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Paradoxical effect of a pequi oil-rich diet on the development of atherosclerosis: balance between antioxidant and hyperlipidemic properties

E.C. Aguilar[1], T.L. Jascolka[2], L.G. Teixeira[1], P.C. Lages[1], A.C.C. Ribeiro[1], E.L.M. Vieira[1], M.C.G. Peluzio[3] and J.I. Alvarez-Leite[1]

[1]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[2]Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[3]Departamento de Nutrição, Universidade Federal de Viçosa, Viçosa, MG, Brasil

Abstract

Pequi is the fruit of *Caryocar brasiliense* and its oil has a high concentration of monounsaturated and saturated fatty acids, which are anti- and pro-atherogenic agents, respectively, and of carotenoids, which give it antioxidant properties. Our objective was to study the effect of the intake of a cholesterol-rich diet supplemented with pequi oil, compared to the same diet containing soybean oil, on atherosclerosis development, and oxidative stress in atherosclerosis-susceptible LDL receptor-deficient mice ($LDLr^{-/-}$, C57BL/6-background). Female mice were fed a cholesterol-rich diet containing 7% soybean oil (Soybean group, N = 12) or 7% pequi oil (Pequi group, N = 12) for 6 weeks. The Pequi group presented a more atherogenic lipid profile and more advanced atherosclerotic lesions in the aortic root compared to the Soybean group. However, the Pequi group presented a less advanced lesion in the aorta than the Soybean group and showed lower lipid peroxidation (Soybean group: 50.2 ± 7.1; Pequi group: 30.0 ± 4.8 µmol MDA/mg protein) and anti-oxidized LDL autoantibodies (Soybean group: 35.7 ± 9.4; Pequi group: 15.6 ± 3.7 arbitrary units). Peritoneal macrophages from the Pequi group stimulated with zymosan showed a reduction in the release of reactive oxygen species compared to the Soybean group. Our data suggest that a pequi oil-rich diet slows atherogenesis in the initial stages, possibly due to its antioxidant activity. However, the increase of serum cholesterol induces a more prominent LDL migration toward the intimae of arteries, increasing the advanced atherosclerotic plaque. In conclusion, pequi oil associated with an atherogenic diet worsens the lipid profile and accelerates the formation of advanced atherosclerotic lesions despite its antioxidant action.

Key words: Pequi oil; Fatty acid; Lipids; Atherosclerosis; Oxidative stress; Cholesterol

Introduction

Atherosclerosis is a multifactorial disease in which genetic, environmental, and lifestyle risk factors drive the lipid abnormalities that determine the progression of atherosclerosis under the action of oxidative stress, inflammation, and thrombogenicity. Diet is one of the most important environmental factors influencing the progression of atherosclerosis. Among dietary nutrients, fatty acids play an important role in the changes in plasma lipid profile. Saturated fatty acids increase low-density lipoprotein (LDL) cholesterol by inhibiting LDL receptor activity and enhancing apolipoprotein B-containing lipoprotein production (1). Replacement of saturated fat with polyunsaturated fat has been shown to decrease total cholesterol (TC) and LDL cholesterol by lowering LDL cholesterol production rates and/or increasing LDL clearance rates. Although replacement of saturated fat with polyunsaturated fat has been shown to decrease high-density lipoprotein (HDL) cholesterol, it decreases LDL cholesterol to even a greater extent, with a consequent reduction of the LDL/HDL ratio. Replacement of saturated fat with monounsaturated fat has also been associated with decreased TC and LDL cholesterol, but the magnitude of HDL reduction is lower than when polyunsaturated fats are the replacement nutrient (2-4).

The potential role of oxidative processes in the development of various diseases has also been studied. In particular, increased reactive oxygen species (ROS) in

Correspondence: J.I. Alvarez-Leite, Departamento de Bioquímica e Imunologia, ICB, UFMG, Av. Antônio Carlos, 6627, 30161-970 Belo Horizonte, MG, Brasil. E-mail: jalvarezleite@gmail.com

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oxidative stress play a central role in LDL oxidation, a key step in the development of atherosclerosis. The particles of oxidized LDL (oxLDL) induce the synthesis of monocyte chemotactic molecules and expression of endothelial adhesion molecules, leading to the attraction and subsequent migration of these cells into the intimae. The monocytes differentiate into macrophages that bind to oxLDL via scavenger receptors and internalize these particles. This event promotes foam cell formation and the consequent development of atherosclerotic plaque (5,6).

Caryocar brasiliense Cambess (Caryocaraceae) is a widely distributed tree that is found primarily in the Brazilian Cerrado, a savannah-like biome. The oil of its fruit, popularly known as pequi, has a high content of lipid-soluble vitamins and saturated and monounsaturated fatty acids. High amounts of carotenoids and phenolic acids are also present in pequi oil, accounting for its antioxidant properties. Although oleic fatty acid is the main component, saturated fatty acids are also present, mainly in the form of palmitic fatty acid (7). Monounsaturated and saturated fatty acids are well known to act as anti- and pro-atherogenic agents, respectively.

Because the composition of fatty acids and antioxidants in pequi oil has the potential to exert beneficial or deleterious influences on atherogenesis, our objective was to determine the effect of the intake of a cholesterol-rich diet supplemented with pequi oil, compared to the same diet containing soybean oil, on lipid metabolism, atherosclerosis formation, and oxidative stress in atherosclerosis-susceptible LDL receptor-deficient mice ($LDLr^{-/-}$).

Material and Methods

The experimental protocol was approved by the Animal Care Committee of the Federal University of Minas Gerais (UFMG; CETEA #225/2008).

Diet and animals

Diets were based on the AIN-93G standard rodent diet, which contains 7% soybean oil. In the soybean diet, the AIN-93G diet was supplemented with 1.25% cholesterol to give it atherogenic properties. In the pequi oil-supplemented diet, the lipid source of the standard diet (soybean oil) was replaced with the same amount of pequi oil. Pequi oil was purchased at the central market of the city of Belo Horizonte, Brazil, and produced by the Cooperativa dos Agricultores Familiares e Agroextrativista Grande Sertão, Montes Claros, MG, Brazil. Both diets contained an antioxidant supplement (0.014% butylated hydroxytoluene, BHT) and were stored at -20°C to avoid oxidation. Twenty-four 6- to 8-week-old $LDLr^{-/-}$ isogenic (C57BL/6 background) knockout mice from Jackson Laboratories (USA) were used in this study. The animals were maintained at the animal facility of the Departamento de Bioquímica e Imunologia (UFMG, Brazil). This strain of laboratory mice is susceptible to the development of hypercholesterolemia and atherosclerosis when fed a cholesterol-rich diet. The mice were divided into two groups: the Soybean group (N = 12) was fed a cholesterol-rich diet containing 7% soybean oil, and the Pequi group (N = 12) was fed a cholesterol-rich diet containing 7% pequi oil. Mice were housed 6 per cage in a temperature-controlled environment with a 12-h light-dark cycle, with free access to food and water. The mice were maintained on their respective diets for 6 weeks. Body weight and food intake were determined weekly. Food intake was measured considering the amount of feed consumed in each cage (2 cages/group) divided by the number of animals/cage. The result represents the average food intake calculated in each experimental week (N = 6/cages). In the 6th week, after an overnight fast and anesthesia, all mice were euthanized by cervical dislocation after exsanguinations through the inguinal plexus. Heart, liver, aorta, and cecal content were collected for later analyses.

Centesimal composition of the diets

Humidity was determined at 105°C and the ash was determined by the calcination of the samples in a muffle at 550°C (8). Protein was determined according to Kjeldahl methods recommended by the AOAC (8), with the amount being calculated by multiplying the nitrogen content by a factor of 6.25. The total lipid content was quantified by extraction using a Soxhlet extractor (8). The carbohydrate content was estimated by calculating the difference between 100 g diet and the sum of the other components.

Analysis of carotenoid content. The carotenoids were extracted from pequi oil in acetone and transferred to petroleum ether. The extracts were subjected to saponification with a 10% KOH solution in methanol and 0.3 g BHT, in the dark for 16 h at room temperature and in an oxygen-free atmosphere, in order to facilitate the subsequent separation, identification, and quantification of carotenoids from the samples (9). Then, the extracts were suspended in methanol and filtered through a 0.45-µm filter. The analysis was performed by the injection of 50 µL of the samples into the chromatographic column. We used the chromatographic conditions proposed by Sant'Ana et al. (10), which included: Shimadzu HPLC chromatographic system with diode array detector, Phenomenex RP-18 C18 chromatographic column (250 x 4.6 mm), the mobile phase consisted of methanol:ethyl acetate:acetonitrile (70:20:10, v/v/v), and flow rate of 2.0 mL/min. The chromatograms were obtained at 450 nm.

Fatty acid profile analysis

After lipid extraction (11), saponification and esterification of the lipid extracts were carried out for gas chromatography analysis. The methodology applied for saponification and esterification of the lipid extract was proposed by Hartman and Lago (12). For chromatography, an aliquot of 1 µL of the lipid extract solution was injected in hexane

(50 mg/mL) into a CG-17A Shimadzu/Class chromatograph (Shimadzu, Brazil), equipped with SP-2560 (biscianopropil polysiloxane) and a silica chromatographic column of 100 m and 0.25 mm in diameter. The carrier gas (mobile phase) was nitrogen. The chromatographic runs began with the column temperature at 100°C, followed by increases of 10°C/min until reaching 180°C. After this period, the column temperature was increased 1°C/min until reaching 240°C, remaining at this temperature for 10 min. The injector and detector temperatures were 250° and 270°C, respectively. Fatty acid identification considered the retention times of a standard fatty acid methyl ester (Supelco™ 37 Component FAME Mix, Supelco, USA).

Blood analysis

Fasting blood samples were collected by inguinal plexus puncture during the euthanasia procedure without anticoagulant for lipid profile determination or using a glucose determination-specific anticoagulant (6 g/L EDTA and 12 g/L potassium fluoride: Glistab, Labtest, Brazil). Next, serum and plasma were obtained after centrifugation at 10,000 *g* for 5 min (centrifuge Fanem Centrimicro 243, Brazil). Serum samples were used for TC, HDL cholesterol and triglyceride analyses using commercial kits (Labtest). Non-HDL cholesterol was calculated as the difference between TC and HDL cholesterol. Plasma glucose concentrations were measured using commercial kits (Labtest).

Hepatic lipids

Total lipids in liver samples were extracted with organic solvents by the method of Folch et al. (11). The lipid extracts were obtained, dried overnight at 37°C, and then gravimetrically quantified and assayed for TC concentration.

Assessment of atherosclerotic lesions

The heart and aorta of each animal were first perfused with phosphate-buffered saline (PBS) and then with 4% phosphate-buffered formalin, pH 7.4. The hearts were removed and fixed with 4% paraformaldehyde for 6 h and then stored overnight in a 30% sucrose solution at 4°C. Next, the hearts were embedded in tissue freezing medium (Leica Microsystems, Germany). Images of the sections (10-µm thickness) were obtained after staining with hematoxylin-eosin. Eight sections per heart, with 40-µm intervals between sections, were used to quantify lesion areas in the aortic roots using the Image-Pro Plus software (Mediacybernetics, USA).

Extracellular collagen in lesions of the aortic root was visualized by Gomori trichrome staining. The deposition of collagen was assessed in four sections per mouse with intervals of 40 µm between sections (13). The result is reported as percent lesion area covered by collagen.

Atherosclerosis was also assessed in the thoracic and abdominal aorta. The aortas were cleaned of adventitial fat, opened longitudinally and stained with Sudan IV. Lesion areas were assessed using the Image-Pro Plus software and are reported as percent total luminal aorta surface (13).

Determination of lipid peroxidation in liver

Lipid peroxidation was determined by the detection of thiobarbituric acid reactive substances (TBARS) (14) in 100 mg liver after homogenization. The results were normalized for the protein in the homogenate and are reported as µmol malondialdehyde/mg protein. Protein was assessed by the method of Lowry et al. (15).

Anti-oxidized LDL autoantibody

Levels of anti-oxLDL autoantibody in plasma from LDLr $^{-/-}$ mice were examined by ELISA as described by Lee et al. (16).

ROS levels in macrophages

LDL is only minimally oxidized in blood. The main source of free radicals for LDL oxidation is the respiratory burst secondary to macrophage activation in the intimae of arteries. For this reason, we performed an *ex vivo* experiment to evaluate macrophage respiratory burst after chronic ingestion of a pequi oil-supplemented diet. To assess ROS production by macrophages, LDLr $^{-/-}$ mice were fed soybean- or pequi oil-supplemented diets for 2 weeks. After this period, the animals were sacrificed and peritoneal resident macrophages were harvested with cold sterile PBS. The macrophages were plated in triplicate at a concentration of 1 x 10^6 cells per well and stimulated with zymosan (1 x 10^7 particles/50 µL). For evaluation of the respiratory burst, changes in chemiluminescence were measured over a 60-min period, using a luminol reagent. The baseline production of ROS was also monitored in macrophages without zymosan stimulus. ROS production was calculated by measuring the area under the curve obtained for macrophages of both experimental groups incubated with or without zymosan (17).

Antioxidant enzyme assay

Determination of superoxide dismutase (SOD) activity in the liver homogenates of mice was based on its ability to scavenge superoxide (O_2^-) radicals, decreasing the rate of auto-oxidation of pyrogallol, according to the method of Dieterich et al. (18). Catalase activity was assayed using the Nelson and Kiesow protocol (19). The decomposition of H_2O_2 due to catalase activity was assessed by the decrease in the absorbance of H_2O_2 at 240 nm.

Statistical analysis

Data were analyzed statistically by the Kolmogorov-Smirnov test to verify normal distributions, and the Grubbs' test was used for the detection of outliers. Student *t*-tests were used to compare the Soybean and Pequi groups. Statistical significance was set at $P < 0.05$. The analysis was carried out using the Prism software version 5.0 (GraphPad Software, USA). Data are reported as means ± SEM.

Results

Diet composition

Diets were analyzed for their nutrient and fatty acid compositions. The results showed that both diets had the same proportion of lipids and proteins (8.85 and 16.32%, respectively; Table 1). The proportion of polyunsaturated fatty acids (PUFA) was higher in the diet containing soybean oil (58.71 and 5.58% for soybean and pequi oils, respectively), whereas monounsaturated fatty acids (MUFA) were higher in the diet containing pequi oil (57.89%) than in the diet containing soybean oil (19.69%), mainly due to the oleic acid content, which represented 56.98% of the MUFAs. The saturated fatty acid (SFA) concentration was also greater in pequi oil (36.53%) than in soybean oil (15.49%). The carotenoids composition of pequi oil was 35.64 mg/100 g oil, being 18.62 mg/100 g oil of β-carotene and 17.03 mg/100 g oil of β-cryptoxantine. The vitamin A content was 2261.9 RAE/100 g oil.

Food intake, body weight, plasma glucose, and lipid profile

There was no difference in food intake, weight gain or blood glucose levels between groups (Table 2).

The cholesterol-rich diet containing pequi oil was responsible for increases in total cholesterol, non-HDL cholesterol and triacylglycerols, without significant changes in HDL cholesterol (Table 2). The cholesterol-rich diet containing pequi oil was also related to a higher lipid accumulation in the liver that was not due to a higher cholesterol concentration since hepatic cholesterol was high but similar in both groups (Table 2).

Table 1. Composition of soybean and pequi oil-rich isocaloric diets.

Parameter	Soybean diet	Pequi oil diet
Humidity (g/100 g diet)	9.55	9.25
Protein (g/100 g diet)	16.3	16.32
Carbohydrate (g/100 g diet)	62.87	63.87
Lipids (g/100 g diet)	8.85	8.85
Fatty acids (g/100 g oil)		
C16:0	11.91	34.45
C16:1	0	0.75
C18:0	3.42	1.9
C18:1	18.22	56.98
C18:2	43.41	3.48
C18:3	0.78	2.09
C20:0	0	0.18
C20:2	5.24	0
C20:3	0.35	0
C20:4	0.52	0
C20:5	0.81	0
C20.1	0	0.17
C22:2	7.63	0
C22:1	1.47	0
Unkown	6.24	0
PUFA (g/100 g oil)	58.71	5.58
MUFA (g/100 g oil)	19.69	57.89
SFA (g/100 g oil)	15.49	36.53

PUFA = polyunsaturated fatty acids; MUFA = monounsaturated fatty acids; SFA = saturated fatty acids.

Atherosclerosis development

We investigated atherosclerotic lesions at two sites, aortic root and aorta, because lesions at these sites are generally in different stages of atherosclerosis formation. The lesion developed faster in the aortic root and was in a more advanced stage after 6 experimental weeks. In the aorta, however, the lesions initiated later, showing only an early lesion at the end of 6 weeks.

In the aortic root, the atherosclerotic lesions were significantly more prevalent in the mice fed the pequi oil-supplemented diet (Figure 1). The Soybean group had intermediate lesions covered by a fibrous cap and composed of several layers of foam cells and cellular infiltration. In contrast, the Pequi group had lesions in a more advanced stage with the presence of a thick fibrous cap and deposition of cholesterol crystals in areas of necrosis.

Because deposition of collagen in atherosclerotic lesions is an important factor in plaque stability, we also assessed the percentage of collagen in the atherosclerotic lesions. Although the lesions were more prevalent in the Pequi group, the collagen content was similar for the two

Table 2. Food intake, body weight gain, plasma glucose and serum lipids, and hepatic lipids in female LDLr$^{-/-}$ mice fed a soybean or pequi oil-rich diet for 6 weeks.

	Soybean group	Pequi group
Food intake (g·animal^{-1}·day^{-1})[a]	3.5 ± 0.06	3.5 ± 0.1
Body weight gain (g)	3.9 ± 0.3	4.3 ± 0.4
Plasma glucose (mM)	6.8 ± 0.5	7.9 ± 0.6
Serum lipids (mM)		
Total cholesterol	9.8 ± 0.7	24.4 ± 1.7*
HDL cholesterol	0.9 ± 0.08	0.7 ± 0.06
Non-HDL cholesterol	8.9 ± 0.8	22.7 ± 1.3*
Triacylglycerols	1.3 ± 0.2	2.0 ± 0.2*
Hepatic lipids (mg/g)		
Total lipids	123 ± 11	191 ± 17*
Total cholesterol	290 ± 48	210 ± 20

Data are reported as means ± SEM (N = 12, except for food intake N = 6). [a]For more details about food intake calculations, see Material and Methods. HDL cholesterol = high-density lipoprotein cholesterol; Non-HDL cholesterol = total cholesterol - HDL cholesterol. *P < 0.05 compared to the Soybean group (Student *t*-test).

groups (Figure 1).

When lesions in the aorta were examined, the Pequi group presented a significant reduction in atherosclerotic lesions compared to those in the aortic root (Figure 2). The reduction of the atherosclerotic lesions in the aorta was unexpected due to the pro-atherogenic lipid profile of the Pequi group. Because pequi oil is rich in antioxidant agents, we investigated the effect of pequi oil intake on oxidative stress.

Hepatic lipid peroxidation and antioxidant enzyme activity

Although the intake of a pequi oil-supplemented diet increased the lipid content in the liver, this diet resulted in a significant decrease in hepatic lipid peroxidation, as evaluated by TBARS determination (Table 3). However, the antioxidant enzymes SOD and catalase were similar in both groups (Table 3), suggesting that pequi oil acts directly as an antioxidant rather than indirectly by activating antioxidant enzymes.

Anti-oxidized LDL antibody

The anti-oxLDL antibody concentrations indirectly indicate the circulating concentration of oxLDL. Oxidized LDL is an important risk factor for atherosclerosis because it is responsible for macrophage activation and atherosclerosis development. However, it also indicates the serum oxidative status. In this study, although there was a significant increase in the concentration of LDL cholesterol by pequi oil-supplemented diet ingestion, we found lower levels of circulating oxLDL antibody in pequi oil-fed animals (Figure 3), supporting the antioxidant effect of pequi oil seen in the liver.

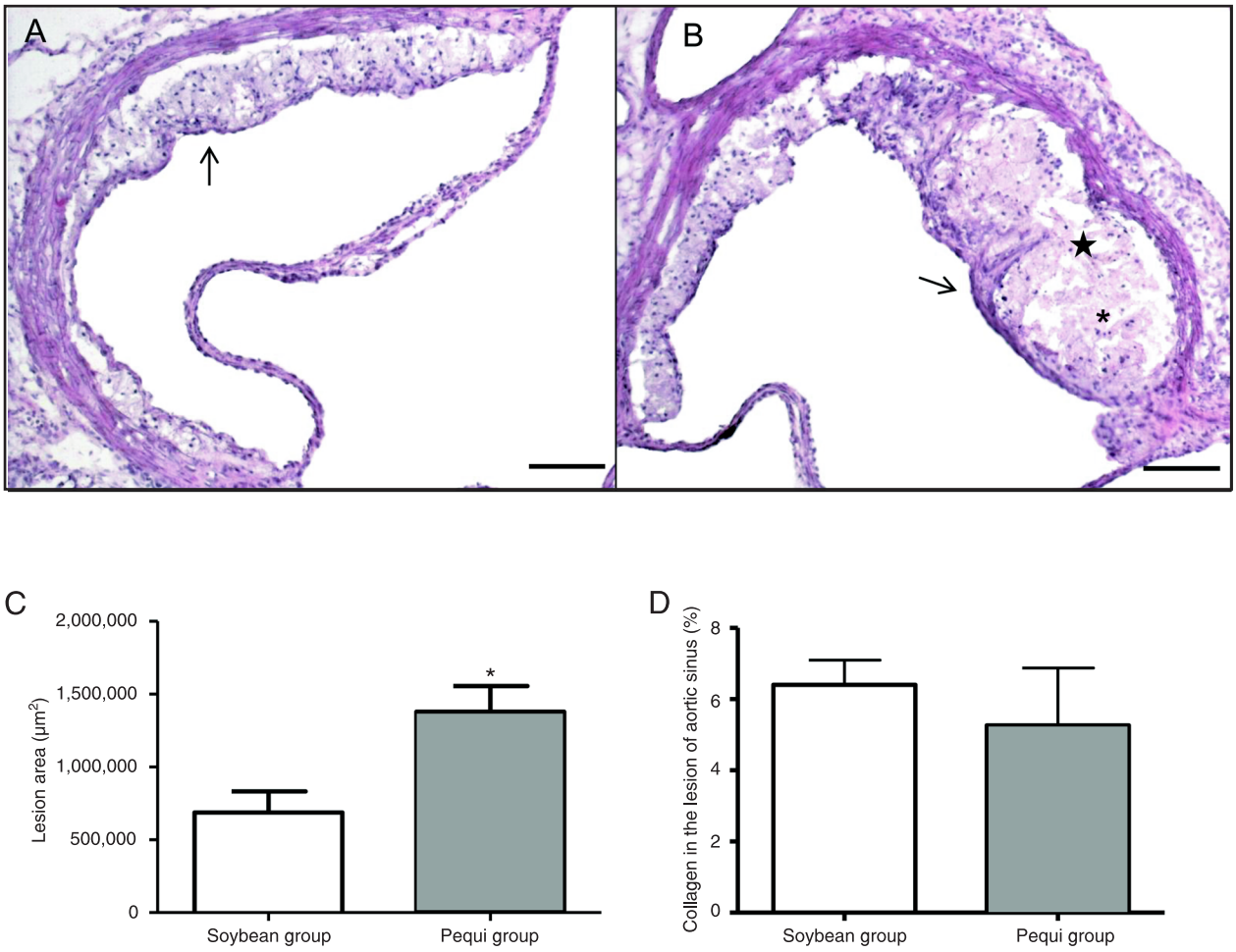

Figure 1. Atherosclerotic lesions in the aortic root of female LDLr$^{-/-}$ mice after 6 weeks on a cholesterol-rich diet containing soybean oil or pequi oil. *A* and *B*, Histology of the aortic root showing characteristics of the lesions in the Soybean and Pequi groups, respectively. Histological sections stained with hematoxylin-eosin, 100X. The arrow indicates the presence of a fibrous cap, the star indicates regions with evidence of cholesterol crystals and the asterisk indicates the region of necrosis. *C*, Graphic representation of the lesion area. *D*, Collagen deposition (%). Data are reported as means ± SEM (N = 10-12/group). *P < 0.05 compared to the Soybean group (Student *t*-test). Magnification bars = 100 µm.

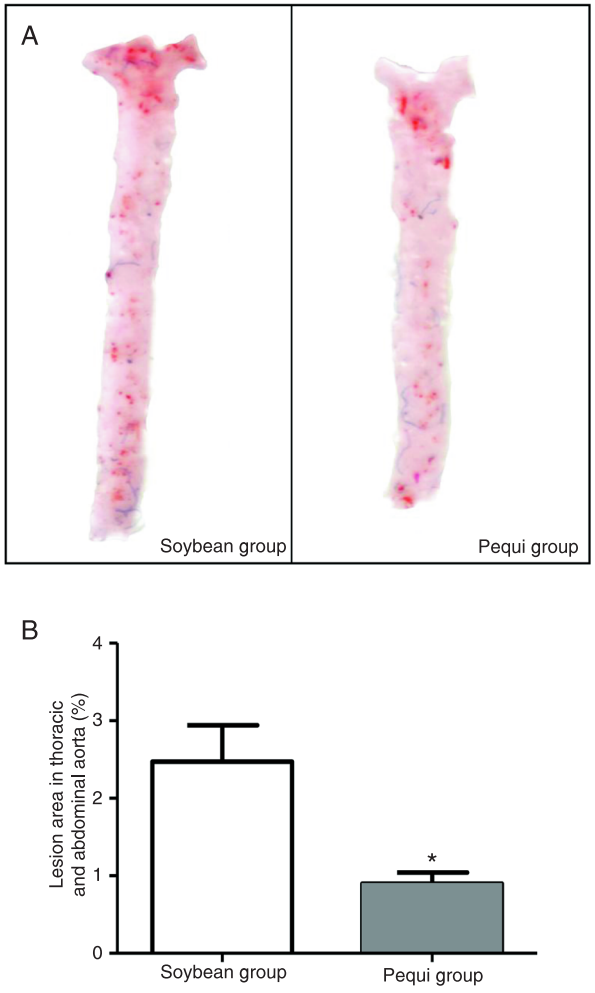

Figure 2. Lesion area in aortas of female LDLr $^{-/-}$ mice after 6 weeks on a cholesterol-rich diet containing soybean oil or pequi oil. *A*, Photograph of aortas stained with Sudan IV. *B*, Percentage of lesion area. Data are reported as means ± SEM (N = 10/group). *P < 0.05 compared to the Soybean group (Student *t*-test).

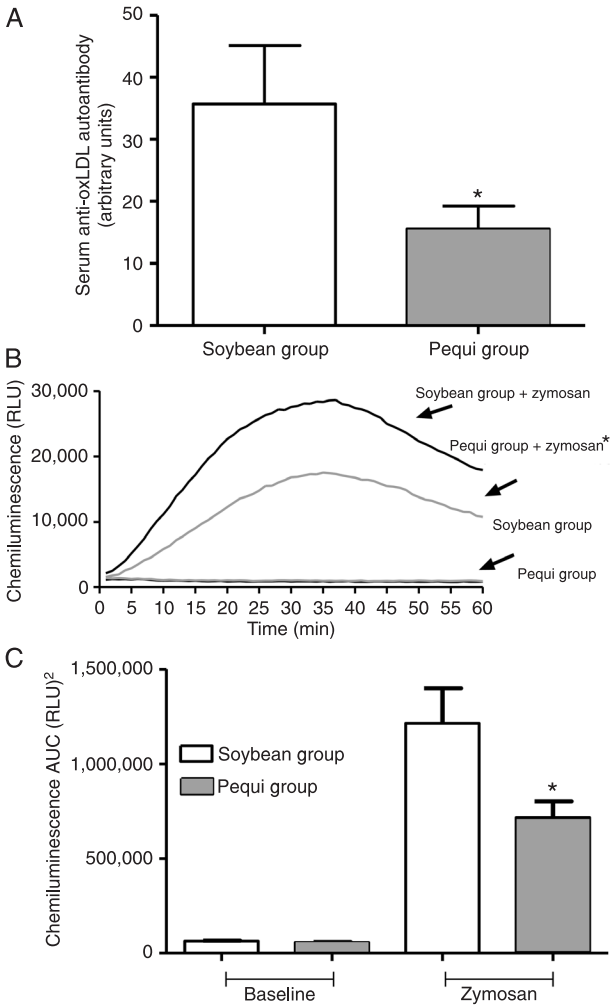

Figure 3. Serum levels of anti-oxLDL autoantibody and production of reactive oxygen species (ROS) by resident peritoneal macrophages from LDLr $^{-/-}$ mice after 2 weeks on a soybean diet or a pequi oil diet. *A*, Serum anti-oxLDL autoantibody (*P < 0.05, Student *t*-test). *B*, Kinetic profile of chemiluminescence of ROS production for 1 h measured at intervals of 60 s (reported as relative light units - RLU). *C*, Area under the curve (AUC) of ROS production in the Soybean and Pequi groups without stimulation (baseline) or stimulated with zymosan. oxLDL = oxidized LDL. The AUC is reported as means ± SEM (N = 5). *P < 0.05 for the Pequi group + zymosan *vs* Soybean group + zymosan (Student *t*-test).

Table 3. Hepatic lipid peroxidation (thiobarbituric acid reactive substances - TBARS), hepatic superoxide dismutase (SOD) activity and hepatic catalase activity in female LDLr $^{-/-}$ mice fed a soybean or pequi oil diet for 6 weeks.

	Soybean group	Pequi group
TBARS (µmol MDA/mg protein)	50.2 ± 7.1	30.0 ± 4.8*
SOD (U/g protein)	0.71 ± 0.03	0.70 ± 0.02
Catalase ($\Delta E \cdot min^{-1} \cdot g\ protein^{-1}$)	18.2 ± 1.3	18.1 ± 1.5

Data are reported as means ± SEM (N = 12 for both groups). *P < 0.05 compared to the Soybean group (Student *t*-test).

ROS production in peritoneal macrophages

Our results showed that baseline ROS levels produced by resident (non-activated) peritoneal macrophages were similar in both groups. However, the respiratory burst induced by zymosan incubation was reduced in macrophages of pequi oil-fed animals, compared to the Soybean group (Figure 3), suggesting that pequi oil had a modulating effect.

Discussion

Our studies showed a paradoxical effect of pequi oil on atherogenesis. After 6 weeks of pequi oil-supplemented diet intake, lesions were increased in the aortic root but were reduced in the aorta when compared to those of animals that ingested a soybean control diet. In contrast, blood lipids presented a more atherogenic profile in the Pequi group, whereas the antioxidant status was improved. The fatty acid composition of pequi oil (approximately 36% SFA) could be responsible for the poorer lipid profile, as increased SFA intake is a known risk factor for dyslipidemia and atherosclerosis. Palmitic fatty acid, the predominant SFA in pequi oil, is one of the most atherogenic fatty acids and is widely linked to both hypertriglyceridemia and hypercholesterolemia, especially associated with LDL cholesterol (20). The proportion of MUFA in pequi oil did not appear to be adequate to counteract the effects of SFA on blood lipids. Regarding soybean oil composition, our results agree with other studies demonstrating that it is composed of approximately 15% SFA, 20% MUFA, and more than 40% of the PUFA linoleic acid (18:2 ω-6) (21-24). Therefore, although pequi oil has more than 50% PUFA in its composition, the high proportion of SFA gives the pequi oil a fatty acid distribution that is more atherogenic than soybean oil, resulting in the hypercholesterolemia seen in the pequi oil diet group. The higher concentration of total lipids in the livers of animals in the Pequi group may also be related to the high intake of SFA, since it favors the synthesis of triacylglycerol for subsequent secretion of very LDL (25,26).

Several studies have shown that the antioxidant activity of pequi extracts has the ability to scavenge free radicals, reduce lipid peroxidation and prevent oxidative damage to DNA (20,27-29). *In vivo* studies carried out in human runners also detected a reduction in plasma lipid peroxidation after pequi oil intake (27,30,31), which is in agreement with the reduction of lipid peroxidation in the liver seen in the present study. Moreover, the release of free radicals was reduced in residential macrophages in the Pequi oil group compared to control after zymosan stimulation. All of these data support the beneficial effect of pequi in reducing oxidative stress, which is a key factor in the genesis of atherosclerosis. Although native LDL can initiate the immune response and the formation of foam cells, oxLDL, which may be increased by oxidative stress, is a more powerful atherogenic stimulus, activating endothelial cells and inducing scavenger receptor expression in macrophages. Thus, a cholesterol-rich diet containing pequi oil was once again associated with atheroprotective actions, as the reduction of oxidative stress suggests a reduced LDL oxidation in the bloodstream.

The most intriguing result obtained in the present study regards the differential effect of a cholesterol-rich diet containing pequi oil on the aortic root and the aorta. In the aorta, we found only lesions in the initial stages (fatty streaks) in both groups, and these lesions were significantly reduced in the Pequi oil group compared to the Soybean group. In contrast, a cholesterol-rich diet containing pequi oil was associated with advanced lesions in the aortic root, characterized by a necrotic core and cholesterol crystal deposition. Site-specific differences in the development of atherosclerosis have been previously described (32-35). In a study by Reardon et al. (35), a diet containing sunflower oil reduced aortic root lesions but did not alter the development of lesions in the aorta in $LDLr^{-/-}$, $RAG^{-/-}$ double-knockout mice. Acín et al. (34), using a cholesterol-rich diet supplemented with olive oil, also found increased atherosclerotic plaque in the aortic valve; however, in the aorta, the lesions were decreased after this supplementation. The authors attributed this result to the antioxidant properties of olive oil. Thus, because the development of atherosclerotic lesions in the aorta begins later than in the aortic root, and therefore has lesions in the initial phase (32), this site could be more susceptible to the antioxidant effects of pequi oil. In addition, smoking selectively raised lesions in the abdominal aorta of young humans but did not influence lesions in the coronary artery (36). Thus, it can be deduced that innumerable factors lead to a site-selective susceptibility to the development of lesions.

Historically, atherosclerosis analyses were dictated by a narrow focus on the aortic sinus or root in the vast majority of published studies of murine atherosclerosis. The aortic sinus is not characteristically involved in human atherosclerosis, although the aorta is frequently involved in both human and mouse atherosclerosis. The very rapid heart rate typical of mice (550 bpm in mice compared to 70 bpm in humans) may account for this difference because flow in the murine aortic sinus is likely to be much more disrupted than in the human aortic sinus. For this reason, the clinical relevance of lesions in the aortic valve is still discussed. Our data showed that, except for the poorer lipid profile, other risk factors for atherosclerosis, such as oxLDL, oxidative stress and macrophage liberation of free radicals, were improved by pequi oil. These results are in agreement with the finding that pequi oil reduces atherosclerotic lesions in the aorta, which is a more relevant atherosclerotic site for humans than the aortic valve.

Our data suggest that a cholesterol-rich diet supplemented with pequi oil confers an important antioxidant effect, thereby reducing oxidative stress, including oxLDL antibodies. However, the increase in total and non-HDL cholesterol could be involved in the enhanced lesions seen in the aortic root of mice fed pequi oil-supplemented diet. Therefore, evaluating the clinical relevance of such effects of pequi oil should be a goal for future clinical studies.

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References

1. Dietschy JM. Dietary fatty acids and the regulation of plasma low density lipoprotein cholesterol concentrations. *J Nutr* 1998; 128: 444S-448S.
2. Siri-Tarino PW, Sun Q, Hu FB, Krauss RM. Saturated fatty acids and risk of coronary heart disease: modulation by replacement nutrients. *Curr Atheroscler Rep* 2010; 12: 384-390.
3. German JB, Dillard CJ. Saturated fats: what dietary intake? *Am J Clin Nutr* 2004; 80: 550-559.
4. Nicolosi RJ. Dietary fat saturation effects on low-density-lipoprotein concentrations and metabolism in various animal models. *Am J Clin Nutr* 1997; 65: 1617S-1627S.
5. Ishigaki Y, Katagiri H, Gao J, Yamada T, Imai J, Uno K, et al. Impact of plasma oxidized low-density lipoprotein removal on atherosclerosis. *Circulation* 2008; 118: 75-83.
6. Witztum JL. The role of oxidized LDL in atherosclerosis. *Adv Exp Med Biol* 1991; 285: 353-365.
7. FDA. A review of *Caryocar brasiliense* (Caryocaraceae) - an economically valuable species of the Central Brazilian Cerrados. *Econ Bot* 1995; 49: 40-48.
8. Association of Official Analytical Chemists (AOAC). *Official methods of analysis of the Association of Official Analytical Chemists*. Vol. 2. Washington: AOAC; 1998.
9. Mercadante AZ. Chromatographic separation of carotenoids. *Arch Latinoam Nutr* 1999; 49: 52S-57S.
10. Sant'Ana HMP, Stringheta PC, Brandão SCC, Azeredo RMC. Carotenoid retention and vitamin A value in carrot (*Daucus carota* L.) prepared by food service. *Food Chem* 1998; 61: 145-151.
11. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957; 226: 497-509.
12. Hartman L, Lago RC. Rapid preparation of fatty acid methyl esters from lipids. *Lab Pract* 1973; 22: 475-476.
13. Portugal LR, Fernandes LR, Pietra P, V, Santiago HC, Gazzinelli RT, Alvarez-Leite JI. Influence of low-density lipoprotein (LDL) receptor on lipid composition, inflammation and parasitism during *Toxoplasma gondii* infection. *Microbes Infect* 2008; 10: 276-284.
14. Wallin B, Rosengren B, Shertzer HG, Camejo G. Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtiter plate: its use for evaluation of antioxidants. *Anal Biochem* 1993; 208: 10-15.
15. Lowry O, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.
16. Lee TS, Yen HC, Pan CC, Chau LY. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 1999; 19: 734-742.
17. O'Dowd Y, Driss F, Dang PM, Elbim C, Gougerot-Pocidalo MA, Pasquier C, et al. Antioxidant effect of hydroxytyrosol, a polyphenol from olive oil: scavenging of hydrogen peroxide but not superoxide anion produced by human neutrophils. *Biochem Pharmacol* 2004; 68: 2003-2008.
18. Dieterich S, Bieligk U, Beulich K, Hasenfuss G, Prestle J. Gene expression of antioxidative enzymes in the human heart: increased expression of catalase in the end-stage failing heart. *Circulation* 2000; 101: 33-39.
19. Nelson DP, Kiesow LA. Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C (with molar extinction coefficients of H_2O_2 solutions in the UV). *Anal Biochem* 1972; 49: 474-478.
20. Khouri J, Resck IS, Poças-Fonseca M, Sousa TMM, Pereira LO, Oliveira ABB, et al. Anticlastogenic potencial and antioxidant effects of an aqueous extract of pulp from the pequi tree (*Caryocar brasiliense* Camb.). *Genet Mol Res* 2007; 30: 442-449.
21. Oliveros LB, Videla AM, Ramirez DC, Gimenez MS. Dietary fat saturation produces lipid modifications in peritoneal macrophages of mouse. *J Nutr Biochem* 2003; 14: 370-377.
22. Diniz YS, Cicogna AC, Padovani CR, Santana LS, Faine LA, Novelli EL. Diets rich in saturated and polyunsaturated fatty acids: metabolic shifting and cardiac health. *Nutrition* 2004; 20: 230-234.
23. Grundy SM, Denke MA. Dietary influences on serum lipids and lipoproteins. *J Lipid Res* 1990; 31: 1149-1172.
24. Trautwein EA, Kunath-Rau A, Dietrich J, Drusch S, Erbersdobler HF. Effect of dietary fats rich in lauric, myristic, palmitic, oleic or linoleic acid on plasma, hepatic and biliary lipids in cholesterol-fed hamsters. *Br J Nutr* 1997; 77: 605-620.
25. Jackson KG, Bateman PA, Yaqoob P, Williams CM. Impact of saturated, polyunsaturated and monounsaturated fatty acid-rich micelles on lipoprotein synthesis and secretion in Caco-2 cells. *Lipids* 2009; 44: 1081-1089.
26. Pai T, Yeh YY. Stearic acid modifies very low density lipoprotein lipid composition and particle size differently from shorter-chain saturated fatty acids in cultured rat hepatocytes. *Lipids* 1997; 32: 143-149.
27. Miranda-Vilela AL, Akimoto AK, Alves PC, Pereira LC, Goncalves CA, Klautau-Guimaraes MN, et al. Dietary carotenoid-rich pequi oil reduces plasma lipid peroxidation and DNA damage in runners and evidence for an association with MnSOD genetic variant -Val9Ala. *Genet Mol Res* 2009; 8: 1481-1495.
28. Paula-Junior W, Doonatti L, Fadel-Picheth CMT, Weffort-Santos AM. Leishmanicidal, antibacterial, and antioxidant activities of *Caryocar brasiliense* Cambess leaves hydroethanolic extract. *Braz J Farmacogn* 2006; 16: 625-630.
29. Roesler R, Catharino RR, Malta LG, Eberlin MN, Pastore G. Antioxidant activity of *Caryocar brasiliense* (pequi) and characterization of components by electrospray ionization mass spectrometry. *Food Chem* 2008; 110: 711-717.
30. Miranda-Vilela AL, Pereira LC, Goncalves CA, Grisolia CK. Pequi fruit (*Caryocar brasiliense* Camb.) pulp oil reduces exercise-induced inflammatory markers and blood pressure of male and female runners. *Nutr Res* 2009; 29: 850-858.
31. Miranda-Vilela AL, Lordelo GS, Akimoto AK, Alves PC, Pereira LC, Klautau-Guimaraes MN, et al. Genetic polymorphisms influence runners' responses to the dietary ingestion of antioxidant supplementation based on pequi oil (*Caryocar brasiliense* Camb.): a before-after study. *Genes Nutr* 2011; 6: 369-395.
32. VanderLaan PA, Reardon CA, Getz GS. Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. *Arterioscler Thromb Vasc Biol* 2004; 24: 12-22.
33. Goel R, Schrank BR, Arora S, Boylan B, Fleming B, Miura H,

et al. Site-specific effects of PECAM-1 on atherosclerosis in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 2008; 28: 1996-2002.
34. Acín S, Navarro MA, Carnicer R, Arbones-Mainar JM, Guzman MA, Arnal C, et al. Dietary cholesterol suppresses the ability of olive oil to delay the development of atherosclerotic lesions in apolipoprotein E knockout mice. *Atherosclerosis* 2005; 182: 17-28.
35. Reardon CA, Blachowicz L, Gupta G, Lukens J, Nissenbaum M, Getz GS. Site-specific influence of polyunsaturated fatty acids on atherosclerosis in immune incompetent LDL receptor deficient mice. *Atherosclerosis* 2006; 187: 325-331.
36. McGill HC Jr, McMahan CA. Determinants of atherosclerosis in the young. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. *Am J Cardiol* 1998; 82: 30T-36T.