Effects of Tomato and Soy Germ on Lipid Bioaccumulation and Atherosclerosis in ApoE$^{-/-}$ Mice

Brendon W. Smith, Rita J. Miller, Kenneth R. Wilund, William D. O'Brien Jr, and John W. Erdman Jr

Abstract: Dietary patterns with cardiovascular benefits have been recommended, but the relative contributions of individual foods and food components, alone or in combination, remain undefined. Male ApoE$^{-/-}$ mice were fed either a purified AIN-93G control diet, a Western diet (WD), or a WD with 10% tomato powder (TP), 2% soy germ (SG), or the combination, for 4 wk (n = 10 per group). Plasma total cholesterol and triglycerides were measured with enzymatic colorimetric kits, and serum amyloid A (SAA) was measured by ELISA. Liver lipids were extracted with chloroform:methanol, and triglycerides, free and esterified cholesterol measured with enzymatic colorimetric kits. Expression of Cyp27a1, Cyp7a1, Abcg5, and Abcg8 in the liver was determined by quantitative polymerase chain reaction. Sections of the aortic root and aorta were cut and stained with hematoxylin and eosin (H&E) to assess extent of atherosclerotic lesions. WD-fed animals had greater liver and adipose weights, plasma cholesterol and SAA, hepatic lipids, and atherosclerosis than AIN-93G animals. TP and SG did not decrease atherosclerosis as measured by H&E-stained sections of the aortic root, aortic arch, and descending aorta. The TP diets further increased plasma cholesterol, but also led to increased expression of the Abcg5/8 transporters involved in cholesterol efflux. Addition of SG alone to the WD attenuated WD-induced increases in plasma cholesterol, liver lipids, and gonadal adipose weight. The results of this study do not support the use of either TP or SG for reduction of atherosclerosis, but suggest some beneficial effects of SG on lipid metabolism in this model of cardiovascular disease.

Keywords: atherosclerosis, cardiovascular disease, diet, nutrition, soy, tomatoes

Practical Application: Cardiovascular disease (CVD) is the leading cause of death in the United States, claiming nearly 800000 lives per year. Diet has been acknowledged as an important determinant of CVD risk, but effective combinations of specific nutritional components remain to be identified. This study evaluated the cardiovascular benefits of tomato and soy germ (SG). Neither intervention decreased atherosclerosis, but SG favorably affected some CVD risk factors.

Introduction

Cardiovascular disease (CVD) claims nearly 800000 lives per year in the United States (Go and others 2013), and remains the leading cause of death despite significant medical advances. Atherosclerosis is a complex pathological process that drives the development of CVD, characterized by entrance of cholesterol-containing lipoprotein particles into the arterial wall, with subsequent inflammation and formation of lipid-rich plaques (Libby and others 2011). Atherosclerosis progresses silently throughout life, manifesting itself during adulthood in heart attacks and strokes. Diet has been acknowledged as an important determinant of CVD risk (Mozaffarian and others 2011). In epidemiological studies, adherence to a healthy diet rich in fruits, vegetables, legumes, nuts, and whole grains has been shown to reduce risk of CVD by up to 80% (Stampfer and others 2000) and reduce mortality if CVD is already present (Iestra and others 2005). Although the benefits of this dietary pattern are clear, effective combinations of specific nutritional components remain to be identified. Evidence points to potential cardiovascular benefits of tomato (Willcox and others 2003; Sesso 2006) and soy germ (SG; Clerici and others 2007; Clerici and others 2011), but there is a need for controlled animal studies to evaluate their effects on atherosclerosis.

Although it would be interesting to study the impact of either tomato or SG individually, evaluation of both in combination is a particularly novel aspect of this study. Tomato was selected based on previous literature suggesting its beneficial cardiovascular effects, and provided as tomato powder (TP). Consumption of lycopene, a bioactive tomato carotenoid pigment, has been shown to improve endothelial function in a randomized controlled trial (Kim and others 2011), and an inverse relationship between serum lycopene concentration and arterial stiffness has also been noted (Kim and others 2010). Whole tomatoes contain many other phytochemicals and micronutrients that can benefit cardiovascular health (Canene-Adams and others 2005), so tomatoes can be provided in the diet as a more practical approach than lycopene supplementation. Additionally, previous work has demonstrated greater health benefit to consuming whole tomatoes instead of lycopene supplements (Canene-Adams and others 2007). Although some studies have suggested cardiovascular benefits of tomato consumption, most experts agree that the literature is not conclusive and that more research is needed (Arab and Steck 2000; Sesso 2006; Riccioni 2009; Mordente and others 2011).

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Although most soy foods such as tofu, soy milk, and soybean protein isolates are very high in the isoflavone genistein, SG, the hypocotyledon of the soy bean, is especially rich in daidzein and glycitein and low in genistein (ratio of 200:156:45). The SG is generally removed during soy processing to produce soy isolates and concentrates and is sold as a dietary supplement. SG was selected as an additional dietary ingredient based on clinical trial data (Clerici and others 2007; Clerici and others 2011), as well as prior literature on the hypocholesterolemic effects of other soy products (Erdman 1995; Potter 1995). Although SG has not been previously tested for its effects on atherosclerosis, numerous studies have suggested anti-atherosclerotic effects of soy isoflavones (Nagarajan 2010), including reduced monocyte activation (Nagarajan and others 2006; Babu and others 2012), and increased excretion of dietary cholesterol (Potter 1995). These effects may be dependent on direct activation of estrogen receptor α by isoflavones (Adams and others 2002), and other studies have shown that isoflavones without soy protein reduce atherosclerosis in cholesterol-fed rabbits (Yamakoshi and others 2000) and ApoE$^{-/-}$ mice (Sato and others 2007). It has also been suggested that the effects of soy isoflavones are mediated through increases in LDL receptor activity (Kirk and others 1998). We hypothesized that TP and SG, especially in combination, would decrease atherosclerosis and lipid bioaccumulation in ApoE$^{-/-}$ mice.

Table 1–Composition of experimental diets.

Ingredient (g/kg)	AIN-93G	WD	WDSG	WDTP	WDTPSG
Casein	200	195	184.9	180.6	170.5
L-Cystine	3	3	3	3	3
Corn starch	397.5	55.46	55.46	43.26	43.26
Maltodextrin	132	60	60	60	60
Sucrose	100	340	340	298.8	298.8
Soybean oil	70	20	15.2	15.14	10.34
Cellulose	50	50	44.9	22.66	17.58
Mineral mix	35	43	43	43	43
Vitamin mix	10	19	19	19	19
Choline bitartrate	2.5	3	3	3	3
TBHQ	0.014	0.04	0.04	0.04	0.04
Anhydrous milkfat	0	210	210	210	210
Cholesterol	0	1.5	1.5	1.5	1.5
Tomato powder	0	0	0	100	100
Soy germ	0	0	20	0	20
Total	1000	1000	1000	1000	1000
Fat (%, w/w)	7.2	23.2	23.2	23.2	23.2
Protein (%, w/w)	17.4	17.0	17.0	17.0	17.0
CHO (%, w/w)	58.3	44.7	44.7	44.7	44.7
kcal/g	3.68	4.55	4.55	4.55	4.55
Fat (%, kcal)	17.6	45.8	45.8	45.8	45.8
Protein (%, kcal)	18.9	14.9	14.9	14.9	14.9
CHO (%, kcal)	63.4	39.3	39.3	39.3	39.3

WD, Western diet; WDSG, Western diet with 2% soy germ; WDTP, Western diet with 10% tomato powder; WDTPSG, Western diet with 10% tomato powder and 2% soy germ.

Materials and Methods

Experimental diets

TP (Drum Dried -20; FutureCeuticals, Momence, Ill., U.S.A.) and SG (SoyLife Complex Regular; Frutarom, Londerzeel, Belgium) were added to a Western diet (WD; TD.10885; Harlan Teklad, Madison, Wis., U.S.A.). Composition of the experimental diets is given in Table 1. All WDs were balanced for total energy, protein, fat, digestible carbohydrates, and fiber. For a non-Western control diet comparison, an AIN 93G diet was included in the trial ($n = 10$).

Carotenoid analysis

To minimize degradation of the heat-labile carotenoids, diets were pelleted without heat and air-dried overnight. Diets were analyzed for carotenoid content prior to and after pelleting. Additionally, 12 g of diet were left in a mouse cage in the animal facility with bedding (no water bottle or animal) for 3 d to simulate experimental conditions. Powdered diet was left in plastic feed bowls on the cage floor, and pellets were left in the hopper on top of the cage. Carotenoids were extracted from diets with hexane and quantified by HPLC using a 150 × 4.6 mm YMC

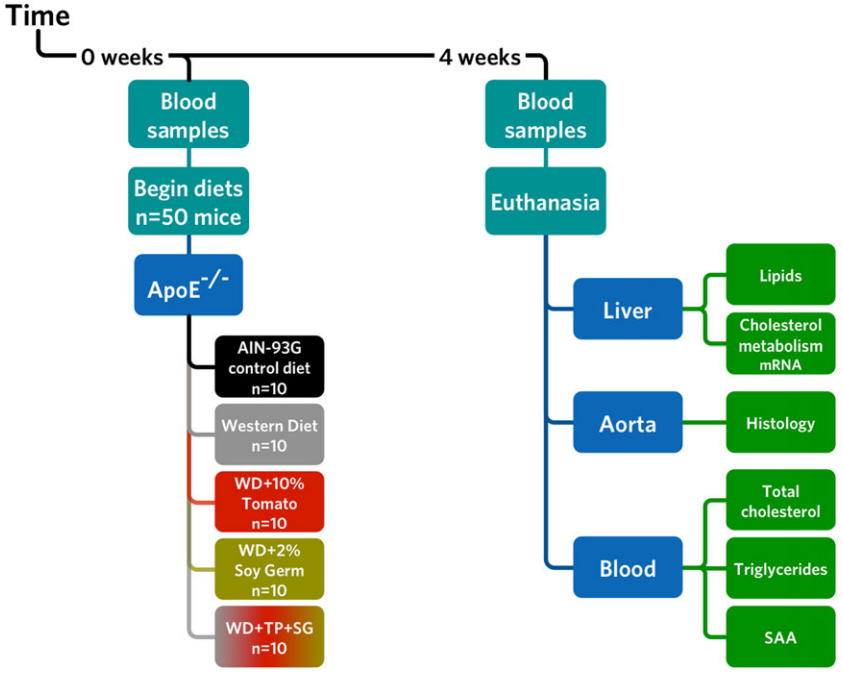

Figure 1–Experimental design. Male ApoE$^{-/-}$ mice (8-weeks old) were fed their respective diets for 4 wk, and tissues were then collected and analyzed.

C30 column (3 μm particle size) with a modified Yeum gradient method (Yeum and others 1996) as previously described (Boileau and others 2000).

Animals

The Univ. of Illinois Institutional Animal Care and Use Committee approved all live animal procedures. Male ApoE$^{-/-}$ mice ($n = 50$, 8-weeks old, Figure 1) were allowed to acclimate to the animal facility for ≥2 d, and 120 μL baseline blood samples were collected from the submandibular region by puncture with a 5 mm lancet (Goldenrod; MEDIpoint Inc., Mineola, N.Y., U.S.A.) into an EDTA-coated capillary tube (Microvette; Sarstedt AG & Co., Nümbrecht, Germany), centrifuged at 2500 × g and 4 °C for 10 min, aliquoted and frozen at –50 °C. Mice were then randomized to 1 of 5 experimental diets: AIN-93G control (AIN), WD, WD with 2% SG (WDSG), WD with 10% TP (WDTP), or WD with 10% TP and 2% SG (WDTPSG), with 10 mice per diet, and food replenished 3 times per wk (35 to 40 g total, adequate for freely intake) for 4 wk. Mice were individually housed and given free access to water. Feed intake was measured each time the animals were fed and body weights were measured weekly. After 4 wk, mice were fasted overnight (10 to 14 h), and 300 μL blood samples were collected from the submandibular region. Mice were then euthanized with CO_2 and perfused with 20 mL cold PBS through the left ventricle, and the vena cava was cut to allow drainage of fluid. Liver and gonadal adipose were removed, sectioned, frozen by immersion in liquid nitrogen, and stored at –50 °C. A portion of the liver was added to 5 mL RNA preservation solution (RNAlater; QIAGEN, Valencia, Calif., U.S.A.) for 24 h at 4 °C, then blotted dry and stored at –50 °C. The heart was excised with 1 mm aorta attached. The bottom half was removed with careful attention to the orientation of the aorta. The top half was then immersed in OCT inside a 12 × 12 × 20 mm tapered cryomold (Electron Microscopy Sciences, Hatfield, Pa., U.S.A.), frozen in 2-methylbutane surrounded with dry ice, wrapped in parafilm, and stored at –50 °C. The rest of the aorta was dissected into 14 to 15 pieces, fixed in neutral buffered formalin (NBF), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

Histology

Serial 10 μm cryosections of the aortic root (Daugherty and Whitman 2002) were cut from OCT-embedded samples at –20 °C using a cryostat (Leica Microsystems, Nussloch, Germany) and mounted on charged microscope slides (Superfrost Plus Gold; Fisher Scientific, Pittsburgh, Pa., U.S.A.). A set of 6 microscope slides was created for each sample, with 6 sections per slide. Sections were allowed to dry and adhere to the slides for 30 to 60 min, fixed in NBF for 5 min, dipped in water for 1 min, allowed to dry for 60 min, and stored at –20 °C until staining. Slides were then stained with H&E. Images were captured using an Olympus BX51 microscope with DP25 camera. Six serial sections with all 3 aortic valve leaflets visible were quantified by manually tracing lesion area using the DP2 program (Olympus America Inc., Center Valley, Pa., U.S.A.). Only foam cell lesions underneath the valve leaflets were included. To quantify the extent of atherosclerotic lesions in the remainder of the aorta (from 1 mm distal to the aortic root to the renal bifurcation), the aorta was isolated and sectioned as described above. Lesion area was determined for each of the 14 to 15 pieces and summed for total lesion area. The arch lesion area was based on sections of the first 3 pieces.

Blood analysis

Total cholesterol and triglycerides were measured using enzymatic colorimetric kits (Wako Chemicals, Richmond, Va., U.S.A.) with samples plated in triplicate and human control sera (Wako) included in each assay to evaluate the reproducibility of measurement. Intra- and interassay coefficients of variation for mouse plasma samples were 2.3% and 2.4% for total cholesterol, and 2.7% and 4.0% for triglycerides. Intra- and interassay coefficients of variation for control sera were 2.4% and 2.9% for total cholesterol, and 1.3% and 3.4% for triglycerides. Serum Amyloid A (SAA) was measured by ELISA (Life Technologies, Grand Island, N.Y., U.S.A.). The antibodies in the assay react with murine SAA1 and SAA2. Intra- and inter-assay precision were 5.2% and 7.7%.

Liver lipid analysis

Total lipids were extracted from an average of 0.42 g liver with chloroform:methanol and quantified by weight as previously described (Folch and others 1957; Smith and others 2012). Lipids were then resuspended in the cholesterol assay buffer by vortexing and homogenization, and assayed for triglycerides and free/esterified cholesterol with enzymatic colorimetric kits (BioVision, Milpitas, Calif., U.S.A.).

Gene expression analysis

Total RNA was isolated from liver tissue using TRIzol (Life Technologies) and the RNeasy kit (QIAGEN). RNA purity was determined by spectrophotometry at 260, 280, and 230 nm (BioSpectrometer Basic with μcuvette G1.0 1 mm; Eppendorf, Hamburg, Germany). RNA integrity was determined by agarose gel electrophoresis, with ethidium bromide (Sigma-Aldrich, St. Louis, Mo., U.S.A.) used as a fluorescent agent to visualize RNA. An average of 11.0 mg liver tissue yielded 544 ng/μL RNA, with average 260/280 of 1.9 and 260/230 of 2.2. Complementary DNA was synthesized from RNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), with reactions performed in a thermal cycler (Eppendorf). Quantitative polymerase chain reaction was carried out in an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, Calif., U.S.A.) using Taqman probes (Life Technologies; assay IDs: Mm00470430_m1 for sterol 27 hydroxylase [Cyp27a1], Mm00484150_m1 for cholesterol 7α hydroxylase [Cyp7a1], Mm00446241_m1 for ATP-binding cassette transporter G5 [Abcg5], Mm00445980_m1 for ATP-binding cassette transporter G8 [Abcg8], Mm02342430_g1 for peptidylprolyl isomerase A [Ppia]) in duplex, with the reference gene (Ppia) in a primer-limited formulation, and the QuantiNova probe kit (QIAGEN). Gene expression was normalized to Ppia and the AIN group using the $\Delta\Delta C_T$ method (Schmittgen and Livak 2008).

Statistical analysis

Statistical analysis was performed in SAS 9.3 (SAS Inst., Inc., Cary, N.C., U.S.A.) for Windows, and graphs were created in SigmaPlot 12.3 for Windows. Differences among means were determined using analysis of variance (ANOVA) and analysis of covariance (ANOCOVA) in the general linear mixed models procedure, with repeated measures testing for serial measurements. Covariance structures were compared graphically and quantitatively, and an appropriate covariance model was fit. Normality was assessed with the Shapiro–Wilk W-test in the univariate procedure. Data that did not meet the necessary assumptions were transformed. Each model was first run with all fixed effects and covariates of interest, and then reduced to eliminate nonsignificant

variables. When a significant result was reported for a fixed variable with more than 2 categories, specific comparisons were made using estimate statements based on the least squares means, and significance values were adjusted for multiple comparisons with the Tukey–Kramer or Bonferroni methods. Regression models were evaluated for normality (Shapiro–Wilk W), heterogeneity of residual variances (statistically by Spearman rank-order correlation between absolute values of residuals and predicted values, and graphically by plotting residuals compared with predicted values), serial correlation (Durbin–Watson D statistic), and influential observations (Cook's D statistic).

Results and Discussion

Histology

This study evaluated the ability of dietary TP and/or SG to reduce atherosclerosis in ApoE$^{-/-}$ mice. Consumption of TP and or SG did not reduce atherosclerotic lesion area in the aortic root or the rest of the aorta. Aortic root lesion areas were 35500 for AIN, 92400 for WD, 98700 for WDSG, 101000 for WDTP, and 129000 μm^2 for WDTPSG. When normalized to the total inner area of the aortic root, all WD groups had significantly greater percent lesion area than AIN control group, but were not different from each other (3.7% lesion area for AIN, 10.4% and $P < 0.05$ for WD, 10.7% and $P < 0.005$ for WDSG, 12.5% and $P = 0.05$ for WDTP, 13.9% and $P < 0.05$ for WDTPSG; by ANOVA with Bonferroni adjustment for multiple comparisons; Figure 2A). Lesion area in the remainder of the aorta was significantly greater than AIN controls in the WDSG ($P < 0.01$), WDTP ($P < 0.01$), and WDTPSG ($P < 0.05$) groups, but not different among WD groups (Figure 2B). Data were log-transformed to meet the assumption of normality. No differences were seen among groups in aortic arch lesion area. Linear regression analyses revealed weak but significant correlations between lesion area in the aortic root and the rest of the aorta ($F_{0.05}[1,32] = 11.42$, $r^2 = 0.26$, $P < 0.005$ for raw aortic root values; $F_{0.05}[1,32] = 11.00$, $r^2 = 0.26$, $P < 0.005$ for normalized aortic root values), between plasma total cholesterol and aortic root lesion area ($F_{0.05}[1,32] = 8.79$, $r^2 = 0.22$, $P < 0.01$ for raw values; $F_{0.05}[1,32] = 15.39$, $r^2 = 0.32$, $P < 0.001$ for normalized aortic root values; aortic root lesion area dependent variables were log-transformed to correct heterogeneity of residual variances), and between plasma total cholesterol and aorta lesion area ($F_{0.05}[1,42] = 14.98$, $r^2 = 0.26$, $P < 0.001$; the aorta lesion area dependent variable was log-transformed to correct non-normality and heterogeneity of residual variances). Feed intake did not contribute significantly to the normalized aortic root lesion area statistical model ($P = 0.13$), nor was it correlated with normalized aortic root lesion area ($F_{0.05}[1,32] = 3.37$, $r^2 = 0.10$, $P = 0.08$).

The aortic root was the most appropriate region to evaluate for this study, because it is one of the first sites to develop atherosclerotic lesions, and the animals in this study were at an early stage of atherosclerosis (Nakashima and others 1994). The 2 gold-standard methods for atherosclerosis assessment are aortic root frozen sections, and *en face* preparations of the aorta (Daugherty and Whitman 2002; Meir and Leitersdorf 2004). The aortic root method generates a tissue cross-section for evaluation of lesion morphology and composition, but does not demonstrate lesion area in the entire aortic tree. The *en face* method allows quantitative determination of aorta surface area lesion coverage, but does not provide information on lesion depth or morphology. The 2 methods can thus be seen as providing complementary information. The method of measuring lesion area in serial cross-sections of the aortic arch and descending aorta used in this study addressed limitations of the *en face* method by allowing measurement of lesion cross-sectional area throughout the aorta. Some studies have shown plasma total cholesterol levels to be correlated with *en face* lesion area (Veniant and others 1997; Wilund and others 2004), but others have reported that they are not well-correlated (Zhang and others 1994). Studies also report that aortic root lesion

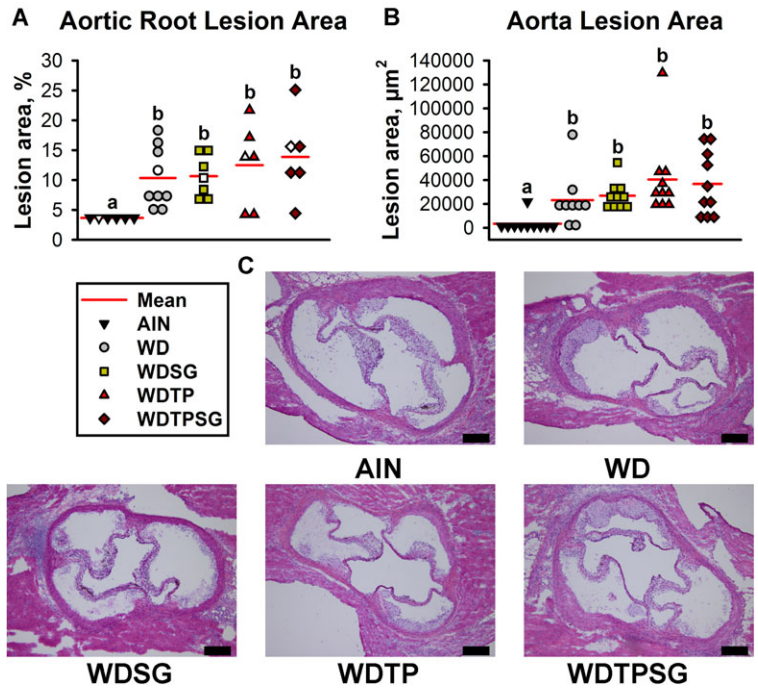

Figure 2–Atherosclerosis in the aorta. (A) Dot density plot of atherosclerotic lesion area in the aortic root. Frozen sections of the aortic root were cut from OCT-embedded tissue samples and stained with H&E. Lesion area was manually traced in 6 serial sections per animal, normalized to the total inner area of the aortic root, and averaged to provide a value for each animal. Each dot represents the normalized lesion area for 1 animal, group means are represented by red lines, and means not sharing a letter are significantly different. Symbols with white fill are the representative samples shown in C. (B) Dot density plot of atherosclerotic lesion area in the aorta. Aortas from the arch to the renal bifurcation were divided into 14 to 15 pieces, fixed in 10% neutral buffered formalin, and embedded in paraffin. Cross-sections of each piece of the aorta were stained with H&E, and lesion area was manually traced. Each dot represents the total lesion area for 1 animal. (C) Representative H&E stained aortic root sections. Scale bars are 200 μm.

Table 2–Lycopene profiles of experimental diets.

Sample	At+5c (%)	Other cis (%)
100% TP	88	12
WD + TP + SG pellet	80	20
WD + TP + SG powder	79	21
WD + TP + SG pellet 3 d	78	22
WD + TP + SG powder 3 d	80	20

Lycopene isomer content was measured by HPLC. For the "3 day" diet samples, 12 g of diet were left in a mouse cage in the animal facility with bedding (no water bottle or animal) for 3 d. Powdered diet was left in plastic feed bowls on the cage floor, and pellets were left in the hopper on top of the cage. Each bar shows the mean + SEM of triplicate measurements. WD, western diet; TP, tomato powder; SG, soy germ.

area is correlated with lesion area in the whole aorta measured by the *en face* method (Tangirala and others 1995; Veniant and others 1997; Meir and Leitersdorf 2004), but this may depend on the stage of atherosclerosis, as lesions appear earliest in the aortic root (Nakashima and others 1994; Meir and Leitersdorf 2004). The weak correlations observed in this study may be due to the early stage of atherosclerosis, when aortic root lesions are most prominent.

Animals and diets

The amounts of TP and SG were chosen based on our previous study (Zuniga and others 2013) that resulted in bioaccumulation of tomato and soy bioactive components and metabolites at levels corresponding to those found in humans consuming soy and tomatoes. TP consumption (10%, w/w) by the mice in this study is comparable to 1 cup tomato sauce, half cup tomato paste, or 6 cups raw tomatoes per day in humans (van Breemen and others 2002; Grainger and others 2008; Zuniga and others 2013). SG consumption (2%, w/w) results in similar serum isoflavone concentrations to men consuming soy foods or isoflavone supplements (Gardner and others 2009; Zuniga and others 2013). The diet pelleting process did not impact carotenoid levels or isomerization (Table 2).

All mice gained weight progressively during the study ($P < 0.0001$ by ANOCOVA with baseline body weight as covariate; Figure 3A). After 2 wk, mice in the WDSG, WDTP, and WDTPSG groups were significantly heavier than AIN diet-fed control mice ($P < 0.01$ after Bonferroni adjustment). These differences persisted after 3 and 4 wk, with WDTPSG animals also having greater body weights than WD animals ($P < 0.05$). WD-fed animals had greater liver weight than AIN animals ($P < 0.01$ by ANOVA; Figure 3B), and no differences were seen among WD groups. WD animals experienced increased gonadal adipose weights (WD $P < 0.05$, WDTP and WDTPSG $P < 0.0001$ by ANOVA), but the WDSG group had an attenuated increase. WDSG adipose weights were not significantly different from AIN control diet group ($P = 0.21$) or WD ($P = 0.69$), but significantly less than WDTP and WDTPSG ($P < 0.05$). Gonadal adipose is a major adipose depot, comprising about 30% of dissectible adipose in BL/6 mice (Chesler and others 2014; The Jackson Laboratory 2014).

Animals consumed 3 to 4 g feed (12 to 18 kcal) per day (Figure 3C and D). Animals in WD groups consumed significantly more kcal per day than the 12.7 kcal/day average for animals fed AIN-93G (14.3 kcal/day and $P < 0.05$ for WD, $P < 0.0001$ for WDSG [16.4 kcal/day], WDTP [15.8 kcal/day], and WDTPSG [17.7 kcal/day] by repeated-measures ANOVA; all feed intake P values adjusted with a Tukey test). WDSG ($P < 0.05$) and WDTPSG ($P < 0.0005$) animals consumed more feed than WD animals, but WDTP animals did not ($P = 0.27$). Among the tomato and soy groups, WDTPSG animals consumed more than WDTP ($P < 0.05$) but not WDSG ($P = 0.35$). Feed intake was greater overall in week 4 than week 1 ($P < 0.05$). The diet × time-point interaction variable was not significant ($P = 0.69$), preventing more detailed subcomparisons.

Blood analysis

There were no differences between groups regarding plasma total cholesterol at baseline ($P = 0.23$ by repeated-measures

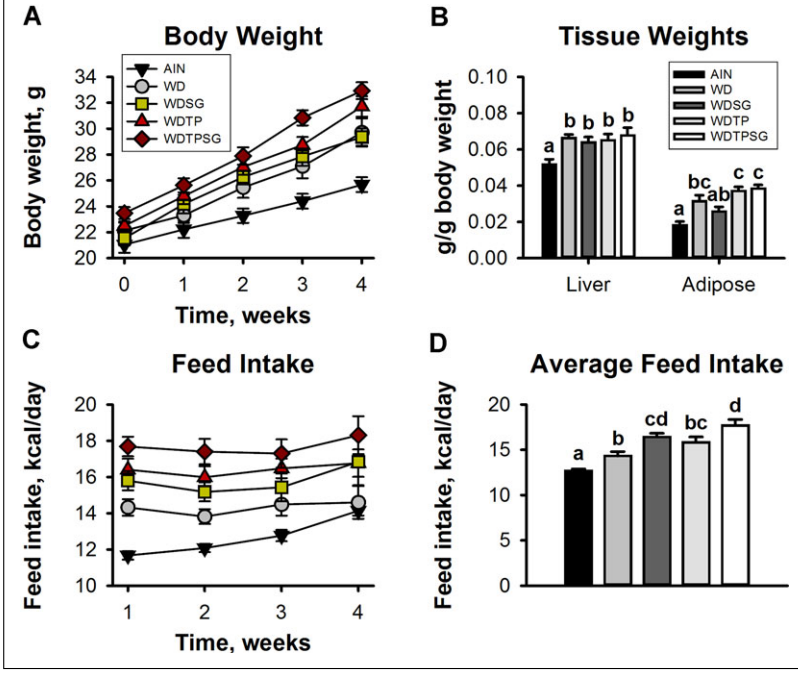

Figure 3–Feed intake, body and tissue weights. Feed intake was measured 3 times per wk, body weights measured weekly, and liver and gonadal adipose weights were measured at euthanasia. Means not sharing a letter are significantly different. Errors bars are SEM.

ANOVA; Figure 4A). After 4 wk on experimental diets, plasma cholesterol levels increased significantly within all groups ($P < 0.0001$ for time and diet × time interaction), and were significantly higher in all WD groups than AIN group ($P < 0.0001$ for all WD animals compared with AIN-93G). Cholesterol levels in WDTP and WDTPSG-fed animals were higher still ($P < 0.0001$), contrary to our hypothesis, but the WDSG group did not experience this increase ($P = 0.47$) despite consuming significantly more diet than WD animals.

Circulating triglycerides in ApoE$^{-/-}$ mice are only moderately higher than in WT mice (Zhang and others 1992; Zhang and others 1994; Veniant and others 1997), and in this study, did not increase further in WD-fed animals (Figure 4B). The results of this study indicate that ApoE$^{-/-}$ mice on WD have elevated liver lipids, elevated blood cholesterol, but not elevated blood triglycerides, as compared to ApoE$^{-/-}$ mice fed the AIN diet. Triglycerides in circulating lipoproteins are removed in extra hepatic tissues by lipoprotein lipase, which is present on capillary endothelial cells (Wang and Eckel 2009), and at the liver by hepatic lipase. Although ApoE$^{-/-}$ mice have defective hepatic lipoprotein uptake, they do not have defective lipolysis, so the triglycerides may be hydrolyzed to free fatty acids and taken up by liver and other tissues, producing atherogenic remnant lipoproteins (Zhang and others 1992; Goldberg and others 2011). Once these dietary fatty acids reach the liver, they may be used to esterify cholesterol for export in lipoproteins (Baum and others 2012). Contributions to hepatic lipid content, which was elevated in WD fed animals, are made by dietary fat intake, by *de novo* lipogenesis in response to dietary fructose (Samuel 2011), and by re-esterification of adipose-derived circulating free fatty acids.

We chose to measure SAA as a circulating biomarker of systemic inflammation. There are several isoforms of SAA, each with their own characteristics. SAA1 and SAA2 are similar isoforms that are responsive to dietary interventions. High fat and cholesterol feeding increase circulating SAA1 (Lewis and others 2004; Tannock and others 2005), whereas green and yellow vegetables decrease SAA1 and SAA2 (Adams and others 2006). SAA1 and SAA2 levels are similar in healthy humans and mice (Godenir and others 1985). SAA3 is another isoform secreted by macrophages (Meek and others 1992) and adipocytes (Chiba and others 2009) that does not contribute to serum levels of SAA1 (Chiba and others 2009). SAA3 is a pseudogene in humans but is an actual acute phase protein in mice. Based on this information, SAA1 and SAA2 were the most appropriate isoforms to measure. In this study, baseline SAA levels were not different among groups at the beginning of the study ($P = 0.54$ by repeated-measures ANOVA with log-transformed data to meet the assumption of normality, Figure 4C), and increased within all groups after 4 wk compared with initial levels ($P < 0.0001$). At 4 wk, WD groups had significantly higher SAA than the AIN-93G group ($P < 0.0001$). Bacterial infection increases circulating SAA to 350 μg/mL in C57BL/6 mice (Amar and others 2007), suggesting that the 60 to 100 μg/mL seen in WD animals in this study is indicative of low-grade systemic inflammation. The lack of effect of TP and SG on SAA suggests the dietary intervention was not able to abrogate systemic inflammation in this mouse model.

Liver lipids and gene expression

As expected, total liver lipids were greater in WD animals than in AIN-93G animals (83.8 compared with 162 mg/g liver, $P < 0.01$ by ANOVA with Tukey–Kramer adjustment for multiple comparisons; Figure 5A). WDTP (167 mg/g, $P < 0.001$) and WDTPSG (185 mg/g, $P < 0.001$), but not WDSG animals (126 mg/g, $P = 0.12$), also had significantly higher liver lipids than AIN control-fed animals. Similarly, liver free cholesterol was elevated in WD ($P < 0.05$ by ANOVA with Tukey–Kramer adjustment for multiple comparisons), WDTP ($P < 0.01$), and WDTPSG ($P < 0.05$) groups, but this increase was attenuated in the WDSG group ($P = 0.24$, Figure 5B).

The attenuated increases in plasma cholesterol, liver lipid accumulation, and adipose weight in WDSG animals led us to hypothesize that SG was affecting cholesterol excretion. The primary

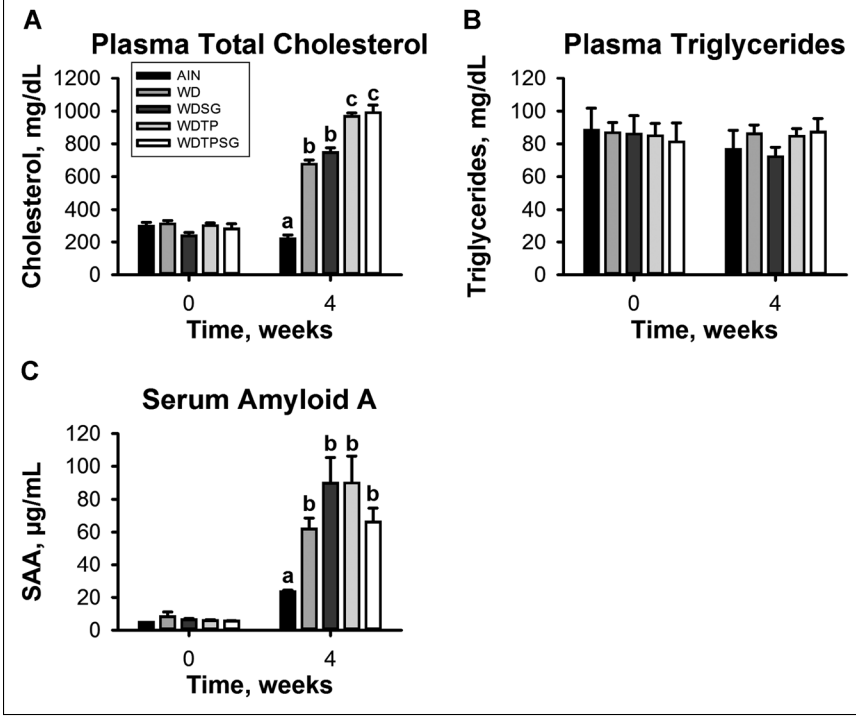

Figure 4–Plasma biomarkers. Plasma total cholesterol and triglycerides were measured with enzymatic colorimetric kits, and serum amyloid A was measured by ELISA. Means not sharing a letter are significantly different. Errors bars are SEM.

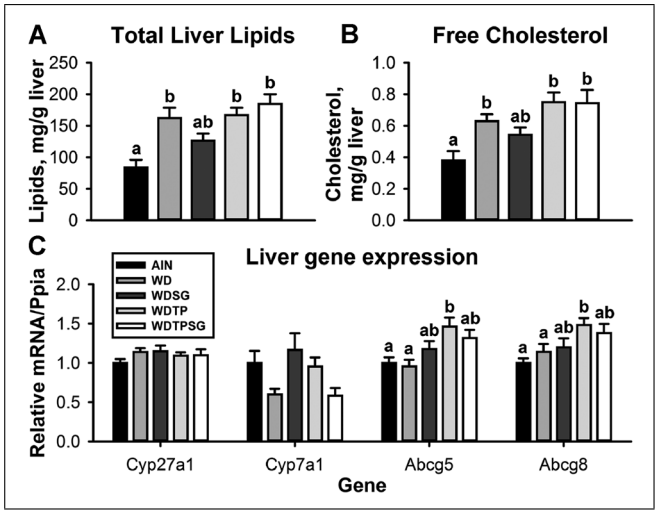

Figure 5–Liver lipids and gene expression. (A) Liver lipids were extracted with chloroform:methanol and total lipids determined by weight. (B) Free cholesterol was measured in the lipid extracts with an enzymatic colorimetric kit. (C) Gene expression was measured by quantitative real-time polymerase chain reaction and normalized with the $\Delta\Delta C_T$ method. Means not sharing a letter are significantly different. Errors bars are SEM.

route of cholesterol disposal from the liver is by secretion into bile, either as bile acids or free cholesterol (Bonamassa and Moschetta 2013). We measured gene expression of Cyp7a1 and Cyp27a1, 2 enzymes involved in bile acid synthesis, and Abcg5/8, which form a heterodimer to efflux cholesterol from the liver into bile. SG did not significantly impact expression of these genes (Figure 5C). The regulation of bile acid metabolic enzymes such as CYP7A1 may occur at post-transcriptional levels, including mRNA stability and enzyme activity (Davis and others 2002). There was a modest, yet significant, induction of Abcg5/8 transcription in the WDTP group ($P < 0.05$ by ANOVA with Tukey–Kramer adjustment for multiple comparisons). This suggests that TP may be able to influence cholesterol efflux, though not enough to overcome the hyperlipidemia of the WD-fed ApoE$^{-/-}$ mouse model.

Conclusions

This study evaluated dietary TP and SG, added alone or in combination to a high fat, high sugar, cholesterol-containing WD, for reduction of atherosclerosis in ApoE$^{-/-}$ mice. It is important to note that addition of TP and SG to a WD for ApoE$^{-/-}$ mice can be used as a dietary approach to attenuate or slow atherosclerosis. Development of atherosclerosis is inevitable in these mice due to their genetic modification, but the rate of progression of atherosclerosis might be modified. Addition of SG alone to the WD attenuated WD induced increases in plasma cholesterol, liver lipids and adipose, while addition of tomatoes alone increased expression of the Abcg5/8 transporters involved in cholesterol efflux. Overall, however, TP and SG did not decrease atherosclerosis as assessed by histopathology. This is probably not due to the amounts of tomato and soy added to the diets, because the dose levels used have been previously shown to result in tissue and blood accumulation of their biologically active components. The lack of effect of the dietary intervention on atherosclerosis is likely due to a combination of factors, with 2 explanations being most probable. First, the dietary intervention may not have been long enough. We selected an earlier timepoint in light of the relatively modest effect sizes of most dietary interventions, but it may be that differences among groups would become more pronounced over a longer period of time. Second, the extreme phenotype of the WD-fed ApoE$^{-/-}$ mouse may have masked any beneficial effects. The addition of TP and SG to an AIN-93G diet instead of a WD should also be considered. This would lead to a milder atherosclerotic phenotype that could be more amenable to dietary interventions. Although soy and its products have established cardiovascular benefits, the cardiovascular effects of tomatoes are currently unclear. This study does not support the use of either tomatoes or SG for reduction of atherosclerosis, but suggests that SG may have positive metabolic effects.

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Authors' contributions

Authors Smith, Wilund, O'Brien, and Erdman designed and planned experiments; Smith and Miller performed animal experiments; Smith performed blood, tissue, and data analyses; Smith wrote manuscript; Miller, Wilund, O'Brien, and Erdman edited manuscript.

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