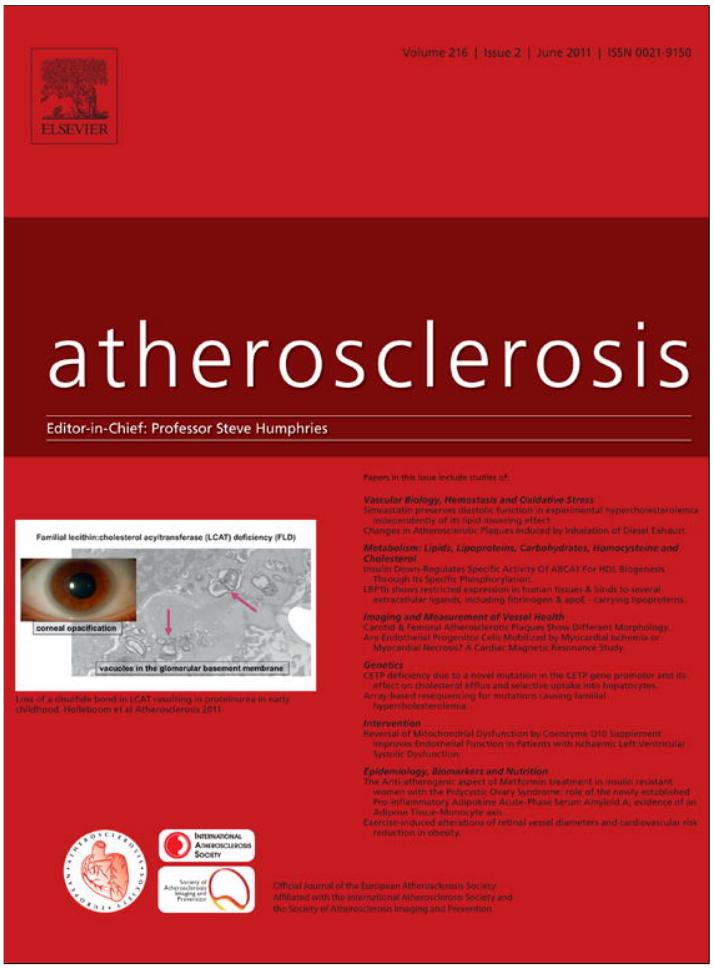

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Açaí juice attenuates atherosclerosis in ApoE deficient mice through antioxidant and anti-inflammatory activities☆

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ABSTRACT

Objective: Açaí fruit pulp has received much attention because of its high antioxidant capacity and potential anti-inflammatory effects. In this study, athero-protective effects of açaí juice were investigated in apolipoprotein E deficient (apoE$^{-/-}$) mice.
Methods and results: ApoE$^{-/-}$ mice were fed AIN-93G diet (CD) or CD formulated to contain 5% freeze-dried açaí juice powder (AJ) for 20 weeks. The mean lesion areas in the aorta for apoE$^{-/-}$ mice fed AJ were 58% less ($P < 0.001$) compared to that for CD fed mice. HDL-cholesterol was higher in AJ fed mice. Biomarkers of lipid peroxidation, including F_2-isoprostanes and isomers of hydroxyoctadecadienoic acids and hydroxyeicosatetraenoic acids were significantly lower in serum and in liver of AJ fed mice. Expression of the two antioxidant enzyme genes, Gpx3 and Gsr, were significantly up-regulated in the aorta from AJ fed mice. The activity of GPX, GSR and PON1 increased in serum and/or liver of mice fed AJ. In the second experiment, ApoE$^{-/-}$ mice were fed CD or AJ for 5 weeks. Serum levels, gene expression and protein levels of the two proinflammatory cytokines TNF-α and IL-6 in the resident macrophages with or without LPS stimulation were lower in mice fed AJ. SEAP reporter assay determined that AJ reduced NF-κB activation.
Conclusion: Reducing lipid peroxidation through boosting antioxidant enzymes and inhibiting pro-inflammatory cytokine production are proposed as major underlying mechanisms for the athero-protective effects of the açaí juice tested in these experimental *in vivo* models.

1. Introduction

Cardiovascular diseases remain the leading cause of death not only in the United States but also in most of the industrialized world [1,2]. It has long been recognized that a diet rich in fruits and vegetables may have beneficial effects on cardiovascular diseases, largely attributed to their antioxidant and anti-inflammatory properties [3–5]. However, *in vivo* experimental evidence that consumption of specific fruits and vegetables reduces the risk of cardiovascular disease based on plausible underlying mechanisms remain scarce.

Açaí (*Euterpe oleracea* Mart.) belongs to the family Arecaceae (palm tree). It is indigenous to South America especially in the Amazon flood plains. Açaí pulp has received much attention in recent years as one of the new "superfruits". It exhibits high antioxidant capacity and has been used as a food ingredient with functional contributions to the diet beyond its basic nutritional composition [6–8]. In a recent paper, diet supplementation with açaí pulp was found to improve biomarkers of oxidative stress resulting in a hypocholesterolemic effect in rats [9]. This suggested that consumption of açaí could improve antioxidant status and provide athero-protective effects in an animal model of hypercholesterolemia. Açaí pulp or açaí juice has also been shown to possess anti-inflammatory activity [10]. Freeze-dried açaí pulp inhibited the activity of cyclooxygenase (COX)-1 and -2 *in vitro*, with greater

Abbreviations: AJaçaí, juice; ApoE$^{-/-}$, apolipoprotein E deficient; GPX, glutathione reductase; GSR, glutathione reductase; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; 12/15-LOX, 12/15-lipoxygenase; IL-6, interleukin-6; ORAC, oxygen radical absorbance capacity; PON1, paraoxonase 1; TNF-α, tumor necrosis factor-alpha.

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Table 1
Body weight, body composition and serum lipid profiles in mice fed CD or AJ.[a]

Parameters	CD	AJ
Body weight (g)[b]	25.0 ± 1.73	27.4 ± 1.61***
Energy Intake (kcal/kg body weight 0.75)	183.8 ± 14.1	194.2 ± 19.8
Body composition[c]		
Body fat (%)	22.4 ± 4.2	24.9 ± 3.7
Lean body mass (%)	62.7 ± 7.6	61.5 ± 4.8
Lipid profile[d]		
Total cholesterol (mmol/L)	13.5 ± 1.0	14.4 ± 1.7
HDL-cholesterol (mmol/L)	0.88 ± 0.06	1.04 ± 0.10**
LDL-cholesterol (mmol/L)	12.4 ± 1.1	13.3 ± 1.7
Triglyceride (mmol/L)	1.30 ± 0.14	1.48 ± 0.24

[a] Data expressed as mean ± SD.
[b] $n = 15$.
[c] $n = 8$.
[d] $n = 6$.
** $P < 0.01$.
*** $P < 0.001$.

efficacy against COX-1 [8]. Açaí extracts inhibited lipopolysaccharide- and interferon-g-induced nitric oxide production by reducing the expression of inducible nitric oxide synthase (iNOS) expression [11]. Consumption of a juice blend containing açai as the main component has demonstrated *in vivo* anti-inflammatory properties in human subjects based on a randomized, double-blind, placebo controlled cross-over study [7]. These findings suggest possible cardio-protective properties of açaí.

In this study, the athero-protective effects of a freeze-dried and frozen açaí pulp juice mixture were studied in the apolipoprotein deficient (apoE$^{-/-}$) mouse model. The apoE$^{-/-}$ mouse model has been a useful model to study the mechanisms of action related to atherosclerosis in cardiovascular research [12]. The underlying mechanisms were also explored with primary emphases on the fruit's antioxidant and anti-inflammatory activities.

2. Materials and methods

2.1. Experimental materials and animal

The açaí juice blend was provided by MonaVie, LLC (South Jordan, UT). This blend contains açaí (*Euterpe oleracea* Mart.) freeze-dried and frozen pulp (Earthfruits, South Jordan, UT) as the predominant ingredient. This juice blend has been characterized as containing polyphenols as major phytochemicals previously [7]. The chromatograms of polyphenol profiles of this açaí juice and freeze-dried açaí pulp were presented as supplemental data (Fig. S1). To precisely control the intake, the juice blend was lyophilized and the dried powder was used to make the pellet diet.

AIN-93G (CD) or AIN-93G incorporated with 5% freeze-dried açaí juice powder (AJ) was made by Harlan Teklad (Madison, WI). To eliminate caloric density as a confounding variable, all diets were formulated to be isocaloric and isonitrogenous. Both diets consisted of 20% casein as the protein source and the diets in different groups had the same levels of protein, essential amino acids, calories, vitamins and minerals. The diet formulation is shown in supplemental data (Table 1).

2.2. Animal protocol

The animal protocol was approved by the Animal Care and Use Committee of the Arkansas Children's Hospital Research Institute. ApoE$^{-/-}$ mice (female, 4 weeks old) of the C57BL/6 genetic background were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were maintained in sterile micro-isolator cages and fed autoclaved-pelleted diet ad libitum and water.

In the first experiment, apoE$^{-/-}$ mice were fed either CD or AJ (15/group) for 20 weeks. Food intake and body weight were recorded weekly. NMR analysis was performed at day 171 to measure body fat and lean body mass content following procedures described before [13]. At the end of 24 weeks, animals were euthanized using CO_2 and serum, plasma and tissue samples (aorta, macrophage, heart and liver) were collected and stored at −70 °C until analysis.

In the second experiment, apoE$^{-/-}$ mice were fed either CD or AJ (15/group) for 5 weeks. Food intake and body weight were recorded weekly. At the end of experiment, animals were euthanized using CO_2 and resident macrophage collected.

2.3. Isolation and treatment of resident macrophages

Resident macrophages were collected using harvest medium (Dulbecco's phosphate-buffered saline) without any eliciting agents. Cells were plated in RPMI-1640 supplemented with 10% (v/v) FBS, 2 mM L-glutamine, penicillin, streptomycin, and sodium pyruvate. Non-adherent cells were removed after 2 h and macrophages used after 48 h. Cells were then treated with or without LPS (Invitrogen, San Diego) for 18 h. Supernate of the cultured macrophages was used to measure protein levels of TNF-a and IL-6, and the cell lysate was used for TNF-α and IL-6 gene expression analyses.

2.4. Serum lipid analysis

Serum total, LDL-, HDL-cholesterol, and triglyceride were determined by colorimetric assay using diagnostic reagents from Synermed (Montreal, Quebec, Canada) [13].

2.5. Aortic lesion analysis

Lesion analysis of the descending aorta (enface analysis) was carried out as described [13] and performed independently by two individuals blinded to the study design. The results are reported as percentage of the total descending aorta area containing lesions.

2.6. LC–MS/MS analysis of lipid peroxidation biomarkers

Analyses of lipid peroxidation markers, including 8-isoprostaglandin $F_{2\alpha}$, hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs), were carried out in an HPLC–MS/MS system based on the method modified from published procedures [14,15]. Sample preparation of serum and liver was based on the method described by our lab [16]. Prior to extraction, three internal standards, 8-isoprostaglandin $F_{2\alpha}$-d_4, 12(S)-HETE-d_8 and 13(S)-HODE-d_6 (Cayman Chemical, Ann Arbor, MI) were added to 30 μL of serum or 100 μL of liver homogenate.

Analysis was carried out using the Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) coupled with a 4000 Q TRAP™ mass spectrometer (Applied Biosystems, Forest City, CA). Separation was performed on a Phenomenex Synergi Max-RP column (150 mm × 3 mm, 4 μm) using a flow rate of 0.4 mL/min. The solvent consisted of (A) 0.2% (v/v) of formic acid in water and (B) methanol. The 19 min gradient was as follows: 22–20% A (1–2 min), 20–20% A (2–18 min), 20–22% A (18–19 min). Multi-reaction monitoring (MRM) mode scan was used for quantitation. The transitions monitored were mass to charge ratio (m/z): m/z 295 → 171 for 9-HODE; m/z 295 → 195 for 13-HODE; m/z 319 → 163 for 8-HETE; m/z 319 → 167 for 11-HETE; m/z 319 → 179 for 12-HETE; m/z 353 → 193 for 8-isoprostaglandin $F_{2\alpha}$; m/z 327 → 184 for 12(S)-HETE-d_8; m/z 299 → 198 for 13(S)-HODE-d_4; m/z 353 → 197 for 8-isoprostaglandin $F_{2\alpha}$-d_4. The mass spectrometer equipped with

an ESI-Turbo V source was operated in the negative ion mode. Major parameters were optimized as follows: ion spray voltage, −4.5 kV; 50 for curtain gas (CUR), 400 °C for source temperature. 30 and 50 for nebulizing (GS1) and turbo spray gas (GS2). The entrance potential (EP), declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were optimized individually with each standard. The analysis was controlled by Analyst v1.4.2 (Applied Biosystems, Forest City, CA) and internal standards 12(S)-HETE-d_8, 13(S)-HODE-d_4 and 8-isoprostane-d_4 were used to for quantification.

2.7. RT-PCR array analyses of aorta samples

Pooled aorta samples (4/group) were frozen in liquid nitrogen and quickly pulverized to a fine powder using a Bessman stainless steel pulverizer (Spectrum Laboratories, Rancho Dominguez, CA). The aorta powders were prepared for mRNA isolation with a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quantity and purity was determined using the NanoDrop ND-2000 (Nanodrop Technologies, Wilmington, DE). RNA integrity was assessed by determining the RNA 28S/18S ratio using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). PCR-array was performed using custom real-time PCR array (SA Biosciences, Frederick, MD) according to the method described previously [16].

2.8. Antioxidant enzyme activity

GSR and GPX activities were measured in homogenized liver or plasma using the Glutathione Reductase Assay Kit and Glutathione Peroxidase Assay Kit from Arbor Assays (Ann Arbor, MI) and Cayman (Ann Arbor, MI), respectively, following the procedures provided by the manufacturers. Serum paraoxonase-1 (PON1) activity was measured by the Paraoxonase Assay Kit from Invitrogen (Carlsbad, CA), following procedures provided by the manufacturer.

2.9. Real-time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and treated with RNase-free DNase. Reverse transcription reaction and quantitative real-time PCR were described previously [17]. Real-time PCR primers (from Integrated DNA Technologies, Coralville, IA) were as follows: β-actin sense (GGCTATGCTCTCCCTCACG), β-actin antisense (CGCTCGGTCAGGATCTTCAT), TNF-α sense (ACAAGGCTGCCCCGACTAC), TNF-α antisense (TGGAAGACTCCTCCCAGGTATATG), IL-6 sense (TGGAGTCACAGAAGGAGTGGCTAAG), and IL-6 antisense (TCTGACCACAGTGAGGAATGTCCAC). A two-step PCR with denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min for 40 cycles was conducted in iCycler (BioRad) to determine the threshold cycle (Ct) value. Expression of TNF-α and IL-6 was calculated using $^{\Delta\Delta}$Ct method using threshold cycles for β-actin as normalization reference. All real-time PCR reactions were carried out at least twice from independent cDNA preparations. RNA without reverse transcriptase served as a negative control.

2.10. Protein measurements

The cytokine assays in serum were performed with an ultra-low-noise charge-coupled device (CCD) Sector® Imager 2400 provided by the Meso Scale Discovery (MSD) (Gaithersburg, MD). The MSD assay kits were purchased from MSD. TNF-α and IL-6 in the supernatant of cultured macrophages was determined by ELISA using Duoset ELISA kits (R&D, Minneapolis, MN) according to the manufacturer's instructions. The optical density was determined using a BMG Polarstar microplate reader at 450 nm. A mean value of triplicate samples for each experiment and two separate experiments were used for analysis.

2.11. SEAP secretion assay

RAW-Blue cells (Invitrogen, San Diego, CA) are derived from RAW264.7 macrophages with chromosomal integration of a SEAP reporter construct inducible by NF-κB and AP-1. RAW-Blue mouse macrophage cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT) and zeocineosin (200 μg/ml). All cell culture reagents were purchased from Invitrogen (San Diego, CA).

RAW-Blue cells (1×10^5 cells/well) were treated with 5% supernates from 48 h cultured resident macrophages for 18 h. The supernatants were then collected for SEAP secretion assay. QUANTI-Blue™ powder was dissolved in endotoxin-free water and sterile filtered (0.22 μm) (QuantiQuanta-blue substrate). RAW-Blue cell supernatant (40 μL/well) was added to QuantiQuanta-blue substrate (160 μL/well) and incubated at 37 °C for 0.5–1 h. Absorbance was measured at 620 nm in a PolarStar microplate reader (BMG Labtech, Durham, NC).

2.12. Statistical analysis

The animals were randomly assigned to treatment groups and the data were normally distributed and the variances of the two groups were equal. Thus, we used an independent group *t*-test to compare the means of the two diet groups. Data were expressed as mean value ± SD. Student's *t*-test was used to analyze differences between groups. A value of $P < 0.05$ was considered as significant difference unless otherwise mentioned. Statistical analyses were performed using SigmaStat statistical software (SigmaStat 3.5).

3. Results

3.1. Results from the first animal experiment

3.1.1. Growth and body composition

Mean body weight of AJ fed mice was higher ($P < 0.001$) than that of CD fed mice (Table 1). NMR analysis showed that body fat content and lean body mass did not differ significantly between CD and AJ fed mice (Table 1).

3.1.2. Plasma lipid profile

Plasma levels of total, LDL- and HDL-cholesterol, and triglyceride were analyzed (Table 1). HDL cholesterol levels were significantly greater ($P < 0.01$) in AJ fed group. Total cholesterol, LDL cholesterol and triglyceride did not differ between the groups.

3.1.3. Atherosclerotic lesion measurement

Enface analysis of the descending aorta showed that the mean percentage of lesion areas in AJ fed animals were 58% less than that in CD fed animals ($P < 0.001$, Fig. 1A and B).

3.1.4. Changes of lipid peroxidation markers

Different isomers of HETEs, HODEs along with 8-isoprostaglandin $F_{2\alpha}$ were quantified in serum and liver (Table 2). In serum, 12-HETE was the most abundant isomer. 12-HETE, 8-HETE, 11-HETE, 9-HODE and 13-HODE were all significantly lower in the serum of AJ fed mice. 5-HETE, 15-HETE and 8-isoprostaglandin $F_{2\alpha}$ were not detected in serum. All forms of HETEs, HODEs and 8-isoprostaglandin $F_{2\alpha}$ were detected and quantified in liver. Except for 15-HETE, 8-isoprostaglandin $F_{2\alpha}$, all

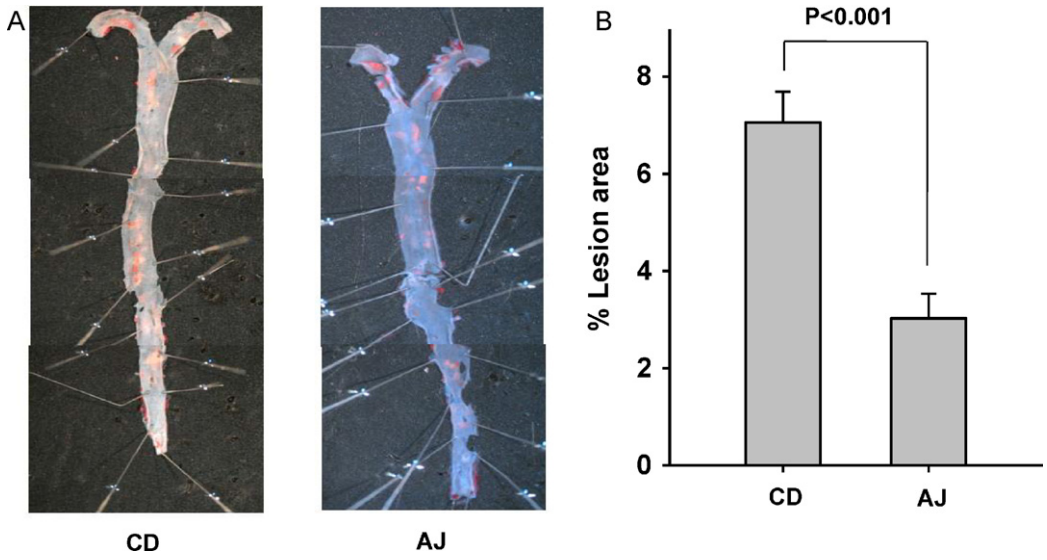

Fig. 1. AJ diet reduced the extent of early atherosclerotic fatty streak lesions in apoE$^{-/-}$ mice. Representative photomicrographs of enface analysis showing the lesion areas in the descending aorta in apoE$^{-/-}$ mice fed CD or AJ diet (A). Percentage of the lesion areas in AJ fed animals were 58% lower than that in CD fed animals ($n = 7$, $^{***}P < 0.001$) (B).

forms of HETEs and HODEs were significantly lower in the liver homogenates of AJ fed mice.

3.1.5. RT-PCR array analyses

Of the genes expression analyzed by RT-PCR array, two antioxidant enzyme genes, glutathione reductase (Gsr) and glutathione peroxidase 3 (Gpx3) were significantly up-regulated in AJ fed animals ($P < 0.05$) (Fig. 2A).

3.1.6. AJ intake increased certain antioxidant enzymes in serum or liver

GSR activities in both serum and liver and GPX activity in serum were greater ($P < 0.05$) in AJ fed animals (Fig. 2B–D). GPX activity in liver did not differ between the two groups due to huge variations (data not shown). PON1 activity in serum of AJ animals was higher than that in CD animals ($P < 0.01$) (Fig. 2E).

Table 2
The results of HETEs, HODEs and F_2-isoprostane in serum and liver of mice fed CD and AJ.[a]

	CD	AJ
Serum (ng/mL)[b]		
5-HETE	ND	ND
8-HETE	66.9 ± 46.7	40.7 ± 11.3*
11-HETE	5.50 ± 2.12	0.61 ± 0.14*
12-HETE	671.4 ± 533.4	370.6 ± 130.2*
15-HETE	ND	ND
9-HODE	1.49 ± 0.67	1.00 ± 0.50*
13-HODE	5.72 ± 3.28	3.88 ± 2.54*
F_2-isoprostane	ND	ND
Liver (ng/g tissue)[c]		
5-HETE	86.8 ± 21.5	63.4 ± 20.1*
8-HETE	86.6 ± 18.4	52.9 ± 10.3***
11-HETE	112.7 ± 29.5	71.6 ± 27.8*
12-HETE	330.8 ± 47.8	159.9 ± 33.6***
15-HETE	141.8 ± 44.1	112.9 ± 35.2
9-HODE	2083.1 ± 825.6	1181.0 ± 378.0*
13-HODE	1432.4 ± 473.6	979.5 ± 249.3*
F_2-isoprostane	448.4 ± 144.2	297.8 ± 78.1*

[a] Results are expressed as mean ± SD.[b] $n = 7$ for CD and $n = 4$ for AJ.[c] $n = 8$ for CD and $n = 7$ for AJ.
* $P < 0.05$.
*** $P < 0.001$.

3.2. Results from the second animal experiment

3.2.1. IL-6 and TNF-α in plasma

The IL-6 levels in the plasma of mice fed AJ and CD were 8.5 ± 4.7 pg/mL and 16.2 ± 6.5 pg/mL ($n = 6$, $P < 0.05$), respectively. The level of TNF-α was also lower in plasma of mice fed AJ comparing to that of mice fed CD (0.23 ± 0.31 pg/mL vs. 0.94 ± 0.45 pg/mL, $n = 6$, $P < 0.05$).

3.2.2. Gene expression and protein levels of IL-6 and TNF-α in resident macrophages

Gene expression and protein levels of IL-6 and TNF-α were lower in resident macrophages from mice fed AJ ($P < 0.05$) (Fig. 3A and B). When the cells were stimulated by LPS, gene expression and protein levels of IL-6 and TNF-α elevated in both diet groups, nevertheless, the values were all lower in mice fed AJ ($P < 0.05$) (Fig. 3C and D).

3.2.3. SEAP secretion assay

The supernates of cultured resident macrophages without stimuli were used in SEAP reporter assay. The 48 h cultured supernates of macrophages from AJ fed mice resulted in lower value compared with the CD fed mice (supplemental data Fig. S2), indicating a reduction in the ability of AJ to activate the NF-κB pathway.

4. Discussion

In this study, we provide direct experimental evidence showing that a diet containing an açaí juice at the dose of 5% developed significantly less atherosclerotic lesions in the apoE$^{-/-}$ mice model (Fig. 1). Descending aorta was used for measuring lesion areas because the lesions developed throughout the aorta and its principal branches in this model [18]. AJ diet did not change body composition despite a significant gain in body weight, nor did it alter total cholesterol, LDL-cholesterol or triglyceride levels (Table 1). However, HDL-cholesterol levels were higher in the AJ fed animals. Clinical and epidemiological studies have reported an inverse and independent association between serum HDL-cholesterol levels and coronary heart disease risk [19]. Thus, elevating HDL could be one of the mechanisms contributing to the observed athero-protective effects of AJ, though how AJ increased HDL is yet to be determined.

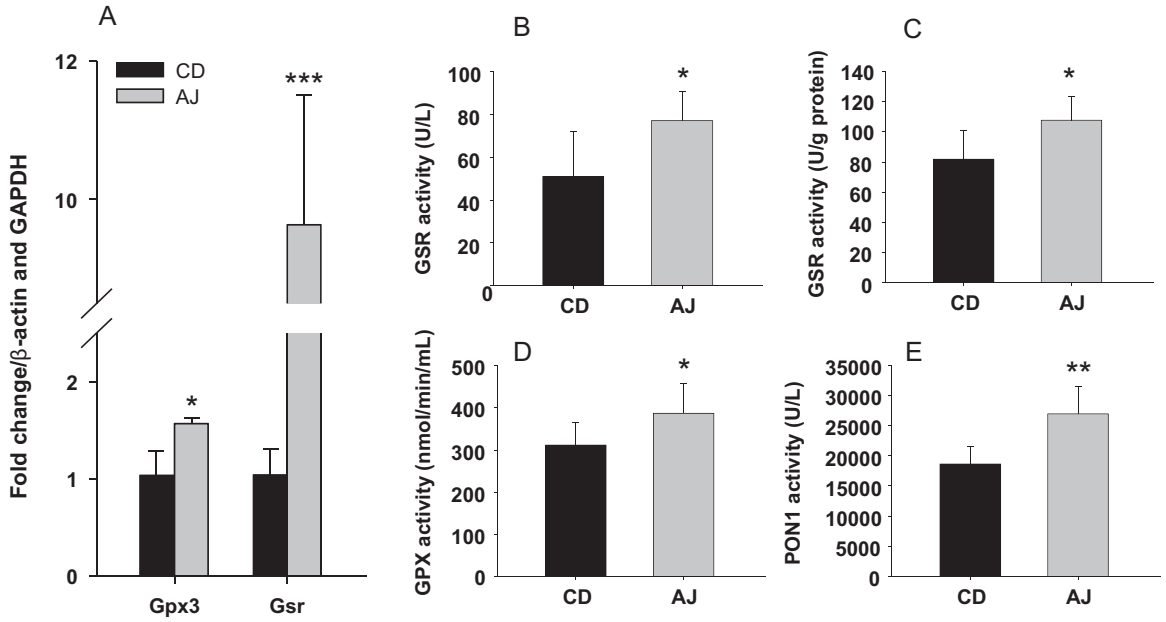

Fig. 2. RT-PCR array analysis indicated that expression of two antioxidant enzymes, glutathione reductase (Gsr) and glutathione peroxidase 3 (Gpx3) were up-regulated in pooled aorta of AJ mice ($n = 4$/group) (A). Enzyme activities of GSR in plasma ($n = 6$) (B), GSR in liver (C) ($n = 6$), GPX in plasma ($n = 6$) (D) and paraoxonase 1 (PON1) ($n = 6$) (E) in mice fed CD or AJ diets. Values are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Atherosclerosis has been recognized as a chronic inflammatory disease in which oxidative stress plays a pivotal role in its initiation and progression [20,21]. The use of natural antioxidants and plant compounds that affect athero-protection has been considered as being of meaningful clinical relevance [5]. The athero-protective activities of açaí juice and the possible underlying mechanisms were investigated with primary emphases on reducing oxidative stress and anti-inflammatory activities.

Low density lipoprotein (LDL) oxidation is a key step in the early stage of the development of atherosclerosis [22]. The oxidation of

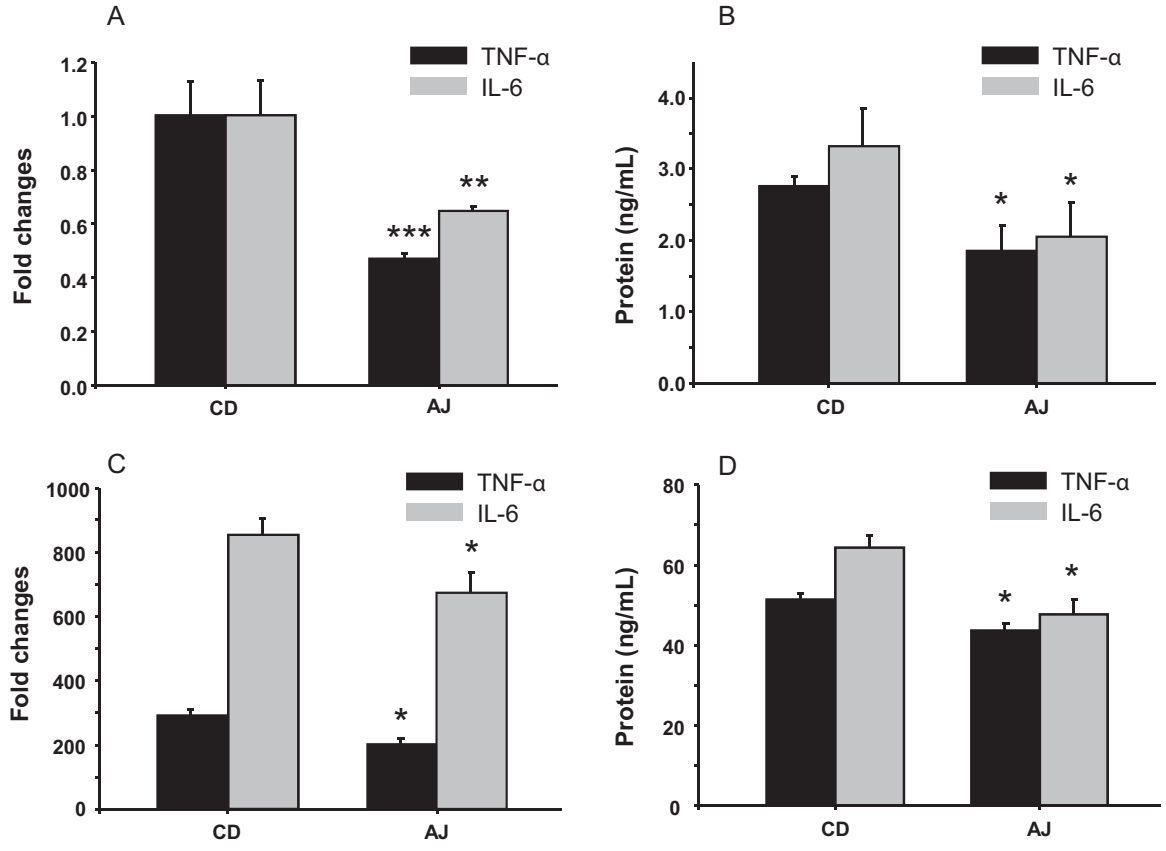

Fig. 3. Gene expression and protein levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in resident macrophages without (A and B) and with LPS stimulation (C and D). Values are expressed as mean ± SD, $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

fatty acids proceeds by either enzymatic or non-enzymatic mechanisms [23]. Various lipid oxidation products have been used as biomarkers of lipid peroxidation [24,25]. 8-isoprostaglandin $F_{2\alpha}$ and its isomers, which are formed by the non-enzymatic, free-radical mediated oxidation of arachidonic acid, are regarded as the "gold standard" for assessing lipid peroxidation [26]. However, because these markers are formed by free radical-mediated oxidation, the yields from the parent lipids are minimal. Other important lipid peroxidation products include HETEs from arachidonic acid and HODEs from linoleic acid, both of which are formed by enzymatic (lipoxygenase and cytochrome P450) and non-enzymatic pathways [27]. Compared to 8-isoprostaglandin $F_{2\alpha}$, HETEs and HODEs are yielded by much simpler mechanisms from more abundant parent lipids *in vivo*. In this study, an LC-MS/MS method was used to simultaneously measure 8-isoprostaglandin $F_{2\alpha}$ and different isomers of HETEs and HODEs. The 8-isoprostaglandin $F_{2\alpha}$ was found to be significantly lower in the liver of mice fed AJ ($P < 0.01$) (Table 2), but was not detected in the plasma probably due to an extremely low concentration. Except for 15-HETE in the liver, all other forms of HETEs and HODEs were found to be significantly lower in the serum and liver of AJ fed animals (Table 2). Taken together, our data clearly indicate that AJ significantly reduced lipid peroxidation, which could be driven by either enzymatic or non-enzymatic pathways.

The results from RT-PCR array of 17 genes related to oxidation/antioxidant enzymes showed that mRNA expression of two antioxidant enzyme genes Gsr and Gpx3 were significantly up-regulated in the aorta of AJ-fed mice (Fig. 2). The activities of GSR in serum and liver and GPX in serum were also increased. The activities of these two enzymes in aorta failed to be detected because of the low enzyme activities. Glutathione peroxidase (GPX) and glutathione reductase (GSR) are considered as important antioxidant enzymes in vascular systems [28]. Gpx3 is an important intracellular antioxidant enzyme that reduces hydrogen peroxide and lipid peroxides to water and lipid alcohols by oxidizing glutathione (GSH) to glutathione disulfide (GSSG) [29]. GSR reduces GSSG to the sulfhydryl form GSH [30]. Hence, these two antioxidant enzymes may act synergistically to reduce lipid peroxidation. Another serum antioxidant enzyme PON1 was also elevated in AJ fed mice. PON1 is associated with HDL and has been implicated in the prevention of LDL lipid peroxidation. It has been proposed to play a major role in protecting against oxidative stress and its consequent cardiovascular disease development [31].

Due to limited sample size/volume, we were unable to study other mechanisms in the first experiment. In a separate experiment, the apoE$^{-/-}$ mice were fed CD or AJ for 5 weeks. Two pro-inflammatory cytokines IL-6 and TNF-α were significantly lower in serum of mice fed AJ. Further tests of gene expression and protein levels of these two cytokines were then performed in resident macrophages based on a consensus that macrophages are the major cell types in vascular systems in producing both of these cytokines [32]. The recruitment and activation of macrophages is considered as the most important early event in the development of atherosclerotic lesion. Activated macrophages release various pro-inflammatory cytokines including IL-6 and TNF-α that amplify the local inflammatory response in the lesion [33]. In agreement with the observation in serum, IL-6 and TNF-α were all significantly lower at mRNA and protein levels in macrophages of mice fed AJ. Not only did AJ reduce basal levels of these two pro-inflammatory cytokines, it also increased the resistance of secretion of these two cytokines by macrophages in response to inflammatory stimuli such as LPS. Both TNF-α and IL-6 are predictive of current as well as future CVD and cardiovascular mortality [34]. A recent study proposed that TNF-α and IL-6 as predictors of the severity of coronary artery disease [35]. Therefore, reducing pro-inflammatory cytokine TNF-α and IL-6 might be another important mechanism underlying the protective effects of AJ. Moreover, it has been recognized that nuclear factor κB (NF-κB) plays a crucial role as transcription factor in regulating many of the pro-inflammatory cytokine genes [36]. The results from SEAP Reporter assay suggested that AJ treatment reduces NF-κB activation. This may partly be explained by the lowered level of TNF-α, which is also the activator of NF-κB activation. However, exactly what components involved in the NF-κB activation pathway were affected by AJ warrants further investigation.

In conclusion, this study provides the first reported indication that açaí juice protects against atherosclerosis in the hyperlipidemic apoE deficient model. We also presented evidence that the athero-protective effect of the açaí juice is in part due to reduced lipid peroxidation, which may be due to increasing the levels and activity of two antioxidant enzymes. The data support that possibility that the açaí juice may also exert a protective effect against the development of atherosclerosis by inhibiting pro-inflammatory cytokines through regulating inflammatory mediators. We are currently working to identify bioactive compounds in the açaí juice that could produce these effects as well as the mechanisms underlying their actions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.02.035.

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