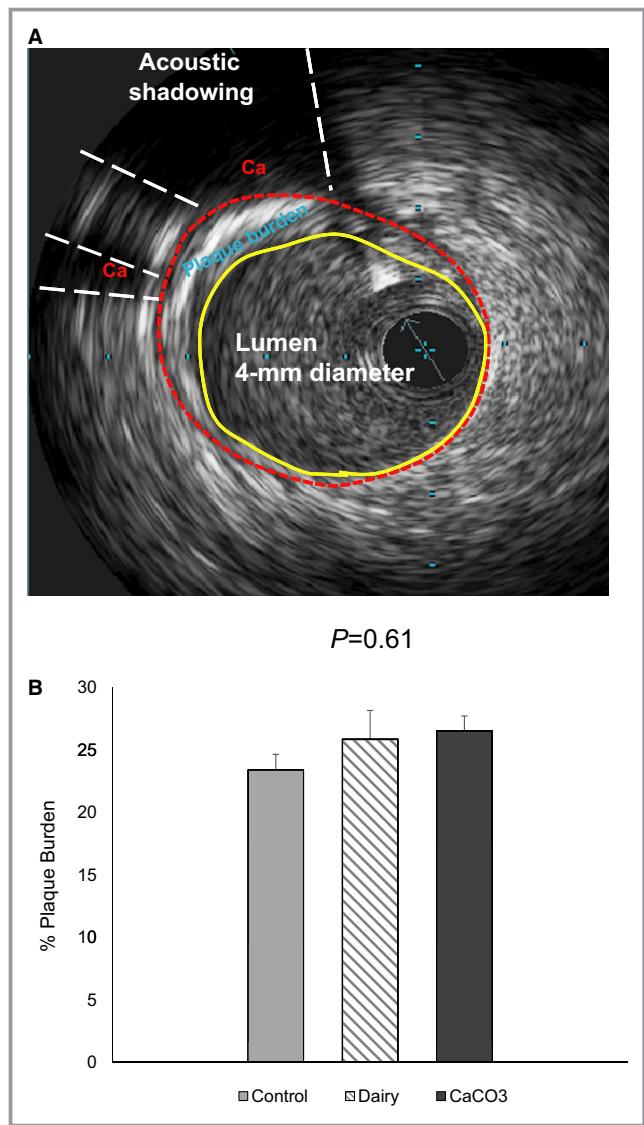


Figure 2. Intravascular ultrasound to assess plaque burden in Ossabaw swine LAD coronary artery. A, An intravascular ultrasound image showing stenotic plaque and calcified plaque (Ca, acoustic shadowing) in the LAD coronary artery. B, Percentage of plaque burden analysis of the proximal 1.5-cm LAD region. Data are means \pm SEM. LAD indicates left anterior descending.

131.5 mmol/L NaCl, 5.0 mmol/L KCl, 1.2 mmol/L NaH_2PO_4 , 1.2 mmol/L MgCl_2 , 2.5 mmol/L CaCl_2 , 11.2 mmol/L glucose, 20.8 mmol/L NaHCO_3 , 0.003 mmol/L propranolol, and 0.025 mmol/L EDTA. Proximal portions of LAD coronary artery \approx 15 mm in length were excised from anatomically identical regions of each pig heart. The LAD segments were placed in ice-cold Krebs bicarbonate buffer solution for immediate analysis of vasoactive responses using in vitro wire myography. Additional segments of the coronary arteries, including the mid-distal LAD coronary artery, midproximal right coronary artery, and circumflex coronary artery, were biopsied and stored in formalin for histopathology and tissue

^{41}Ca tracer measurements. A diagram of the heart tissue sampling distributed for each experimental method is shown in Figure 3.

In Vitro Wire Myography

Detailed procedures used to assess vasoactive responses of arterial rings have been published previously.^{26–28} Proximal LAD segments in ice-cold Krebs bicarbonate buffer were immediately cleaned of adipose and connective tissue and cut into 4 rings ≈ 2 to 3 mm in length. The rings were mounted on a 4-channel in vitro wire myograph (Myobath II; World Precision Instruments) and submerged in individual organ baths filled with the Krebs buffer solution (pH 7.4) maintained at 37°C and bubbled with 5% CO₂ and 95% O₂. Following an equilibration period, resting tension was set between 5 and 6 g for each ring segment; this resting tension was previously demonstrated to be optimal for porcine coronary arteries.^{28,29}

Bradykinin and sodium nitroprusside were used to examine endothelium-dependent and -independent vasorelaxation, respectively. Following preconstriction with prostaglandin F_{2 α} (30 $\mu\text{mol/L}$), increasing doses of bradykinin (10⁻¹¹ to 10⁻⁶ mol/L) or sodium nitroprusside (10⁻¹⁰ to 10⁻⁴ mol/L) were added to each bath. Vessels were washed with the Krebs buffer solution and allowed to return to baseline tensions before proceeding to the subsequent dose-response protocol.

Histopathology

Two or 3 cross-sections from formalin-fixed tissues including the midproximal right and circumflex coronary artery, mid-

distal LAD coronary artery, and the aortic root were examined microscopically for each pig. Slides of artery cross-sections were prepared for paraffin embedding, cut at 5- μm thickness, and stained with hematoxylin and eosin stain and von Kossa stain using standard techniques. Each arterial cross-section was examined by quadrant (Figure 4A and 4B) for the presence of atherosclerotic or calcified plaque. The proportion of quadrants with atherosclerotic or calcified plaque was determined to assess plaque burden of each artery.

$^{41}\text{Ca}:\text{Ca}$ Measurements in Serum and Tissue

Formalin-fixed arteries were cleaned of connective tissue and fat and dried overnight. Dried coronary artery samples weighing 2.5 to 37.4 mg were digested in concentrated nitric acid for metal analysis and diluted with ultrapure deionized water. Small aliquots of the digested tissue samples were further diluted with 0.5% lanthanum as LaCl₃ for total calcium determination by atomic absorption spectroscopy. Serum obtained from the serial kinetic blood draws and the digested coronary artery samples were analyzed for $^{41}\text{Ca}:\text{Ca}$ using accelerator mass spectrometry.²¹

Compartmental Kinetic Modeling with ^{41}Ca

Kinetic data including values for $^{41}\text{Ca}:\text{Ca}$ and total calcium of the serum and coronary arteries were used to fit a compartmental model²⁰ with the Windows version of Simulation, Analysis, and Modeling (WinSAAM) computer software.³⁰ The model consists of 3 compartments considered to represent exchangeable calcium in serum, extravascular fluid, and bone, respectively.²¹ A single loss pathway was incorporated to

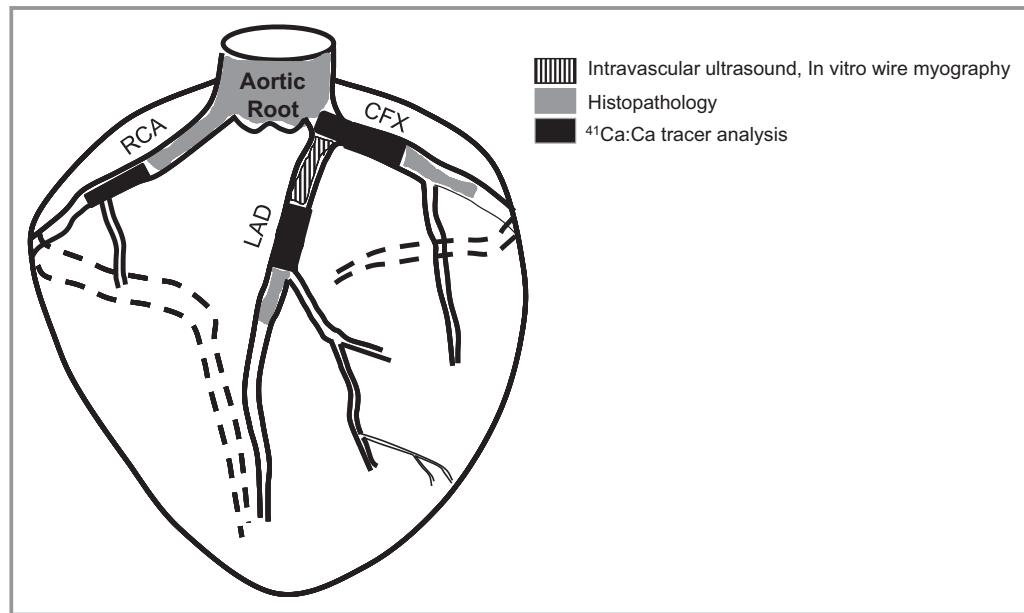


Figure 3. Diagram of tissue sampling and distribution for analysis. CFX indicates circumflex; LAD, left anterior descending; RCA, right coronary artery.

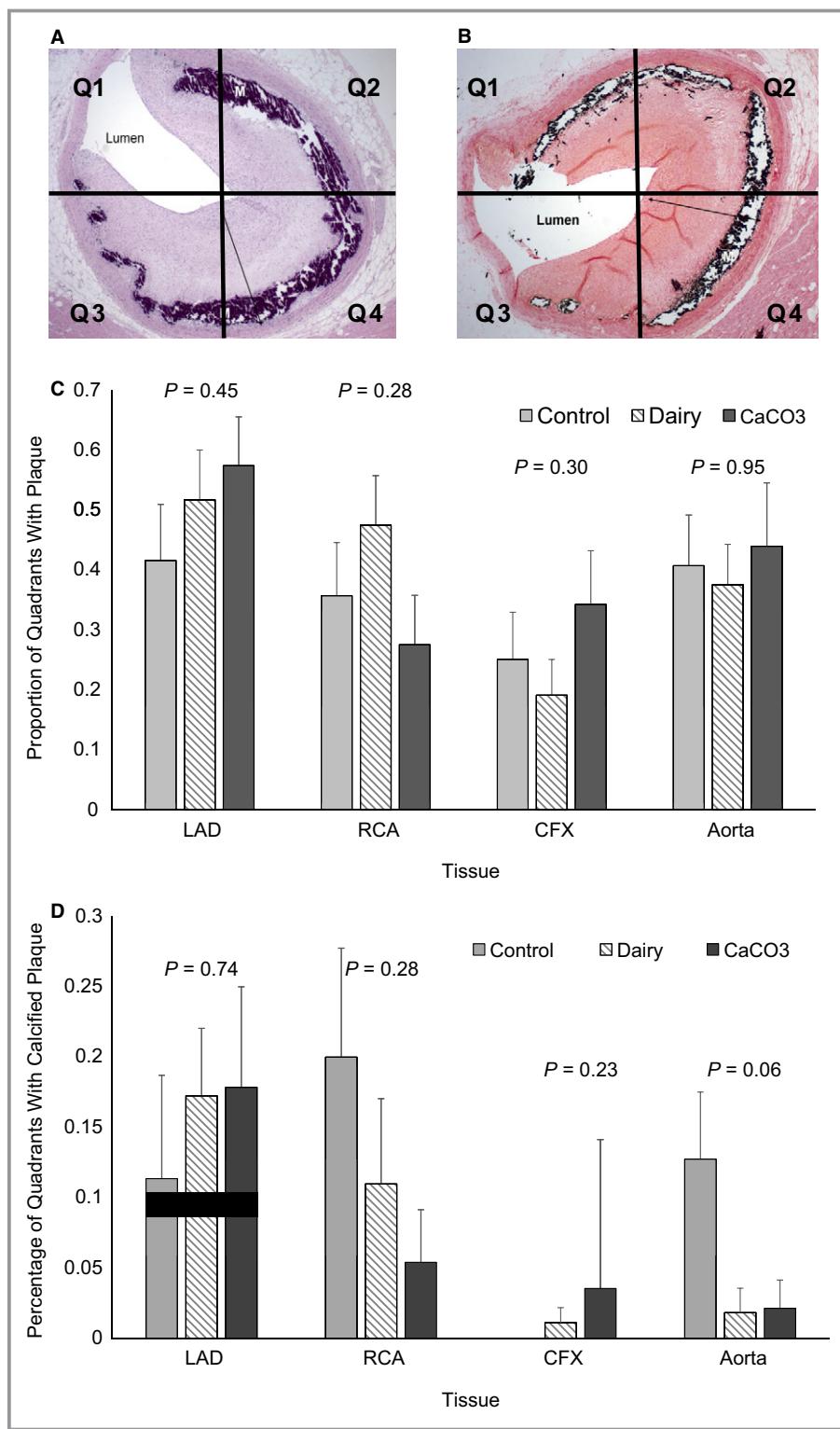


Figure 4. Histopathology of swine coronary arteries to assess atherosclerotic and calcified plaque coverage. Cross-sections of coronary arteries and aorta were stained with hematoxylin and eosin stain (A) and von Kossa stain (B) and examined by quadrant for presence of atherosclerotic plaque and calcified plaque. C, Proportion of total quadrants examined with atherosclerotic plaque present. D, Proportion of quadrants examined with calcified plaque present. Data are expressed as mean \pm SEM. Note: The control group had no detectable calcification in the CFX. CFX indicates circumflex; LAD, left anterior descending; RCA, right coronary artery; Q, quadrant.

account for calcium deposition in bone, urinary excretion from the kidney, and endogenous intestinal excretion. The pigs were assumed to be in steady state because the amount of calcium fed was constant over the 6-month intervention period. The model was first fitted to the serum ^{41}Ca :Ca data by allowing the transfer coefficients and initial volumes of distribution to be adjustable in WinSAAM. The ^{41}Ca :Ca ratio was determined as the tracer in the first compartment divided by the steady state mass of that compartment (serum calcium concentration times volume of distribution). The tissue data were fitted by pathways from the plasma-containing compartment to pools representing the LAD, right, and circumflex coronary arteries, as previously described.²⁰ The initial amount of calcium in the coronary arteries was made adjustable so that the amount measured at sacrifice corresponded to the calculated mass.

Data Analysis

The study was powered at 80% to detect differences of 0.46 (10^{-10}) in our primary end point, tissue ^{41}Ca :Ca levels, for $n=8$ per group.

Data from the in vitro wire myography studies were calculated as the percentage of relaxation from prostaglandin $\text{F}_{2\alpha}$ -induced constriction. Resultant concentration response curves for the 2 agonists tested (Figure 5) were fitted to a 4-parameter logistic equation using nonlinear regression, and EC_{50} (the effective concentration producing 50% of the maximal response) values were derived using Origin 9.1 (OriginLab Corporation).

Relationships between dietary treatment groups and outcome measures were analyzed using ANOVA with Student-Newman-Keuls post hoc analysis with commercially available software (SAS 9.2). $P<0.05$ was set for attaining statistical significance. Data are expressed as mean \pm SEM.

Results

Weights and Biochemistries

Reference data for lean female pigs (unpublished data from the laboratory of M. Sturek) that were approximately matched for age and sex confirmed fundamental characteristics of metabolic syndrome in the study pigs (Table 2). Similar reference data for lean male Ossabaw pigs have been published previously.^{31,32}

Although pigs in the dairy group tended to consume more food per day, differences in total food consumption were not statistically significant among groups (control, 1143 ± 39 g/day; dairy, 1322 ± 26 g/day; CaCO₃, 1248 ± 84 g/day; $P=0.08$). The average daily calcium intake was similar between the dairy and CaCO₃ treatment groups, and as expected, both of these groups consumed significantly more calcium than the control group (control, 4.05 ± 1.30 g/day;

dairy, 23.20 ± 0.25 g/day; CaCO₃, 21.61 ± 1.56 g/day; $P<0.0001$). Body weights were similar among groups at baseline (control, 58.85 ± 1.01 kg; dairy, 56.15 ± 1.43 kg; CaCO₃, 57.23 ± 0.86 kg; $P=0.26$) and at the end of the 6-month treatment (Table 3).

There were no treatment differences in systolic blood pressure, diastolic blood pressure, and pulse rate (Table 3). Fasting concentrations of serum triglycerides, C-reactive protein, calcium, phosphorous, and creatinine were not significantly different among treatment groups. The dairy group had significantly elevated total cholesterol, LDL-C:HDL-C ratio, and calculated LDL-C compared with control and CaCO₃ groups, whereas the CaCO₃ group had significantly elevated HDL-C compared with the control and dairy groups (Table 3).

In Vivo Imaging

Ventricular stroke volume and ejection fraction at rest under isoflurane anesthesia did not differ significantly by treatment group (stroke volume: control, 46.53 ± 9.52 mL; dairy, 59.05 ± 8.14 mL; CaCO₃, 50.23 ± 7.05 mL; ejection fraction: control, 0.49 ± 0.10 ; dairy, 0.58 ± 0.06 ; CaCO₃, 0.43 ± 0.04). All pigs that underwent CT scans ($n=10$) had CAC scores of 0 (ie, undetectable CAC). Data obtained from intravascular ultrasound revealed no treatment effect on percentage of plaque burden of the proximal LAD (Figure 2).

Serum Calcium Response to Feeding

The serum calcium AUC₀₋₇₂₀ and C_{max} data for both of the high-calcium diets in response to the test diets were not significantly different from the control diet, likely because of high variability in the response (AUC₀₋₇₂₀: control, 115.30 ± 67.13 mg·min/dL; dairy, 218.40 ± 117.52 mg·min/dL; CaCO₃, 259.00 ± 91.75 mg·min/dL; C_{max}: control, 0.12 ± 0.05 mmol/L; dairy, 0.19 ± 0.05 mmol/L; CaCO₃, 0.21 ± 0.07 mmol/L). The T_{max} was not significantly different among groups (control, 193.1 ± 81.5 minutes; dairy, 338.6 ± 101.6 minutes; CaCO₃, 345.0 ± 101.7 minutes).

Vasoactive Responses of the Coronary Artery

Characteristics of the LAD ring segments and vasoactive responses are summarized in Table 4. Bradykinin and sodium nitroprusside, the endothelium-dependent and -independent vasodilators used, produced significant concentration-dependent relaxation responses of porcine proximal LAD segments preconstricted with prostaglandin $\text{F}_{2\alpha}$ (Figure 5); however, there were no significant differences in response due to diet. Both bradykinin and sodium nitroprusside produced a concentration-dependent response with similar EC₅₀ and maximal relaxation response across treatments.

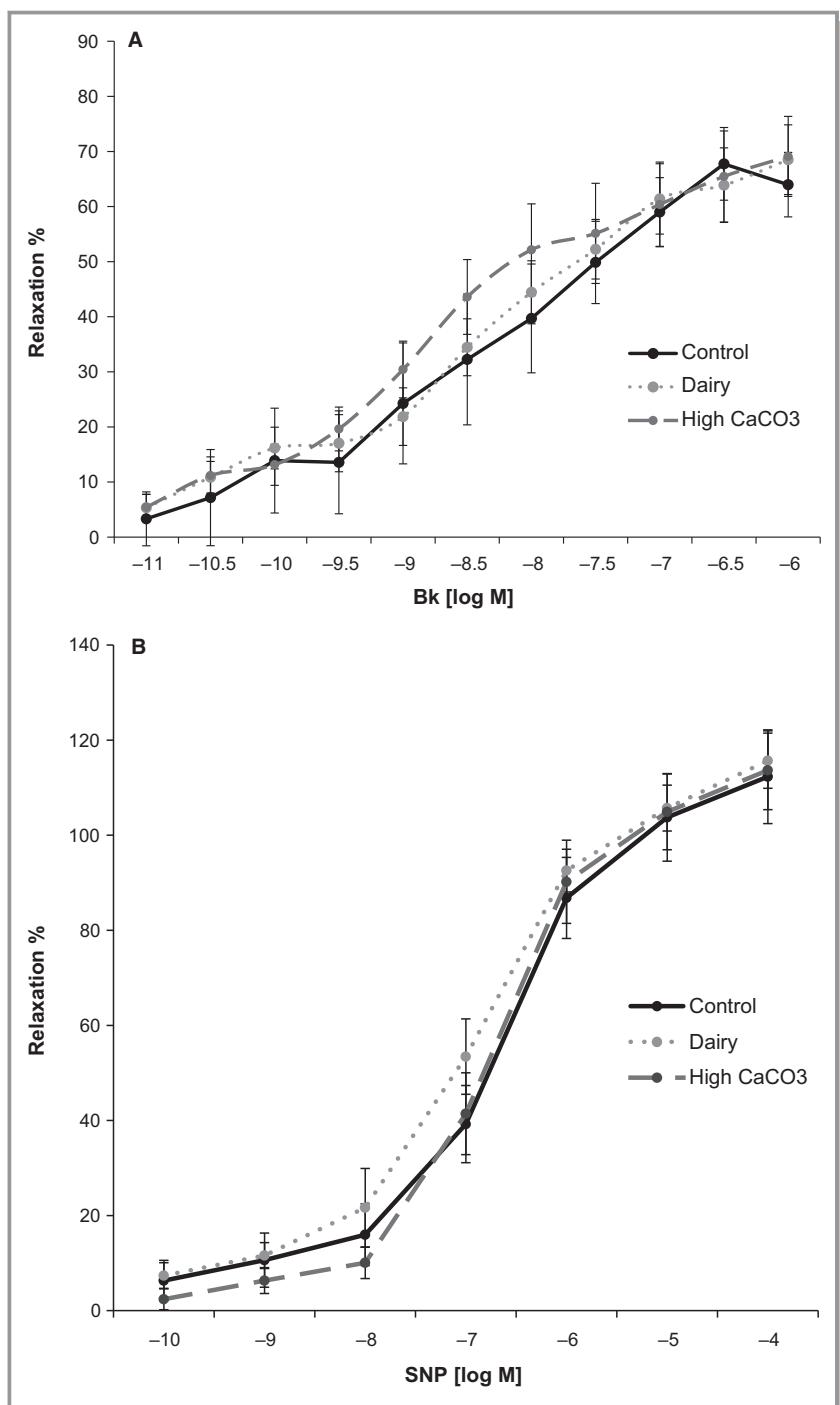


Figure 5. Relaxation responses of swine LAD coronary artery in response to Bk and SNP by in vitro wire myography. A, Log concentration-response curves for Bk in swine LAD coronary artery ring segments. Relaxation responses, shown as mean \pm SEM, are expressed as a percentage of the PGF_{2 α} -induced contraction. B, Log concentration-response curves for SNP in LAD segments. Relaxation responses, shown as mean \pm SEM, are expressed as a percentage of the PGF_{2 α} -induced contraction. Bk indicates bradykinin; LAD, left anterior descending; PGF_{2 α} , prostaglandin F_{2 α} ; SNP, sodium nitroprusside.

Histopathology

The proportion of coronary artery quadrants examined with visible atherosclerotic plaque or calcified atherosclerotic

plaque did not differ among dietary groups (Figure 4). There was a trend for more quadrants containing calcified plaque in the aortas of pigs consuming the control

Table 2. End-of-Treatment Parameters of Metabolic Syndrome in Ossabaw Swine From the Present Study Compared With Values From Age- and Sex-Matched Lean Reference Ossabaw Swine*

	Lean Reference Pigs Mean (\pm SEM)	Study Pigs Mean (\pm SEM)	<i>P</i> Value
	n=8	n=22	
Body weight, kg	45 (\pm 3)	93 (\pm 3) [†]	<0.0001
Fasted glucose, mmol/L	3.5 (\pm 0.1)	3.3 (\pm 0.2) [†]	0.50
Total-C, mmol/L	1.42 (\pm 0.1) [‡]	14.4 (\pm 0.8)	<0.0001
TG, mmol/L	0.2 (\pm 0.0) [‡]	1.2 (\pm 0.2)	0.0012
LDL-C, mmol/L	0.4 (\pm 0.1)	12.2 (\pm 0.8)	<0.0001
HDL-C (mmol/L)	0.9 (\pm 0.1)	1.8 (\pm 0.2)	0.002
Systolic blood pressure, mm Hg	122 (\pm 8)	133 (\pm 4)	0.19
Diastolic blood pressure, mm Hg	78 (\pm 7)	94 (\pm 3)	0.02

HDL-C indicates high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; Total-C, total cholesterol.

*Data for lean age- and sex-matched reference pigs are unpublished and were provided by the laboratory of Michael Sturek at the Indiana University School of Medicine (Indianapolis, IN).

[†]n=23.

[‡]n=9.

diet versus the CaCO₃ or dairy diets (control, 0.13 \pm 0.05, n=8; dairy, 0.02 \pm 0.02, n=8; CaCO₃, 0.02 \pm 0.02, n=7; *P*=0.06).

⁴¹Ca Kinetics

The total calcium content of the LAD, circumflex, and right coronary artery samples (in milligrams of calcium per gram of dry weight of tissue) did not differ across treatment groups (Table 5). Similarly, the ratio of ⁴¹Ca:Ca of the coronary arteries did not differ across the treatment groups (Table 5). Overall, 27 of the coronary artery samples (47% of the total samples) had values below the limit of detection due to the small amount of calcium present in the sample (0.020 to 6.780 mg); therefore, 2.5 mg Ca was added to all of these samples so that their ratios could be determined. The samples that had large enough ratios for successful assay averaged 0.387 mg Ca per sample, whereas those that ran below the detection limit averaged 0.172 mg Ca per sample. Because the amount of calcium in the original sample and the precise amount added to the sample was known, determination of ⁴¹Ca:Ca after a successful measurement was simple. A sample had to have twice the ratio of the average of the blank ratios to be considered above the detection limit. One pig from the control group was excluded from final kinetic analysis because it had calcium transport rates to the

coronary artery pools that were several hundred times higher than those of the other pigs. Pigs from the present study appeared to have coronary artery ⁴¹Ca:Ca similar to the pigs with metabolic syndrome from the pilot study and elevated coronary artery ⁴¹Ca:Ca values compared to those of the lean pigs from the previous study,²⁰ suggesting active CAD development because calcium is a component of atherosclerotic plaque. There were no differences in fractional transfer coefficients among treatment groups (data not shown), indicating that the serum curves for ⁴¹Ca:Ca did not differ (Figure 6). Because the serum is the source of ⁴¹Ca in arteries and the serum curves did not differ, the ⁴¹Ca:Ca ratios in the arteries correlate with, and thus represent, rates of transfer of calcium from blood to the artery.

Because of concerns with normality, the nonparametric Kruskal-Wallis procedure was also used to assess outcomes; however, similar results were observed with both the parametric and nonparametric procedures, so results of the original ANOVA procedure are reported throughout the paper.

Discussion

In this randomized feeding trial, elevated calcium intakes, intended to reflect human intakes of 2000 to 2500 mg/day, compared with adequate calcium intakes did not result in significantly worsened indices of cardiovascular function, coronary artery plaque burden, or CAC in Ossabaw miniature swine fed an atherogenic diet for 6 months. This was true whether the predominant source of calcium in the feed was nonfat dairy or CaCO₃.

The rationale for using the atherogenic diet was to induce the disease process; however, the study was designed so that outcomes were measured sufficiently early in disease development to determine whether disease severity was greater in pigs fed high calcium. Although the pigs showed the presence of CAD, there were no differences in severity between those on adequate versus high calcium diets. Pigs demonstrated similar end-of-treatment body weights, organ weights, serum triglycerides, and systolic and diastolic blood pressures, regardless of dietary condition. Serum HDL-C was elevated in the CaCO₃ group, whereas LDL-C, total cholesterol, and the ratio of LDL-C:HDL-C were elevated in the dairy group compared with the other 2 treatments. The elevation of selected lipid parameters observed in the dairy group is likely a result of the tendency for the pigs in this group to consume more of the atherogenic diet per day. In support of this hypothesis, serum total cholesterol concentration was demonstrated to be positively correlated with average daily food intake ($r=0.66$; *P*=0.002). The absence of greater CAD in the dairy group is especially profound because the dairy group had higher LDL-C, which is the major driving influence in CAD. There was no observed dietary effect on vascular function

Table 3. End-of-Treatment Phenotypic Characteristics of the Ossabaw Swine by Treatment Group

Parameter	Control (n=8)	Dairy (n=7)	CaCO ₃ (n=7)	P Value
Body weight, kg	88.70 (± 2.66)	97.53 (± 2.93)	93.96 (± 6.22)	0.31
Heart weight, g	207.14 (± 6.12)	220.39 (± 7.83)	216.47 (± 8.50)	0.44
Heart weight:body weight, g/kg	2.37 (± 0.08)	2.25 (± 0.07)	2.26 (± 0.08)	0.48
Systolic blood pressure, mm Hg	140 (± 4)	127 (± 5)	133 (± 9)	0.41
Diastolic blood pressure, mm Hg	95 (± 3)	92 (± 6)	95 (± 5)	0.93
Resting heart rate, bpm	81 (± 9)	86 (± 7)	80 (± 6)	0.84
Total-C, mmol/L	13.40 (± 0.92)	18.80 (± 1.06) [†]	12.96 (± 0.98)	0.001
HDL-C, mmol/L	1.62 (± 0.12)	1.63 (± 0.24)	2.30 (± 0.30) [*]	0.09
LDL-C, mmol/L	10.55 (± 0.99)	16.56 (± 1.14) [†]	10.48 (± 1.06)	0.001
LDL-C:HDL-C	6.91 (± 1.34)	11.84 (± 1.45) [*]	4.85 (± 1.34)	0.008
TG, mmol/L	1.11 (± 0.28)	1.55 (± 0.40)	1.13 (± 0.25)	0.61
Blood glucose, mmol/L	3.36 (± 0.23)	3.59 (± 0.19)	3.06 (± 0.28)	0.47
Creatinine, mmol/L	62.76 (± 7.96)	79.56 (± 7.96)	61.88 (± 9.72)	0.17
C-reactive protein, mg/L	52.08 (± 16.68)	61.89 (± 19.31)	41.81 (± 13.30)	0.71
Serum total Ca, mmol/L	2.62 (± 0.03)	2.62 (± 0.05)	2.61 (± 0.02)	0.97
Serum phosphorous, mmol/L	2.39 (± 0.05)	2.31 (± 0.05)	2.35 (± 0.11)	0.92
Parathyroid hormone, pg/mL [‡]	17.81 (± 8.80)	6.30 (± 0.93)	6.38 (± 0.08)	0.32
25-hydroxyvitamin D, nmol/L	52.50 (± 3.26)	52.60 (± 5.41)	52.00 (± 1.93)	0.99

Blood chemistry values were from fasted samples. Data are mean (\pm SEM). bpm indicates beats per minute; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; Total-C, total cholesterol.

* $P<0.05$, different from others. [†] $P<0.01$, different from others.

[‡]n=2 pigs per group.

determined by endothelium-dependent and -independent responses to bradykinin and sodium nitroprusside measured using *in vitro* wire myography. Plaque burden and percentage

of atherosclerotic and calcified plaque coverage in the coronary arteries did not differ among groups, as measured by intravascular ultrasound and histopathology, respectively.

Table 4. Ring Segment Characteristics and Relaxation Responses to Bk and SNP Using Ossabaw Swine LAD

Variables	Control (n=7)	Dairy (n=8)	CaCO ₃ (n=7)	P Value
Resting tension, g	5.52 (± 2.15)	5.50 (± 1.08)	5.44 (± 0.92)	0.99
PGF _{2α} tension, g	8.95 (± 2.02)	8.76 (± 1.43)	7.67 (± 0.97)	0.86
Length, mm	2.63 (± 0.28)	2.19 (± 0.13)	2.16 (± 0.13)	0.17
Outer diameter, mm	2.87 (± 0.28)	2.49 (± 0.09)	2.76 (± 0.10)	0.07
Inner diameter, mm	1.10 (± 0.12)	1.06 (± 0.12)	1.21 (± 0.12)	0.67
Thickness, mm	1.78 (± 0.17)	1.44 (± 0.15)	1.57 (± 0.10)	0.29
Inner diameter/thickness	0.68 (± 0.16)	0.82 (± 0.19)	0.80 (± 0.13)	0.75
EC ₅₀ Bk, log M	7.51 (± 0.62)	7.21 (± 0.42)	7.36 (± 0.56)	0.92
EC ₅₀ SNP, log M	7.12 (± 0.49)	6.93 (± 0.16)	6.66 (± 0.23)	0.61
Max Bk (% relaxation)	70.25 (± 7.81)	69.09 (± 8.64)	70.18 (± 8.99)	0.99
Max SNP (% relaxation)	120.10 (± 17.39)	115.82 (± 6.22)	114.23 (± 10.82)	0.94

Data are mean (\pm SEM). Bk indicates bradykinin; EC₅₀, half-maximal effective concentration; LAD, left anterior descending; PGF_{2 α} , prostaglandin F_{2 α} ; SNP, sodium nitroprusside.

Table 5. Calcium Content of Arteries

	Control	Dairy	CaCO ₃	P Value
Total artery calcium (mg/g tissue)				
LAD	6.91±1.21 (7)	6.38±1.16 (7)	5.95±1.44 (7)	0.38
RCA	5.41±1.23 (7)	2.92±0.35 (7)	5.82±2.82 (7)	0.10
CFX	4.84±1.42 (7)	4.00±1.65 (7)	3.94±0.58 (7)	0.87
Averaged for each pig	5.72±0.83 (7)	4.43±0.78 (7)	5.24±1.54 (7)	0.31
Artery ⁴¹ Ca:Ca ($\times 10^{-10}$)				
LAD	2.19±0.96 (5)	1.36±0.35 (4)	2.07±1.50 (3)	0.95
RCA	1.06±0.55 (5)	1.06±0.16 (5)	0.97±0.10 (4)	0.37
CFX	1.69±0.99 (2)	1.13±0.31 (6)	0.62±0.12 (3)	0.80
Averaged for each pig	1.90±0.63 (7)	1.21±0.25 (7)	1.17±0.33 (7)	0.44

Values are mean±SEM (n). CFX indicates circumflex; LAD, left anterior descending; RCA, right coronary artery.

All pigs that underwent CT scans had a CAC score of 0 because the disease was not advanced to a stage at which the calcified plaque was detectable by CT scan, the current cornerstone of CAC assessment in the clinical setting; however, coronary artery calcium tracer accumulation, detectable by accelerator mass spectrometry, demonstrated that coronary artery ⁴¹Ca:Ca was similar among treatment groups. Kinetic modeling demonstrated that these ratios were correlated with rates of calcium transport from the blood to the coronary arteries. These findings do not support the theory that high calcium intakes may adversely affect cardiovascular health by accelerating calcium deposition in the coronary arteries.

In the present study, pigs randomized to the high-calcium diets had fasted serum total calcium after 6 months similar to that of pigs on the control calcium diet. Serum calcium AUC_{0–720} and C_{max} appeared slightly elevated in the dairy and

CaCO₃ groups after feeding; however, this difference was not significant, likely due to the large variability in response observed between individual pigs. Although large doses of supplemental calcium (typically >500 mg) may elevate serum calcium above baseline levels for several hours in humans,^{33,34} this change is transient. In healthy adults, prolonged elevation of serum calcium is prevented by the combined hormonal actions of vitamin D, parathyroid hormone, and calcitonin that work to maintain serum calcium within a tightly regulated homeostatic range.³⁵ In healthy adults, 1000 mg of a single dose of calcium citrate has been demonstrated to acutely elevate serum calcium concentrations and subsequently improve pulse rate and the augmentation index relative to baseline.³⁶ The cardiovascular effects of this acute elevation in circulating calcium, if recurring on a daily basis over an extended period of time, are not well understood.

Through the use of the swine model, we were able to achieve controlled high-calcium feeding over a 6-month period and to use invasive methodology with the capability to detect early CAC. Relative to rodent and rabbit models of CAD, swine possess more anatomical and physiological similarities to humans including similar organ size and lipid metabolism.³⁷ Notably, having evolved on the isolated Ossabaw Island, the Ossabaw pig exhibits a thrifty genotype that allows for efficient accumulation of adipose tissue, dyslipidemia, hypertension, insulin resistance, and CAD when fed an atherogenic diet for several months.^{18,31,32}

An additional strength of this study was the use of a novel calcium tracer with sufficient sensitivity to detect atom quantities of early CAD-induced coronary artery calcium accumulation. A method with this sensitivity is crucial because >99% of calcium in the body is found in bone and relatively very little is deposited in soft tissues. Because the ⁴¹Ca tracer dose was given 1 month prior to the conclusion of the study,

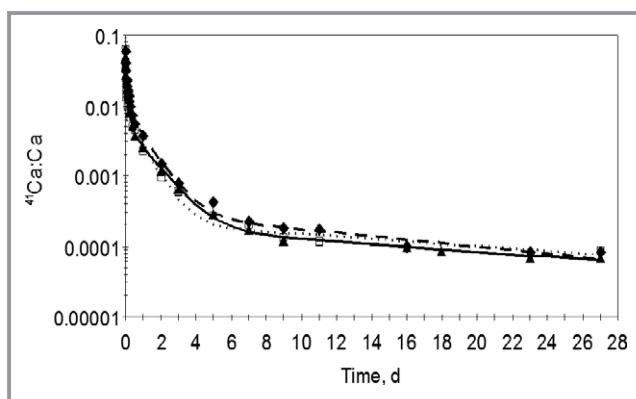


Figure 6. Serum ⁴¹Ca:Ca in a representative study pig from each treatment group over the 27 days after the tracer dose. The lines were calculated by the compartmental model,²⁰ and the symbols represent observed data. Solid line with triangles (control); dashed line with diamonds (dairy); dotted line with squares (CaCO₃).

coronary artery $^{41}\text{Ca}:\text{Ca}$ is a reflection of dietary-induced CAD and subsequent calcium deposition in these tissues during the final month of intervention. All pigs that underwent CT scans had no detectable CAC, whereas measurable amounts of ^{41}Ca tracer were found in the tissues, indicating that ^{41}Ca deposition can detect vascular calcification much earlier than the CT scan. Because the precise location of coronary artery plaque accumulation in disease states may differ, representative samples of 3 coronary arteries were examined. Limitations include the likely inability to generalize the findings to patients with chronic kidney disease, who often experience accelerated vascular calcification,³⁸ which is a likely result of prolonged elevation of serum phosphorous and bouts of hypercalcemia that occur when the kidneys inefficiently excrete these minerals. In addition, this study focused on mild to moderate atheromas, and because calcification progression is quite nonlinear and rather exponential, it cannot be excluded that more advanced atherosomas with complex calcium aggregates could exhibit different behavior than those in the present study.

Conclusions

The majority of evidence to date suggesting associations between supplemental calcium and cardiovascular risk in otherwise healthy adults comes from prespecified secondary analysis of studies designed to investigate bone outcomes or associational studies of calcium intake and CAC scores. Concern exists about the cardiovascular safety of calcium supplementation because no dose-response relationship or underlying physiological mechanism to explain this relationship has been demonstrated. Achieving recommended calcium intakes to promote bone health is often facilitated by calcium supplementation because dietary calcium intake remains low in many populations. This study used a novel calcium isotope and kinetic modeling to sensitively explore the causal relationship of an atherogenic diet enriched with calcium with early CAC in an animal model with a disease process that was more comparable to humans than models such as rodent or rat. The findings support the hypothesis that high consumption of calcium as nonfat dairy or CaCO₃ does not pose a cardiovascular risk through the acceleration of calcium deposition in the coronary arteries.

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Disclosures

Connie Weaver is on the scientific advisory board for Pharmavite LLC and Pfizer Consumer Health Care. All other authors: none.

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