Biochemical and Molecular Action of Nutrients

Pomegranate Juice Supplementation to Atherosclerotic Mice Reduces Macrophage Lipid Peroxidation, Cellular Cholesterol Accumulation and Development of Atherosclerosis[1]

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ABSTRACT   Inhibition of lipid peroxidation contributes to the attenuation of macrophage cholesterol accumulation, foam-cell formation and atherosclerosis. Evidence suggests that nutritional antioxidants such as pomegranate juice (PJ) can contribute to the reduction of oxidative stress and atherogenesis. The goals of the present study were to determine whether such beneficial effects of PJ exist when supplemented to apolipoprotein E-deficient (E^0) mice with advanced atherosclerosis and to analyze the antiatherosclerotic activity of a tannin-fraction isolated from PJ. Mice (4-mo-old) were supplemented with PJ in their drinking water for 2 mo and compared with age-matched placebo-treated mice, as well as to young (4-mo-old) control mice, for their mouse peritoneal macrophage (MPM) oxidative state, cholesterol flux and mice atherosclerotic lesion size. PJ supplementation reduced each of the proatherogenic variables determined in the present study compared with age-matched placebo-treated mice. It significantly induced serum paraoxonase activity and reduced MPM lipid peroxide content compared with placebo-treated mice and control mice. PJ administration to E^0 mice significantly reduced the oxidized (Ox)-LDL MPM uptake by 31% and MPM cholesterol esterification and increased macrophage cholesterol efflux by 39% compared with age-matched, placebo-treated mice. PJ consumption reduced macrophage Ox-LDL uptake and cholesterol esterification to levels lower than those in 4-mo-old, unsupplemented controls. PJ supplementation to E^0 mice with advanced atherosclerosis reduced the lesion size by 17% compared with placebo-treated mice. In a separate study, supplementation of young (2-mo-old) E^0 mice for 2 mo with a tannin fraction isolated from PJ reduced their atherosclerotic lesion size, paralleled by reduced plasma lipid peroxidation and decreased Ox-LDL MPM uptake. PJ supplementation to mice with advanced atherosclerosis reduced their macrophage oxidative stress, their macrophage cholesterol flux and even attenuated the development of atherosclerosis. Moreover, a tannin-fraction isolated from PJ had a significant antiatherosclerotic activity.   J. Nutr. 131: 2082–2089, 2001.

KEY WORDS: • pomegranate • lipid peroxidation • LDL • HDL • macrophages • antioxidants • atherosclerosis

Macrophage cholesterol accumulation leading to foam cell formation is the hallmark of early atherosclerosis (1,2). Cholesterol accumulation in macrophages can result from an unbalanced cellular cholesterol flux, i.e., increased uptake of atherogenic lipoproteins and/or decreased cholesterol efflux from the cells (3–5).

Oxidized (Ox)[3]-LDL are taken up by macrophages at an enhanced rate via scavenger receptors (4), leading to the formation of lipid-laden foam cells and accelerated atherosclerosis (3,4,6). Moreover, oxidative stress has been shown to affect not only the LDL lipids, but also cellular lipids, including those found in arterial macrophages (7,8). We have previously shown that such lipid-peroxidized macrophages exhibit an increased ability to oxidize LDL and to take up Ox-LDL (7,8), thus leading to cellular accumulation of cholesterol and oxidized lipids. Serum paraoxonase is an HDL-associated esterase and it was shown to protect from oxidation lipids in lipoproteins and in lesions by its hydrolytic and peroxidative-like properties (9–11).

Because induction of oxidative stress on LDL and on arterial cells plays an important role in atherogenesis, its inhibition by nutritional antioxidant should slow the progression of

the disease. Enrichment of LDL, as well as of arterial cells with nutritional antioxidants such as vitamin E or polyphenolic flavonoids, protects the LDL against lipid peroxidation and attenuates the development of atherosclerosis (12–15). Pomegranate juice (PJ), which is very rich in flavonoids, was recently shown to be antiatherogenic, and this effect is probably related to its potent antioxidative activity (16).

PJ supplementation to atherosclerotic apolipoprotein E-deficient (E^0) mice before they develop atherosclerosis significantly inhibited the progression of atherosclerotic lesion (16). No studies, however, have been conducted on the effect of nutritional antioxidants on macrophage cholesterol accumulation in mice that already exhibit advanced atherosclerotic lesions.

PJ contains 85% moisture, 10% sugar, 1.5% pectin, ascorbic acid and polyphenolic flavonoids. The content of soluble polyphenols in PJ varied between 0.2% and 1.0% and include mainly anthocyanins (such as cyanidin-3-glucoside, cyanidin-3, 5-diglucoside and delphindin-3-glucoside), catechins, ellagic tannins, gallic and ellagic acids (17).

E^0 mice are often used to study atherogenesis because they develop severe hypercholesterolemia low cholesterol diet, they exhibit high oxidative stress and develop extensive atherosclerosis by the age of 4 mo (18,19).

In the present study we examine the effect of PJ administration to E^0 mice with advanced atherogenesis on their macrophage lipid peroxidation, cellular cholesterol flux and the development of atherosclerotic lesions. In addition, we analyzed the antiatherosclerotic properties of an isolated antioxidant fraction of PJ.

METHODS

Mouse study

E^0 mice were generously provided by Dr. Jan Breslow (Rockefeller University, NY). Gene targeting in mouse embryonic stem cells was used to create mice that lack apolipoprotein E (18).

To analyze the effect of PJ on the development of the atherosclerotic lesions, 30 male E^0 mice were divided into three groups of 10.

PJ group. A concentrated PJ, obtained by squeezing the entire fruit, was diluted in water (6.25 mL of concentrated PJ in 1 L of water). The PJ solution diluted in water was given to the mice at 4 mo of age for 2 mo, whereas only water was given to the placebo mice. Because mice drank an average of 5 mL/d, they effectively drank 31 μL of PJ/d, which is equivalent to 0.875 μmol of total polyphenols/d. This dose of PJ was used based on results of our previous study (16), showing that it substantially inhibited the development of atherosclerotic lesion.

Placebo group. Mice received no addition to their drinking water from 4 to 6 mo of age.

Control group. Mice at 4 mo of age received no addition to their drinking water. This reference group was included to measure the state of the atherosclerotic lesions in E^0 mice at the beginning of the treatment period.

At the end of the study (6 mo of age for the first two groups and 4 mo of age for the control group) blood, peritoneal macrophages and aortas were obtained.

Serum cholesterol, lipid peroxidation and paraoxonase (arylesterase) activity. Blood was collected from the retroorbital plexus of mice under ether anesthesia. Serum cholesterol was determined using a commercial kit (Ref 352-20; Sigma Diagnostics, St Louis, MO) (20).

To determine plasma lipid peroxidation, plasma samples were incubated in the absence or presence of 100 mmol/L of 2,2′-azobis 2-amidinopropane hydrochloride (AAPH; Wako Chemical Industries Ltd, Osaka, Japan) for 2 h at 37°C. AAPH is a water-soluble azo compound that thermally decomposes to produce peroxyl radicals at a constant rate. Plasma lipid peroxide content was determined by measuring their ability to convert iodide to iodine, measured at 365 nm (21).

Serum paraoxonase is an enzyme with multiple activities. Its activity is commonly determined by its arylesterase activity using phenylacetate as the substrate. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 5 μL of serum, 1.0 mmol/L phenylacetate and 0.9 mmol/L $CaCl_2$ in 20 mmol/L Tris HCl (pH 8.0). Nonenzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. The E_{270} for the reaction was 1310 $(mol/L)^{-1}$ cm^{-1} (22).

Mouse peritoneal macrophages (MPM). MPM were harvested from the peritoneal fluid of E^0 mice 4 d after intraperitoneal injection into each mouse of 3 mL of thioglycolate (24g/L) in saline. The cells ($10–20 \times 10^6$/mouse) were washed and centrifuged three times with phosphate-buffered saline (PBS) at $1000 \times g$ for 10 min, then resuspended at 10^9/L in DMEM containing 10% horse serum (heat-inactivated at 56°C for 30 min) and 1×10^5 U penicillin/L, 100 mg streptomycin/L and 2 mmol/L glutamine. The dishes were incubated in a humidified incubator (5% CO_2, 95% air) for 2 h, washed with DMEM to remove nonadherent cells and the monolayer was incubated under similar conditions for 18 h.

The level of cellular lipid peroxidation in MPM was determined by lipid peroxide determination in a sonicated cell preparation (23).

Lipoprotein preparation. LDL and HDL were prepared from human plasma (drawn into 1 mmol/L of Na_2 EDTA) from fasting normolipidemic volunteers. LDL (d = 1.019–1.063 kg/L) and HDL (d = 1.064–1.21 kg/L) were prepared by discontinuous density gradient ultracentrifugation (24). The lipoproteins were then dialyzed against 150 mmol/L NaCl, 1 mmol/L Na_2 EDTA (pH 7.4). LDL were sterilized by filtration and was used within 2 wk. The protein content of the lipoproteins was determined with the folin phenol reagent (25). LDL was radioiodinated using the iodine monochloride method (26) modified for lipoproteins. ^{125}I-Oxidized LDL were prepared by an overnight dialysis of ^{125}I-labeled LDL (1 g of lipoprotein protein/L) against PBS to remove any residual EDTA, followed by incubation with 10 μmol/L $CuSO_4$ for 18 h at 37°C. Oxidation was terminated by refrigeration and the addition of 0.1 mmol/L Na_2 EDTA. The degree of LDL oxidation was determined by using the thiobarbituric acid reactive substances assay (27), and it ranged from 18 to 25 nmol of mafondialdehyde (MDA) equivalents/mg lipoprotein protein compared with 0.5–1.0 nmol of MDA equivalents/mg lipoprotein protein in native LDL.

Macrophage cholesterol metabolism

Cellular uptake of lipoproteins by macrophages. Uptake of lipoproteins by macrophages was assessed by measuring the degradation of lipoproteins. ^{125}I-Ox-LDL or native ^{125}I-LDL at 10 mg of protein/L were incubated with the MPM at 37°C for 5 h. Lipoprotein cellular degradation was measured in the collected medium as the trichloroacetic acid-soluble, nonlipid radioactivity, which was not due to free iodide (28). Lipoprotein degradation in a cell-free system measured under identical conditions was minimal (<10%) and was subtracted from the total degradation. The remaining cells were washed three times with cold PBS and dissolved in 0.1 mol/L NaOH for protein determination.

Cellular cholesterol esterification. Cellular cholesterol esterification was estimated by measurement of the incorporation of [^{3}H]-oleate into cholesteryl ester. Cells were incubated with medium in 10% fetal calf serum, for 18 h at 37°C. During the last 2 h of incubation, [^{3}H]-oleate in complex with albumin (2.7 mmol/L, 83 mmol oleate/mg albumin, 10 Ci/L) was added to the medium. At the end of the incubation, cellular lipids were extracted with hexane/isopropanol (3:2 v/v) and the cholesteryl ester was separated by thin layer chromatography using hexane/ether/acetic acid (130:30:1.5 v/v/v), scraped into vials containing scintillation fluid and counted in a β-counter (29).

Cellular cholesterol efflux. MPM were incubated with [^{3}H]-labeled cholesterol for 18 h at 37°C followed by cell wash in ice-cold PBS (three times) and further incubation in the absence or presence

of 100 mg of human HDL protein/L for 3 h at 37°C. Cellular and medium [^{3}H]-label were quantitated and HDL-mediated cholesterol efflux was calculated as the ratio of [^{3}H]-label in the medium/[^{3}H]-label in the medium+[^{3}H]-label in cells (30).

PJ fractionation and characterization of antioxidant activities of an isolated fraction. Purification of active compounds from PJ was achieved by HPLC on reverse-phase Sep-Pak. PJ (8 g) was dissolved in a mixture of methanol:water (30 mL; 1:2 v:v), and diethyl ether was added (40 mL). The aqueous phase was separated and mixed with ethyl acetate (200 mL). After phase separation, the organic layer was evaporated. This residue was dissolved in 1 mL water, chromatographed on reverse-phase Sep-Pak column with water (3 × 2 mL) followed by mixtures of water:methanol (10–20%), and a pure fraction was collected.

This fraction was named compound 25 because it appears after 25 min of chromatography on the Sep-Pak column. Compound 25 was hydrophilic with medium solubility in ethyl acetate, suggesting that it is not a lipid or a protein. Furthermore, its maximal absorption was at 254 nm and, therefore, it is not a simple flavonoid. It was shown from infrared spectra to contain many hydroxyl groups. Nuclear magnetic resonance-spectra revealed the presence of quaternary carbons and a few hydrogen atoms, and, hence, compound 25 might be a complexed polyphenol, such as hydrolyzable tannin.

The antioxidant capacity of compound 25, which was isolated from PJ, was tested by its ability to inhibit $CuSO_4$-induced LDL oxidation. LDL were oxidized in the absence or presence of increasing concentrations of compound 25, by its incubation with 5 μmol/L of $CuSO_4$ for 2 h at 37°C. The degree of LDL oxidation was determined by the thiobarbituric acid reactive substances assay (27) and the lipid peroxide content (21).

Mice at 2 mo old were administered either compound 25 (20 μg/d) or PJ [31 μL (equivalent to 0.875 μmol of total polyphenols)/d] for 2 mo and were analyzed for their atherosclerotic lesion size compared with age-matched, placebo-treated mice.

Histopathology of aortic atherosclerotic lesions. After PJ treatment to E^0 mice for 2 mo, the heart and aorta were rapidly removed and immersion fixed in 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer with 0.1 g/L calcium chloride (pH 7.4) at room temperature. The aortic arch was then dissected free from the surrounding fatty tissue and the first 4 mm of the ascending aorta was removed and cut transversely with razor blades into blocks of 1 mm each. After overnight treatment with fixative, the samples were then rinsed and stored in 0.1 mol/L of sodium cacodylate buffer containing 75 g/L sucrose before treatment with an unbuffered 10 g/L aqueous solution of osmium tetroxide for 4 h. This was followed by dehydration in ascending ethanols, before propylene oxide and embedding in epoxy resin (Eponate 12; Pelco Int., Redding, CA). The blocks were then orientated so that transverse sections of the aorta could be cut. After heat polymerization (18 h at 60°C), the blocks were trimmed and 1-μm sections cut with diamond knives on an LKB Nova
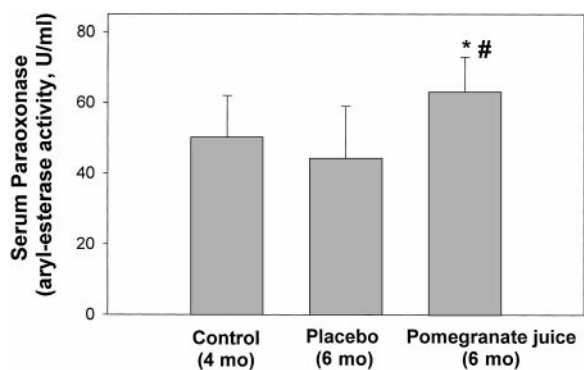
FIGURE 1 Serum paraoxonase activity in 4-mo-old, unsupplemented, control E^0 mice and in 6-mo-old E^0 mice administered a placebo or PJ for 2 mo. Values are means ± SEM, n = 10. *P < 0.01 vs. placebo; #P < 0.01 vs. control.
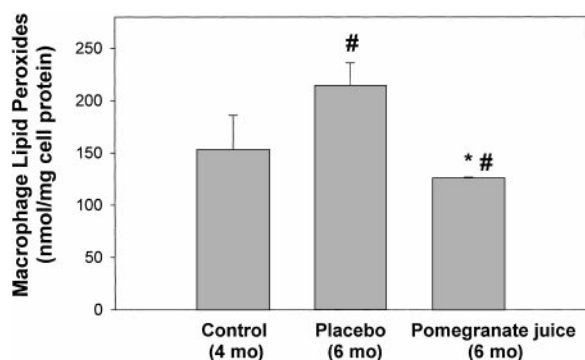
FIGURE 2 MPM lipid peroxidation in 4-mo-old, unsupplemented, control E^0 mice and in 6-mo-old E^0 mice administered a placebo or PJ for 2 mo. Values are means ± SEM, n = 10. *P < 0.01 vs. placebo; #P < 0.01 vs. control.

ultramicrotome (LKB, Bromma, Sweden). When sufficient semithin sections were obtained from all the blocks, the remainder of the blocks was then cut into much thicker sections (150–200 μm) for more macroscopic observation. The lipid content of the lesions of these thicker sections was stained an intense black from the osmium treatment and permits lesion areas to be easily determined histomorphometrically. Only the area of the aortic arch was examined because previous and on-going studies by us and other groups have shown that this area is especially prone to atherosclerosis in E^0 mice and the areas well-defined with a clear starting point (aortic valves). Histomorphometric determinations of lesion size were performed using an Olympus Cue-2 image analysis system with appropriate morphometry software (Olympus Corporation, Lake Success, NY). The system consists of a Zeiss Universal R photomicroscope (×10 objective) fitted with a Panasonic WV-CD50 camera. Measurements were made in standardized windows (fields) with an area of 176,758 μm^2.

Statistics. Student's t-test was used in comparing two means, whereas ANOVA and post-hoc tests were used when more than two groups were compared. Results are given as means ± SEM. Differences were considered significant if P < 0.05.

RESULTS

Serum lipid concentrations, lipid peroxidation and paraoxonase activity. Plasma cholesterol concentrations were not significantly affected by PJ consumption (621 ± 36, 593 ± 58, and 666 ± 47 mg/dL in the control, placebo-treated and PJ-treated mice, respectively). PJ consumption minimally reduced plasma lipid peroxide concentration produced by AAPH by 8% in comparison to control and placebo-treated mice, as shown by values of 547 ± 39, 545 ± 27 and 507 ± 32 mmol lipid peroxides/L in the plasma of control, placebo-treated and PJ-treated mice, respectively (P < 0.05).

A nonsignificant 12% reduction (P = 0.12) in serum paraoxonase was noted in placebo-treated mice (6 mo old), in comparison to the control, 4-mo-old mice (**Fig. 1**). A significant 43% increase in serum paraoxonase activity was obtained in mice that consumed PJ for 2 mo compared with placebo-treated mice. Moreover, serum paraoxonase activity in PJ-treated mice was 26% higher than in control mice (Fig. 1).

Macrophage oxidative state. MPM lipid peroxides content in placebo-treated mice was 40% higher than in control, 4-mo-old mice (**Fig. 2**). PJ consumption for 2 mo reduced macrophage lipid peroxide content by 42% compared with placebo-treated mice (P < 0.05). Furthermore, macrophage lipid peroxide content in MPM harvested from PJ-treated mice was 20% lower than in control, 4-mo-old mice (Fig. 2).
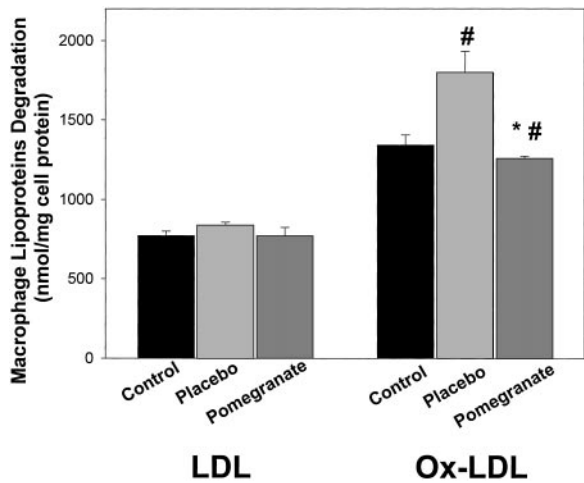
FIGURE 3 Macrophage uptake of Ox-LDL and native LDL in 4-mo-old, unsupplemented, control E^0 mice and in 6-mo-old E^0 mice administered a placebo or PJ for 2 mo. Values are means $\pm$ SEM, n = 10. *$P < 0.01$ vs. placebo; #$P < 0.01$ vs. control.

Lipoprotein uptake by peritoneal macrophages. Degradation of Ox-LDL by MPM harvested from 6-mo-old, placebo-treated mice was 34% greater than in control, 4-mo-old mice (**Fig. 3**). Incubation of MPM, derived from E^0 mice that consumed PJ with ^{125}I-labeled Ox-LDL (10 mg of protein/L) significantly reduced cellular lipoprotein degradation, by 31% and 10%, compared with Ox-LDL uptake by MPM harvested from age-matched, placebo-treated mice or control mice, respectively (Fig. 3). In contrast, the uptake of native LDL by MPM was not significantly affected by age or PJ supplementation (Fig. 3).

Peritoneal macrophage cholesterol esterification rate. MPM harvested from 6-mo-old, placebo-treated mice had a 110% greater cholesterol esterification rate compared with control, 4-mo-old mice (**Fig. 4**). Cholesterol esterification rates in MPM isolated from PJ-treated mice was 80% and 57% lower, respectively, compared with age-matched, placebo-treated mice and control mice (Fig. 4).
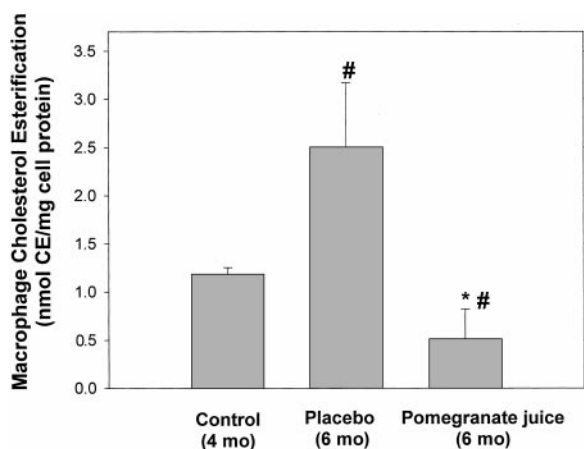

FIGURE 4 Macrophages cholesterol esterification in 4-mo-old, unsupplemented, control E^0 mice and in 6-mo-old E^0 mice administered a placebo or PJ for 2 mo. Values are means $\pm$ SEM, n = 10. *$P < 0.01$ vs. placebo; #$P < 0.01$ vs. control.

FIGURE 5 Cholesterol efflux from their harvested macrophages in 4-mo-old, unsupplemented, control E^0 mice and in 6-mo-old E^0 mice administered a placebo or PJ for 2 mo. Values are means $\pm$ SEM, n = 10. *$P < 0.01$ vs. placebo; #$P < 0.01$ vs. control.
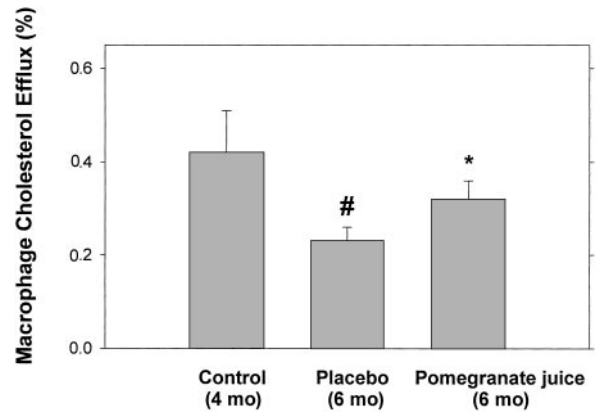
Peritoneal macrophage cholesterol efflux rate. MPM cholesterol efflux was 46% lower ($P < 0.05$) in cells harvested from the 6-mo-old, placebo-treated mice than in control, 4-mo-old mice (**Fig. 5**). In contrast, PJ treatment significantly increased, by 39%, macrophage cholesterol efflux from macrophages compared with cholesterol efflux from MPM harvested from the placebo-treated mice (Fig. 5).

Atherosclerotic lesion size. As illustrated in the photomicrographs of the aortic lesions (**Fig. 6**, A and B), lesions of the placebo-treated E^0 mice were progressively enlarged and became more complex from 4 to 6 mo with increased numbers of lipid-laden macrophage-derived foam cells. The PJ-supplemented mice, in contrast, showed less advanced lesions with fewer foam cells (Fig. 6C) compared with the age-matched, placebo-treated mice.

When measuring their atherosclerotic lesion size, placebo-treated E^0 mice exhibited a substantial increase (by 214%) at 6 mo of age compared with control mice (Fig. 6D). Although the atherosclerotic lesion area in the PJ-treated mice was greater than in the younger control mice (Fig. 6D), it was 17% ($P < 0.01$) smaller than in the lesion area in aortas from age-matched, placebo-treated mice (Fig. 6D).

PJ isolated fraction on atherosclerotic lesion development. We isolated an active antioxidant compound from PJ, referred to as compound 25, which inhibited $CuSO_4$-induced LDL oxidation (**Table 1**). Although this active compound was not fully identified, it has characteristics of complexed tannins.

Because the antiatherosclerotic effect of PJ is more pronounced when PJ was given to young mice, before any significant atherosclerotic development is observed (16), we administered compound 25 and whole PJ to young, 2-mo-old E^0 mice. In comparison to control, nontreated E^0 mice, compound 25 and PJ possessed potent antiatherosclerotic characteristics as shown by significant reductions of 54% and 44%, respectively, in the lesion size (**Fig. 7**, A–D).

Moreover, administration of either compound 25 or whole PJ to 2-mo-old E^0 mice for 2 mo resulted in 25% and 17% reductions, respectively, in the plasma lipid peroxide concentrations (305 $\pm$ 2 μmol/L in control mice and 230 $\pm$ 19 μmol/L and 253 $\pm$ 4 μmol/L in mice treated with compound 25 and PJ, respectively).

In MPM from E^0 mice that consumed compound 25 or whole PJ, cellular degradation rates of Ox-LDL (25 mg/L of Ox-LDL) were decreased by 34% and 25%, respectively, com-

FIGURE 6 Atherosclerotic lesion area and foam cell formation. Photomicrographs of typical foam cells in E^0 mice that were supplemented with PJ for 2 mo (C) are presented in comparison to placebo-treated mice (A), as well as to control, 4-mo-old mice (B). The lesion macrophage lipids in macrophages were stained black by osmium tetroxide. All micrographs are at the same magnification. Each experiment group contains 10 mice. The aortic arch in 4-mo-old, unsupplemented, control E^0 mice and in 6-mo-old E^0 mice administered a placebo or PJ for 2 mo (D). Values are means $\pm$ SEM, $n = 10$. *$P < 0.01$ vs. placebo; #$P < 0.01$ vs. control.
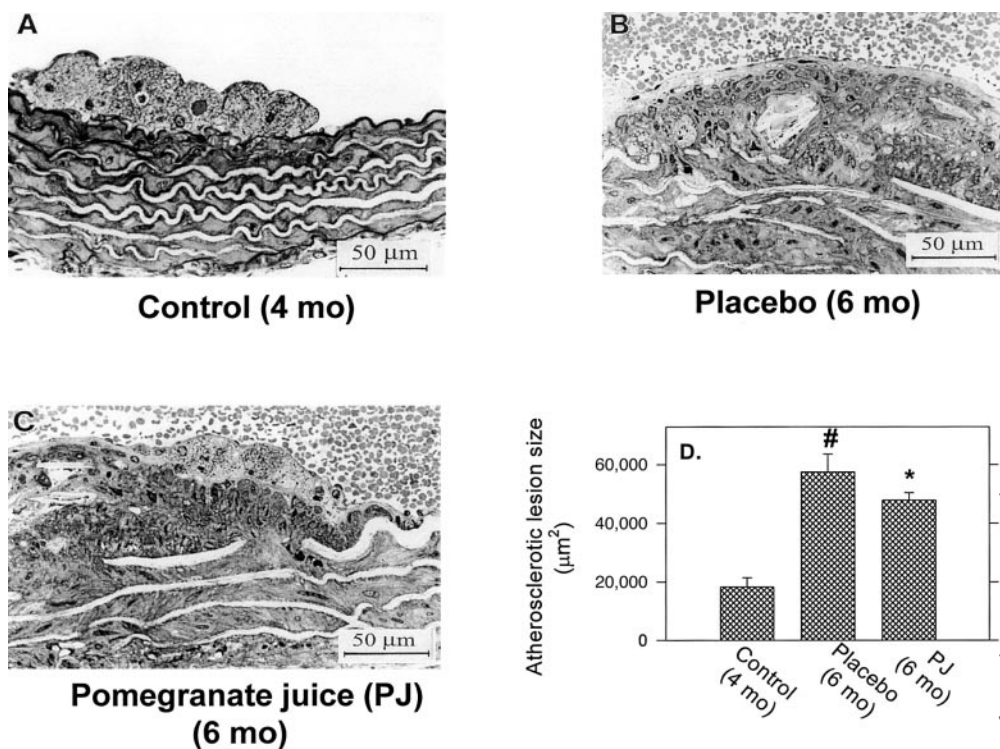
pared with Ox-LDL degradation by control E^0 mice (from 2998 ± 238 ng/mg cell protein in MPM from control mice to 1969 ± 161 ng/mg and 2258 ± 277 nmol/mg in MPM from mice treated with compound 25 or PJ, respectively).

DISCUSSION

At early stage of atherosclerosis, macrophage cholesterol (and oxidized lipid) accumulation and foam cell formation takes place, leading to the development of the complicated atherosclerotic lesion (1–4). Major contributors to cholesterol accumulation in arterial cells during atherogenesis include high plasma cholesterol concentration (31), increased oxidative stress (23,32), reduced serum paraoxonase activity (33), increased uptake of atherogenic lipoproteins by arterial cells (34–36), enhanced macrophage cholesterol esterification rate

TABLE 1

The antioxidant capacity of an isolated fraction (compound 25) from whole pomegranate juice[1]

Compound 25	TBARS[2]	Lipid peroxides
mg/L	*nmol/mg LDL protein*	
0	116 ± 21	2752 ± 189
2.5	99 ± 19	2591 ± 201
5.0	$50 \pm 4^*$	$1120 \pm 111^*$
7.5	$13 \pm 2^*$	$382 \pm 25^*$
10	$11 \pm 1^*$	$152 \pm 14^*$
20	$2 \pm 0.3^*$	$85 \pm 19^*$
25	$2 \pm 0.3^*$	$175 \pm 15^*$

[1] Values are means $\pm$ SEM, $n = 3$. *$P < 0.01$ vs control (0 mg/L compound 25).
[2] TBARS, thiobarbituric reactive substance.

(37) and decreased cholesterol efflux from arterial cells (5,38). Because oxidative stress is believed to play an important role in early atherogenesis, efforts should be made to reduce oxidative stress and its harmful consequences (39–43). Antioxidants, such as those found in some fruits and vegetables, which are rich in polyphenolic flavonoids (red wine, grapes, licorice, ginger and pomegranate), significantly reduce oxidative stress by inhibiting the formation of Ox-LDL and macrophage lipid peroxidation. The capacity of these antioxidants to reduce atherogenesis may be due to the reduction in the oxidative stress (13–16,44).

In this study PJ consumption by atherosclerotic mice significantly reduced cholesterol accumulation and foam cell formation. PJ supplementation to atherosclerotic mice reduced all the proatherogenic variables determined compared with age-matched, placebo-treated mice. A major difference between the present study and our previous study (16) on the effect of PJ supplementation to E^0 mice is that in the present study, PJ consumption started in mice with already advanced atherosclerosis (4-mo-old). Under such conditions of extensive atherosclerosis, the lesions mature and become more resistant to the influence of drug or nutrient interventions. When initiating antioxidant-rich nutrient supplementation after the onset of atherosclerosis, inhibition of the progression of atherogenesis is more difficult than intervention during early stages of atherogenesis, but this condition may be a more realistic reflection of common practice. By using two sets of controls, i.e., the 4-mo-old, control mice and the placebo-treated, 6- mo-old mice, we were able to measure the atherogenic variables in similar mice before PJ supplementation.

In the present study, the reduction in the size of the atherosclerotic lesion by PJ consumption was smaller (only 17%) compared with our previous study (16), where up to a 33% reduction in the lesion size was obtained. Whereas in the present study mice were initially supplemented with PJ at 4 mo of age when they already had extensive atherosclerosis, in our
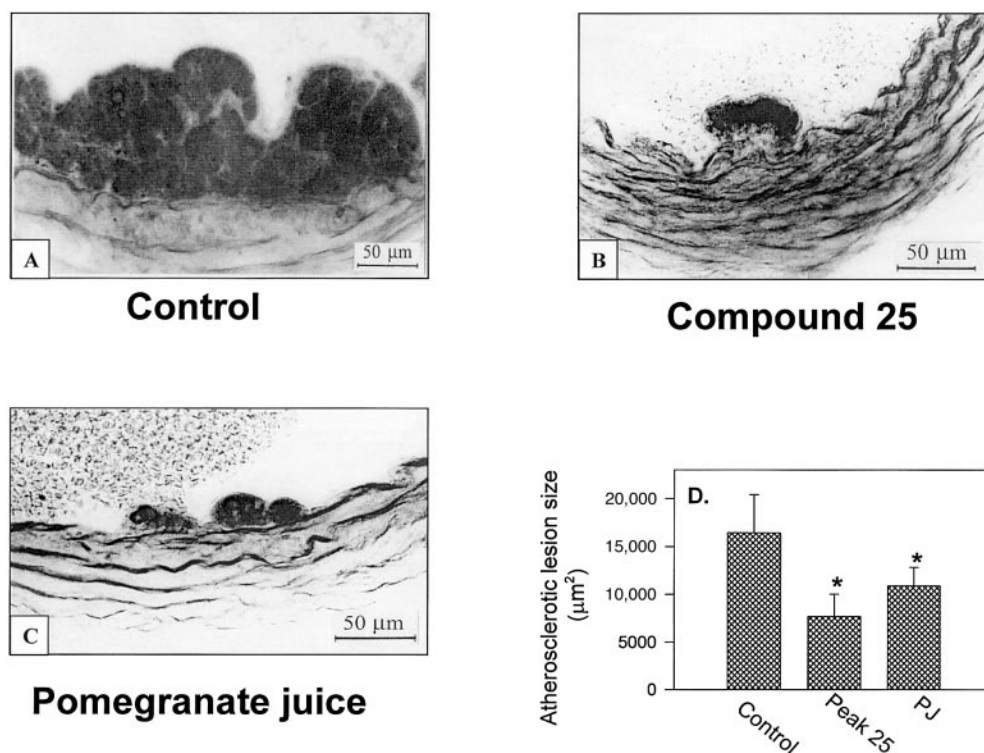
FIGURE 7 Effect of and isolated fraction from PJ (compound 25) and PJ supplementation to young E^0 mice on their arterial foam cell formation and atherosclerotic lesion area. Photomicrographs of typical foam cells in E^0 mice that were supplemented with either compound 25 (C) are presented compared with PJ-treated mice (B) and placebo-treated mice (A). The lesion macrophage lipids were stained black by osmium tetroxide. All micrographs are at the same magnification. Each experiment group contains 10 mice. The aortic arch in 4-mo-old, unsupplemented, control E^0 mice and in 6-mo-old E^0 mice administered a placebo or PJ for 2 mo (D). Values are means $\pm$ SEM, $n = 10$. *$P < 0.01$ vs. control.

previous study (16) treatment began as early as 5 wk of age when no atherosclerosis could be observed. The present study, however, demonstrated that even in the atherosclerotic mice, PJ consumption still showed antiatherogenic activity and could still slow down the progression of atherosclerosis. This may be related to the impressive inhibitory effect of PJ consumption on macrophage cholesterol and cholesteryl ester accumulation as evidenced by attenuation of cholesterol influx and esterification and by an enhanced macrophage cholesterol efflux. However, the relatively small reduction in the atherosclerotic lesion size after PJ consumption, in comparison to the substantial reduction in macrophage lipid peroxides content may indicate that in the advanced atherosclerotic mice, factors in addition to oxidative stress may affect lesion progression. Such factors may include growth factors and cytokines produced and released by arterial cells at later stages of atherogenesis.

As shown in previous studies, the antioxidative effect of PJ consumption could be detected in plasma and in the harvested peritoneal macrophages (MPM), as measured by analysis of their lipid peroxides (16,45). Due to the onset of PJ consumption by the mice when they already presented advanced atherosclerosis, the antioxidant effect of PJ in the plasma was rather small compared with our previous study (16), which showed a substantial decrease in plasma susceptibility to oxidation after PJ supplementation to young, nonatherosclerotic E^0 mice. Of interest is that the increase with age (6 mo vs. 4 mo) in the E^0 MPM lipid peroxide content was reduced by PJ consumption to levels that were even lower than those observed in MPM from control, young E^0 mice.

Furthermore, serum paraoxonase activity was significantly increased by PJ consumption. Because paraoxonase protects against LDL oxidation (11) and hydrolyzes and reduces lipid peroxides and cholesteryl linoleate hydroperoxides in oxidized lipoproteins (46) and in atherosclerotic lesions (47), the beneficial effect of PJ on serum paraoxonase may further contrib-

ute to the reduction in oxidative state found in the PJ-treated E^0 mice both in their plasma and MPM. In addition to its ability to hydrolyze specific oxidized lipids in oxidized lipoproteins, paraoxonase itself is inactivated by lipid peroxides (47). Thus, the increased serum paraoxonase activity may have resulted from the reduced oxidative stress in the serum of PJ-treated mice.

In the present study we have extended the search for possible mechanisms related to the inhibitory effect of PJ consumption on macrophage foam cell formation. Cellular cholesterol flux was analyzed by determining macrophage cholesterol influx (uptake of atherogenic Ox-LDL), as well as macrophage cholesterol efflux (release of cellular cholesterol from MPM by HDL). The present study clearly demonstrated the beneficial effect of PJ consumption by E^0 mice on both fluxes, i.e., inhibition of Ox-LDL uptake by the harvested MPM and stimulation of HDL-induced cholesterol release from these cells. Both of these processes reduced macrophage cholesterol content, foam cell formation and atherosclerosis (1,3–6). PJ reversed the increased macrophage uptake of Ox-LDL, which was associated with aging and the development of atherosclerosis, to levels of lipoprotein degradation rates by MPM that were even lower than those observed in control young mice. Macrophage cholesterol efflux was not affected with mice age (6 mo vs. 4 mo), but PJ supplementation increased the efflux rate, and, thus, could contribute to the reduction in macrophage cholesterol content.

Native LDL cellular uptake (via the LDL receptor), unlike macrophage uptake of Ox-LDL (via the scavenger receptors), was not affected by age (placebo-treated vs. control, young mice) or by PJ consumption. This may be related to the inability of native LDL to cause macrophage cholesterol accumulation, because the LDL receptor is tightly regulated by the cellular cholesterol content (48).

In macrophage foam cells, substantial amounts of the cholesterol are in the esterified form and acyl cholesterol acyl-

transferase activity is increased during atherogenesis (37). Enhanced esterification of the cholesterol leads to the accumulation of cholesteryl ester droplets, a feature that resembles lesion's macrophage foam cells (1,31,49). The present study demonstrated that macrophage cholesterol esterification rate substantially increased with age (6 mo vs. 4 mo). PJ consumption, however, inhibited macrophage cholesterol esterification rate to rates that were slower than those in control mice. Macrophage cholesterol esterification rate was measured using MPM that were harvested from mice after PJ or placebo administration. The decreased cholesterol esterification rate after PJ consumption, thus, may indicate an inhibitory effect of PJ on acyl cholesterol acyltransferase activity, which is in line with antiatherogenicity secondary to decreased cholesteryl ester formation. However, because the MPM are incubated with fetal calf serum that contains plasma lipoproteins, it is also possible that the reduced macrophage cholesterol esterification rate observed after PJ consumption is related to a decreased uptake of atherogenic lipoprotein, and, hence, to a reduced macrophage cholesterol content, which is reflected by a slower cholesterol esterification rate because less substrate (lipoprotein-derived unesterified cholesterol) is available for the added labeled fatty acid.

We have also isolated an active antiatherosclerotic fraction (compound 25) from PJ with significant antioxidative capacities. Compound 25 consumption by E^0 mice significantly reduced the atherosclerotic lesion size and this effect was associated with decreased plasma lipid peroxidation and attenuated MPM uptake of Ox-LDL. Because the anti-atherosclerotic effect of PJ was more pronounced when it was given to the young, nonatherosclerotic mice, we analyzed the effect of the isolated active PJ tannin fraction on lesion progression in the young E^0 mice.

In conclusion, PJ supplementation to E^0 mice possesses very impressive antiatherogenic properties, which could be related to its potent antioxidative activity and beneficial effect on macrophage cholesterol flux, which results in decreased macrophage cholesterol accumulation. The effect of PJ consumption on atherosclerosis was shown not only when supplementation of PJ to E^0 mice started before they developed atherosclerotic lesions, but also in mice with extensive atherosclerosis. The above antiatherosclerotic properties of PJ could be related to the presence of a tannin fraction in PJ with potent antioxidative characteristics.

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