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ORIGINAL ARTICLE

Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation

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Summary Dietary supplementation with polyphenolic antioxidants to animals was shown to be associated with inhibition of LDL oxidation and macrophage foam cell formation, and attenuation of atherosclerosis development.

We investigated the effects of pomegranate juice (PJ, which contains potent tannins and anthocyanins) consumption by atherosclerotic patients with carotid artery stenosis (CAS) on the progression of carotid lesions and changes in oxidative stress and blood pressure.

Ten patients were supplemented with PJ for 1 year and five of them continued for up to 3 years. Blood samples were collected before treatment and during PJ consumption. In the control group that did not consume PJ, common carotid intima-media thickness (IMT) increased by 9% during 1 year, whereas, PJ consumption resulted in a significant IMT reduction, by up to 30%, after 1 year. The patients' serum paraoxonase 1 (PON 1) activity was increased by 83%, whereas serum LDL basal oxidative state and LDL susceptibility to copper ion-induced oxidation were both significantly reduced, by 90% and 59%, respectively, after 12 months of PJ consumption, compared to values obtained before PJ consumption. Furthermore, serum levels of antibodies against oxidized LDL were decreased by 19%, and in parallel serum total antioxidant status (TAS) was increased by 130% after 1 year of PJ consumption. Systolic blood pressure was reduced after 1 year of PJ consumption by 21% and was not further reduced along 3 years of PJ consumption. For all studied

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parameters, the maximal effects were observed after 1 year of PJ consumption. Further consumption of PJ, for up to 3 years, had no additional beneficial effects on IMT and serum PON1 activity, whereas serum lipid peroxidation was further reduced by up to 16% after 3 years of PJ consumption.

The results of the present study thus suggest that PJ consumption by patients with CAS decreases carotid IMT and systolic blood pressure and these effects could be related to the potent antioxidant characteristics of PJ polyphenols.
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Introduction

Oxidative stress, a major contributor to cardiovascular diseases, is associated with lipid peroxidation in arterial macrophages and in lipoproteins.[1–4] Oxidized low density lipoprotein (Ox-LDL) has been shown to be atherogenic and inhibition of LDL oxidation by potent dietary flavonoid antioxidants[4,5] attenuated atherosclerosis development in laboratory animals. Recently, it was shown that vitamin E supplementation to patients with carotid artery stenosis inhibited LDL accumulation in arterial macrophages.[6] Protection of lipids from oxidation can be also achieved by serum paraoxonase 1 (PON1), an HDL-associated esterase that can hydrolyze and reduce specific lipid peroxides in arterial cells and in lipoproteins in coronary and carotid lesions.[7–10]

The medicinal properties of pomegranate are described by all major religions and by folk medicine.[11] Pomegranate juice (PJ) was indeed shown recently to possess impressive antioxidative properties due to its polyphenolics, tannins and anthocyanins.[12] We have recently shown the antioxidative and antiatherogenic characteristics of PJ consumption in atherosclerotic apolipoprotein E deficient (E^0) mice.[13] Also, in healthy humans, PJ consumption was shown to possess potent antioxidative capabilities against lipoprotein oxidation, and increased serum PON1 activity and serum total antioxidant status.[13] In the present study, thus, we analyzed for the first time the effects of PJ consumption by patients with carotid artery stenosis, on their serum oxidative stress in association with the progression of carotid atherosclerotic lesions.

Subjects and methods

Patients

Nineteen patients from the Vascular Surgery Clinic, 5 women and 14 men, aged 65–75 years, non-smokers, with asymptomatic severe carotid artery stenosis (CAS, defined as 70–90% stenosis in the internal carotid arteries) were included in the study. These patients had an abnormal echo Doppler of the carotids, which was performed following a finding of carotid ''bruit'' on physical examination, or complains of headache or dizziness. The patients were randomized to either pomegranate juice or placebo, and they signed an informed consent before the beginning of the study. Ten patients were included in the PJ treated group. Nine patients that did not consume PJ served as a control group. Both groups were matched, with similar serum concentrations of lipids and glucose, and with similar blood pressures (data not shown). Both groups were treated with similar hypocholesterolemic and anti-hypertensive drugs. In each group, 60% of the patients were treated with statins, 60% were treated with angiotensin converting enzyme (ACE) inhibitors, 20% were treated with β-blockers, and 20% were treated with calcium channel blockers. The patients continued their therapy along the study, and their dietary habits and life style did not change during the whole study. Ten patients consumed 50 ml of PJ per day (which contain 1.5 mmoles of total polyphenols) for a period of 1 year, and five out of them agreed to continue for up to 3 years. This PJ concentration was chosen based on our previous study on the beneficial PJ properties in healthy volunteers.[13] Blood samples were collected after 12 h fast. Blood analyses and echo doppler of the carotid arteries were performed at the beginning of the study and 3, 6, 9, 12, 22, 28 and 36 months after PJ consumption. In the control group echo doppler of the carotid arteries was performed at the beginning of the study and after 1 year.

The study was approved by the Helsinki Committee of the Rambam Medical Center, Israel Ministry of Health.

Pomegranate processing

Pomegranates were picked by hand, washed, and stored in tanks. The fruits were crushed, and

squeezed. The juice was filtered, pasteurized, concentrated and stored at −18°C. Each day along the study period, the concentrated PJ was diluted 1:5 (v:v) with water in order to obtain a single strength PJ. The antioxidant composition of the juice include: 1979 mg/l of tannins (1561 mg/l of punicalagins and 417 mg/l of hydrolyzable tannins), 384 mg/l of anthocyanins (delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, delphinidin-3-glucoside, cyanidin 3-glucoside, pelargonidine 3-glucoside), and 121 mg/l of ellagic acids derivatives. The juice contained also 3 mg of vitamin C per 100 ml of PJ.

Atherosclerotic lesion analysis by B-mode ultrasonography

Common carotid artery IMT from B-mode ultrasound is a widely used measure of early atherosclerosis.[14–16] After careful axial scan, longitudinal B-mode images of common carotid artery wall boundaries were obtained with a high resolution color power Doppler ultrasound (ATL 5000 or 3500 Advanced Technological Laboratories, Bothell, WA) with a 5–12 MHz multifrequency transducer. IMT was electronically measured at the far wall of the distal common carotid arteries, about 1 cm from the carotid bifurcation, by assessing the boundaries of intima and media with electronic calipers. Atherosclerotic plaques at the common carotid arteries and the carotid bulb, as well as the proximal and distal internal carotid arteries were imaged and the length and width of the plaque were assessed. On duplex examination of the internal carotid arteries, flow velocities were calculated at the stenotic sites, and expressed by peak systolic velocity (PSV), and end diastolic velocity (EDV). The ultrasound outcome analyses were the change over time in IMT, which was measured in the same preselected carotid artery segments, the change in the plaque dimensions and the change in blood flow velocities. A protocol was adapted in order to ensure that the arteries were examined from the same angle (60°) at all follow-up examinations. Patients had up to eight duplex examinations of the common and internal carotid arteries on each side: there was one ultrasound Doppler examination at baseline, and seven more examinations during PJ consumption. All ultrasound studies were done by the same physician (DG), assuring reproducibility of the site of IMT and plaque measurement, as well as site and interrogation angles on the duplex follow-up examinations. In order to avoid potential introduction of scanner-dependent variabilities, the same ultrasound system was used for individual patients on all follow-up examinations.

Analytical methods

All biochemical determinations were performed in serum. Blood glucose was measured using enzymatic kit (Roche). Total cholesterol, high density lipoprotein (HDL) cholesterol and triglyceride concentrations in serum were measured by diagnostic kits (Raichem). Serum apolipoproteins A-I and B-100 concentrations were determined using specific antibodies by an immunoturbidimetric assay.[17]

Serum paraoxonase 1 (PON1) arylesterase activity

Serum arylesterase activity was measured 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. Non-enzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. The E_{270} for the reaction is 1310 M^{-1} cm^{-1}. One unit of arylesterase activity is equal to 1 μmol of phenylacetate hydrolyzed/min/ml.[18]

Serum total antioxidant status (TAS)

Total antioxidant status was measured in serum with a commercially available kit (Randox Laboratories, Antrim, United Kingdom, catalog no. NX 2332).

Serum anti Ox-LDL antibodies

Serum anti Ox-LDL antibodies concentration was measured in samples collected from the patients before treatment and after 3 or 6 months of PJ consumption, by using the immunoelisa anti-Ox-LDL test (Immco Diagnostics, Inc. Buffalo, NY, USA, Cat No 1158). Results are expressed as Enzyme Units per milliliter (EU/ml).

Serum lipids peroxidation

Serum lipids peroxidation was measured before and after 12, 22, 28 and 36 months of PJ consumption. Plasma samples were incubated without or with 100 mM of 2.2'-azobis, 2-amidinopropane hydrochloride (AAPH, Wako, Japan) for 2 h at 37°C.[19] At the end of the incubation period, the amount of lipid peroxides was measured by the method of

El-Saadani et al.[20] Plasma lipids peroxidation was calculated by subtracting the values obtained in the absence of AAPH.

LDL isolation

Serum samples were drawn from the patients before and after 1, 3, 6, 9 and 12 months of PJ consumption and kept frozen at -70° until all the samples were collected. Blood was collected also from healthy volunteers in the same time periods, for standardization of the assays. LDL was isolated from the frozen plasma samples by discontinuous density gradient ultracentrifugation as previously described.[21] The LDL was washed at $d = 1.063$ g/ml, dialyzed against 150 mmol/l NaCI, 1 mmol/l Na_2 EDTA (pH 7.4) at 4°C. The LDL fractions were then sterilized by filtration (0.45 μm), kept under nitrogen in the dark at 4°C and used within 1 week. The lipoprotein protein concentration was determined by the Lowry assay.[22] Prior to oxidation, LDL was dialyzed against EDTA-free, phosphate buffered saline (PBS) solution at pH 7.4, and at 4°C.

LDL oxidation

LDL (100 μg of protein/ml) was incubated with 5 μmol/l of $CuSO_4$ for 2 h at 37°C. Formation of conjugated dienes was continuously monitored by measuring the increase in absorbance at 234 nm.[23] Lag time required for initiation of lipoprotein oxidation was calculated from the oxidation curve. The amount of LDL-associated lipid peroxides was measured by the method of El-Saadani et al.[20] LDLs isolated from healthy volunteers at the same time periods were used for standardization of the oxidation studies.

Carotid Lesion analyses

Complete atherosclerotic plaques, (including the common, internal and external, carotid parts of the lesion) were collected from 2 groups of patients after endartherectomy. One group included seven patients with CAS that did not consume PJ. The second group included two patients that consumed PJ (for 3 or for 12 months). The two groups were age matched and had similar serum lipids and glucose concentration. Treatment with PJ was the only relevant difference between these two groups. Lesions were washed in saline, dried, and their weight measured. The lesions were cut into small pieces and rinsed in PBS, followed by their sonication in an ultrasonic processor (3×20 s at 80 W). The lesion's cholesterol content was measured in the homogenate samples by enzymatic, colorimetric assay using commercial kit (Sigma Co. Ltd). The lipid peroxide content in the lesion was also measured.[20] Reduced glutathione (GSH) content was measured by the DTNB-GSSG reductase recycling assay.[24]

Statistics

The ANOVA test was performed for all statistical analyses used to compare repeated measurements. Results are given as mean $\pm$ SEM. Assays in each sample were performed in triplicate. All comparisons are shown for data after PJ consumption vs. results obtained before treatment (''0'' time).

Results

Mean intima-media thickness (IMT) of the left and right common carotid arteries from severe carotid artery stenosis (CAS) patients that did not consume pomegranate juice (PJ), increased significantly ($P < 0.01$), by 9%, during 1 year period from 1.52 ± 0.03 to 1.65 ± 0.04 mm. In contrast, mean IMT (of the left and right common carotid arteries) in CAS patients that consumed PJ for up to 1 year was reduced after 3, 6, 9 and 12 months of PJ consumption by 13%, 22%, 26% and 35%, respectively, in comparison to baseline values (''0 time, Fig. 1A).

The inhibitory effect of PJ consumption on carotid PSV was significant only after 1 year, with a reduction in mean PSV of both left and right carotid arteries by 21% (Fig. 1B). Mean carotid EDV of both left and right carotid arteries however, gradually decreased, by 16%, 20%, 31% and 44%, after 3, 6, 9 and 12 months of PJ consumption, respectively (Fig. 1C).

PJ consumption by the patients did not significantly affect the levels of all major serum biochemical markers studied including: glucose and cholesterol in HDL and LDL (Table 1). Serum triglyceride concentration however increased by 16%, as reflected by a similar increase in VLDL cholesterol concentration after 12 months of PJ consumption (Table 1), but these increased levels were still in the normal range. Serum markers for heart, kidney and liver function, as well as homocysteine, Lp (a), and total protein concentrations remained unchanged during the whole study (data not shown). Similarly, blood coagulation and blood cell count were not significantly affected by PJ consumption (data not shown).

The patient's systolic blood pressure was significantly ($P<0.05$) reduced by 7%, 11% ,10%, 10% and 12% after 1, 3, 6, 9, and 12 months of PJ consumption, respectively, compared to values obtained before treatment (Table 1). In contrast, PJ consumption had no significant effect on the patient's diastolic blood pressure (Table 1). In the control group, systolic and diastolic blood pressure values were not significantly changed along 1 year of follow-up ($160\pm7/88\pm4$ vs. $163\pm9/85\pm6$ mmHg at baseline and after 1 year, respectively).

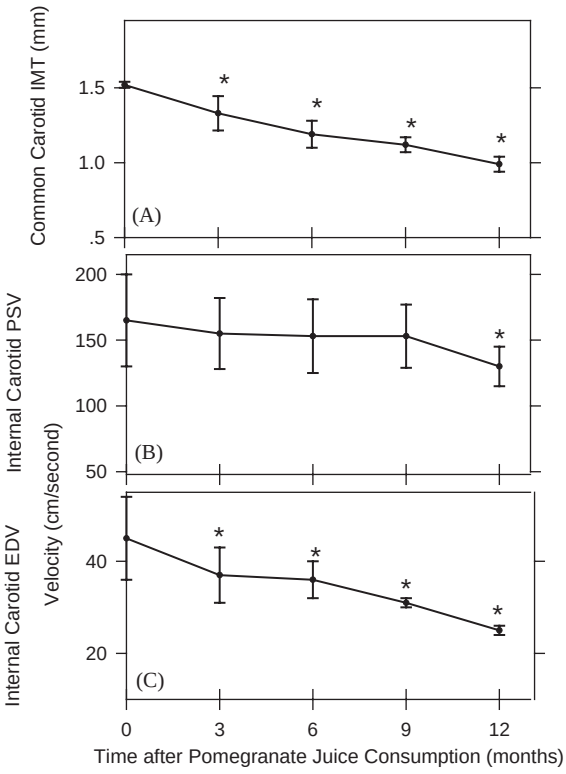

Figure 1 The effect of PJ consumption by patients with carotid artery stenosis (CAS) on carotid IMT, PSV, and EDV. Ten patients with severe carotid artery stenosis were supplemented with PJ for up to 1 year. Common carotid IMT, PSV and EDV were measured in the patients left and right carotid arteries before treatment (Baseline) and during PJ consumption. The mean values of both carotid arteries are presented. (A) Mean common carotid artery IMT. (B) Mean internal carotid artery PSV. (C) Mean internal carotid artery EDV. Results represent mean$\pm$ SEM. *$P<0.01$(after PJ consumption vs. Baseline, "0" time).

In order to analyze the effect of PJ consumption on the patients' serum oxidative state, we measured serum concentration of antibodies against oxidized LDL (Ox-LDL). A significant ($P<0.01$) reduction in the concentration of antibodies against Ox-LDL by 24% and 19% was observed after 1 and 3 months of PJ consumption, respectively, (from 2070 ± 61 EU/ml before treatment to 1563 ± 69 and 1670 ± 52 EU/ml after 1 and 3 months of PJ consumption, respectively, $n=10$). Similarly, total antioxidant status (TAS) in serum was substantially increased, by 130%, from 0.95 ± 0.12 nmol/l at baseline to 2.20 ± 0.25 nmol/l after 12 months of PJ consumption ($n=10$). These results indicate that PJ administration to the patients substantially reduced their serum oxidative status, and could thus inhibit serum lipid peroxidation. Indeed, serum lipid peroxidation, induced by the free radical generator AAPH, was significantly reduced, by 59%, after 1 year of PJ consumption (from 1670 ± 66 to 691 ± 43 nmol of lipid peroxides/ml, $n=10$). The increased resistance of the patients' serum to oxidation after PJ administration could have also resulted from increased serum paraoxonase1 (PON1) activity. Fig. 2A demonstrates a significant ($P<0.01$) increase in serum paraoxonase, measured as arylesterase activity, by up to 83%, after 1 year of PJ consumption ($n=10$). We next isolated LDL from ten patients that consumed PJ for 1 year and analyzed basal oxidative state, as well as

Table 1 The effect of PJ consumption for up to 1 year by patients with carotid artery stenosis (CAS) on their serum biochemical markers, serum lipids levels and on blood pressure.

Time (months)	Glucose (mg/dl)	Cholesterol (mg/dl)	TG (mg/dl)	VLDL-C (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	ApoB-100 (mg/dl)	Apo AI (mg/dl)	Sys. BP (mmHg)	Dial. BP (mmHg)
0	123 ± 9	184 ± 7	118 ± 16	24 ± 3	47 ± 4	113 ± 6	114 ± 3	136 ± 8	174 ± 8	81 ± 3
1	125 ± 8	186 ± 8	148 ± 20*	32 ± 4*	47 ± 3	107 ± 7	102 ± 5	127 ± 7	162 ± 9**	84 ± 3
3	128 ± 10	178 ± 9	144 ± 19*	29 ± 4*	46 ± 4	103 ± 7	102 ± 5	149 ± 10	155 ± 9*	84 ± 2
6	119 ± 9	177 ± 6	145 ± 17*	29 ± 3*	45 ± 2	103 ± 5	99 ± 3	134 ± 7	157 ± 8*	84 ± 2
9	116 ± 10	190 ± 3	144 ± 21*	29 ± 4*	42 ± 3	120 ± 3	95 ± 2	139 ± 6	157 ± 5*	81 ± 1
12	120 ± 11	185 ± 4	137 ± 18*	27 ± 4*	44 ± 3	114 ± 3	97 ± 3	148 ± 6	153 ± 7*	81 ± 2

TG: triglyceride, VLDL-C: VLDL cholesterol, LDL-C: LDL cholesterol, HDL-C: HDL cholesterol, Apo AI: apolipoprotein A-I, Apo B: Apolipoprotein B-100, Sys BP: systolic blood pressure, Dias BP-diastolic blood pressure. *$P<0.01$, **$P<0.05$ (vs. "0" time). Results represent mean$\pm$SEM ($n=10$).

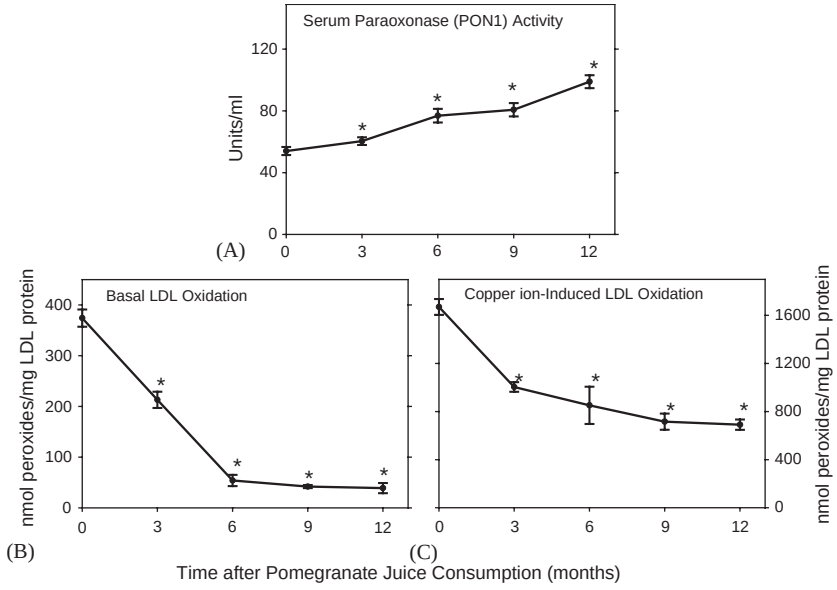

Figure 2 The effect of PJ consumption by patients with carotid artery stenosis (CAS) on serum paraoxonase 1 (PON 1) activity (A), on LDL basal oxidative state (B), and on copper ion-induced LDL oxidation (C). Ten patients with carotid artery stenosis were supplemented with PJ for one year. Blood samples were drawn from the patients before and after 3, 6, 9 and 12 months of PJ consumption. At each time point the patients' serum was collected, their LDL isolated and the oxidative status analyzed. LDL basal oxidative state was determined by the lipid peroxide assay. LDL (100 mg of protein/l) was also incubated with 5 μmol/l of $CuSO_4$ for 2 h at 37°C and the amount of lipid peroxides formed was measured at the end of the incubation period ($n = 10$). Results represent mean SEM. *$P < 0.01$ (after PJ consumption vs. before treatment).

the susceptibility of LDL to copper ion-induced oxidation (Fig. 2B and C). PJ consumption resulted in a significant reduction in the levels of LDL-associated lipid peroxides by up to 90% already after 6 months of PJ consumption (Fig. 2B). The susceptibility of LDL to copper ion-induced oxidation was gradually reduced, as observed by prolongation of the lag time required for the initiation of LDL oxidation (from 30 ± 4 min before treatment to 55 ± 5, 60 ± 2, 64 ± 1 and 65 ± 2 min after 3, 6, 9 and 12 months of PJ consumption, respectively). LDL lipid peroxides content formed during copper ion-induced LDL oxidation was also reduced by up to 59% after 12 months of PJ consumption (Fig. 3C). Continuous supplementation of PJ was required in order to maintain the reduced oxidative stress, as 1 month after termination of PJ consumption, TAS and paraoxonase 1 activity were both reduced from 2.2 ± 0.1 nmol/l and 107 ± 10 U/ml to 1.4 ± 0.1 nmol/l, and to 88 ± 18 U/ml, respectively ($n = 5$). After 1 year, five out of the 10 patients agreed to continue PJ consumption for up to 3 years. Blood samples were taken after 18, 22, 28 and 36 months. Body mass index (BMI) did not change (Table 2) and systolic (but not diastolic) blood pressure remained reduced in comparison to baseline (''0'' time) along the 3 years of PJ consumption (Table 2).

Serum glucose and lipid concentrations were not significantly affected by PJ consumption. Serum PON1 activity increased after 1 year of PJ consumption by 73% and a further 10% increase was obtained after 3 years (Table 2). Similarly, AAPH-induced serum lipids peroxidation that was decreased by 60% after 1 year of PJ consumption was further reduced by 16% after 3 years, as compared to the levels obtained after 1 year on PJ. The reduction in mean IMT, PSV, EDV (mean of both left and right carotid arteries) by 26%, 20%, and 22%, respectively, was noted already after 1 year of PJ consumption, and further consumption for up to 3 years had no additional inhibitory effect on these lesion parameters (Table 2). In two out of the ten patients on PJ (after 3 and 12 months) due to clinical deterioration, carotid endartherectomy operation was performed and their carotid lesions were analyzed and compared to lesions obtained from seven patients that did not consume PJ (not the patients of the placebo group). The cholesterol content in carotid lesions from the two patients that consumed PJ was lower by 58% and 20%, respectively, in comparison to lesions obtained from CAS patients that did not consume PJ (Fig. 3A). Similarly, the lipid peroxides content in lesions obtained from the patients after PJ consumption for 3 or 12 months was significantly reduced by 61%

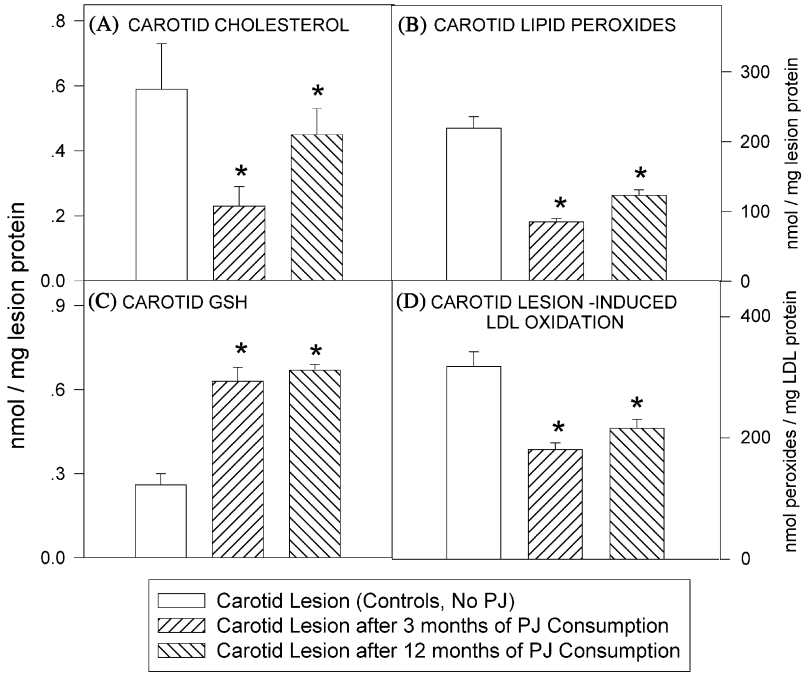

Figure 3 The effect of PJ consumption by patients with carotid artery stenosis on their lesion's cholesterol, oxidized lipids and reduced glutathione (GSH) content and on lesion-mediated oxidation of LDL. Lesions were collected from seven patients with carotid artery stenosis after endartherectomy, and from two patients from the PJ study group that consumed PJ for 3 and 12 months, and had to undergo endartherectomy during the study due to clinical deterioration. The amount of cholesterol (A), lipid peroxides (B), and GSH (C) were measured in lesion homogenates. Lesions (0.3 g) were also incubated with LDL (100 mg of protein/l) in PBS for 20 h at 37°C. The extent of LDL oxidation was measured by the lipid peroxide assay. Lesion-mediated oxidation of LDL was calculated by subtracting the values obtained in control LDL (incubation with no lesion) from those obtained after LDL incubation with the lesions. Three determinations were done on each lesion. Results represent mean ± SEM. *$P < 0.01$ (carotid lesions after PJ consumption vs. control, no PJ carotid lesions).

Table 2 The effect of pomegranate juice consumption for up to 3 years by patients with carotid artery stenosis (CAS) on their blood pressure, serum lipids, oxidative stress and lesion IMT.

Time (months)	BMI Kg/m^2	Sys.BP (mmHg)	Dias. BP (mmHg)	Glucose (mg/dl)	Cholesterol (mg/dl)	TG (mg/dl)	VLDL-C (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)
0	27±2	170±7	78±4	120±7	189±8	120±18	24±4	121±7	46±3
12	28±2	143±7*	81±2	118±11	185±4	137±18	27±3	114±3	44±3
22	27±3	141±5*	77±3	117±5	174±18	175±48*	35±3	100±10	38±3
28	26±2	139±3*	72±2	99±4	176±15	142±45	28±5	107±9	43±5
36	27±2	143±2*	77±3	106±5	191±21	172±50	34±4	118±14	40±3

Time (months)	PON1 (U/ml)	Serum Lipids Oxidation (nmol peroxides/ml)	Mean PSV/ (cm/s)	Mean EDV (cm/s)	Mean IMT(mm)
0	56±5	1980±60	135±6	38±1	1.5±0.2
12	97±10*	790±20*	103±10*	30±12*	1.1±0.1*
22	93±7*	770±30*	100±11*	27±5*	1.0±0.1*
28	105±8*	690±40*	100±10*	29±2*	1.1±0.1*
36	107±10*	660±30*	100±10*	29±2*	1.0±0.1*

PON1: paraoxonase 1, serum lipids oxidation: AAPH-induced serum lipids peroxidation, PSV: peak systolic velocity, EDV: end diastolic velocity, IMT intima media thickness. Results are given as mean ± SEM. *$P < 0.01$ vs. "0" time ($n = 5$). BMI-body mass index, Sys BP: systolic blood pressure, Dias BP: diastolic blood pressure, TG: triglyceride, VLDL-C: very low density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol.

or 44%, respectively, as compared to lesions from patients that did not consume PJ (Fig. 3B). As the balance between lesion pro-oxidants and anti-oxidants levels determines the extent of oxidized lipids, which accumulate in the lesion, we next determined the levels of reduced glutathione (GSH, a major cellular antioxidant). A substantial increase in the lesion GSH content, by 2.5-fold, was observed after PJ consumption for 3 or 12 months, (Fig. 3C). In support to these results, LDL oxidation by lesions derived from the patients after PJ consumption for 3 or 12 months, was significantly ($P<0.01$) decreased by 43% or 32%, respectively, in comparison to LDL oxidation rates obtained by lesions from CAS patients that did not consume PJ (Fig. 3D).

Discussion

The present study clearly demonstrates for the first time that pomegranate juice (PJ) consumption by patients with carotid artery stenosis (CAS) possesses anti-atherosclerotic properties as it significantly reduced common carotid IMT in association with a decrement in systolic blood pressure, and a substantial inhibition of lipids peroxidation in serum and in LDL.

Pomegranate juice was used in our study as the antioxidant of choice, as it is very rich in polyphenols and demonstrates high capability to scavenge free radicals and to inhibit LDL oxidation in vitro and in vivo.[12,13,25,26]

PJ consumption by the CAS patients for as much as 3 years was not toxic as blood chemistry analyses for liver, heart and kidney functions were normal as was recently shown also in rats.[27]

The effect of PJ consumption on carotid lesion progression in CAS patients was assessed by measuring IMT, PSV, and EDV in the right and in the left carotid arteries at baseline and during PJ consumption, for up to 3 years. A marked decrement in IMT, and in blood flow velocities were demonstrated already after 12 months of PJ consumption, compared to values obtained at baseline. Average IMT varies in middle aged men between 0.7 to 1.2 mm, and the progression slope of the mean maximum carotid IMT in untreated patients with carotid artery stenosis was shown to be about 0.02 mm/year.[28] We studied patients with very severe atherosclerosis as shown by their high IMT values. In the present study, PJ consumption for 1 year resulted in a significant reduction in IMT whereas in the control CAS patients (that did not consume PJ), IMT was increased by 9% along 1 year of follow-up.

A reduction in oxidative stress was demonstrated already after 1 month of PJ consumption, (though it was much more pronounced with duration of PJ consumption), but substantial inhibitory effects of PJ consumption on carotid atherosclerosis were demonstrated only after 9–12 months. This may indicate that if the anti-oxidant properties of PJ are responsible for the lesion size regression, then a long period of reduced oxidative stress is required. It might be also that a more profound inhibition of oxidative stress is needed to affect atherosclerotic lesion size. Similarly, it was recently shown that combined supplementation of the antioxidants vitamin E and vitamin C retarded the progression of common carotid atherosclerosis in men.[29,30] Consumption of the ω-3 linolenic acid was also associated with lower prevalence of odds of carotid plaques and lesser thickness of segment-specific carotid IMT.[31] In another study however, vitamin E supplementation reduced the level of circulating oxidized LDL, but did not influence the progression of IMT over a 3-year period.[32] Low plasma levels of the carotenoid anti-oxidant lycopen was shown to be associated with increased IMT,[33] and a positive association was observed between antibodies to oxidized LDL and IMT in healthy aged men.[34] Furthermore, in asymptomatic members of families with familial combined hyperlipidemia, a positive correlation was shown between IMT and LDL susceptibility to oxidation.[35]

The lipid peroxidation hypothesis of atherosclerosis[1–4,36,43] is supported by the presence of oxidized lipids in the atherosclerotic lesion, by an increased oxidative state in LDL derived from atherosclerotic patients[4,37] and by the anti-atherogenicity of potent antioxidants against LDL oxidation.[5,38–40] The ability of PJ to inhibit LDL oxidation (both basal and copper ion-induced) could be related to the high potency of PJ major polyphenols (tannins and anthocyanins) to scavenge free radicals.[12,13] Prodelphinidins in PJ peel were also demonstrated to possess anti-oxidant properties.[41] It has been recently shown that, due to pH reduction, anthocyanins are largely transformed and/or degraded during gastrointestinal digestion.[42] Serum paraoxonase 1 (PON1) activity was shown to be reduced in subjects with hypercholesterolemia, diabetes, and cardiovascular disease.[43–46] We have previously demonstrated increased serum PON1 activity in healthy human volunteers after PJ consumption.[13] Similarly, in the present study, we showed that PJ consumption by patients with CAS also resulted in a most substantial increase in serum PON1 activity (up to 91% after 3 years). Paraoxonase is inactivated by oxidized lipids,[47] and potent antioxidants such as red wine flavonoids, as

well as the licorice-derived isoflavan glabridin, can preserve paraoxonase activity during lipoprotein oxidation.[47] The increase in serum paraoxonase activity after PJ consumption may be a direct effect of PJ, or it might also be the result of the reduction in lipid peroxides by PJ antioxidants. PJ contains very potent anti-oxidants and, unlike other nutrients,[47] it not only preserved serum PON1, but even increased the enzyme activity.[13] The present study, as well as previous studies,[7,13,40] clearly demonstrated that the increase in serum PON1 activity was associated with reduced serum and LDL lipid peroxidation. In another study, however, no association between serum PON1 activities and increased oxidized LDL levels in diabetic patients was found.[48] PON1 in serum is not the only protective factor against lipid peroxidation, as the serum contains additional antioxidants (vitamin E, carotenoids, bilirubin, uric acid etc.) and the antioxidants absorbed from PJ also contribute to protection against oxidative stress in serum and tissues.

Another anti-atherogenic effect of PJ consumption that could lead to decreased IMT is its blood pressure lowering effect, as shown by the reduction in the patients systolic blood pressure after 3 years of PJ consumption, where most of the effect seen already after 1 year of PJ consumption. Similar hypotensive results were recently obtained upon PJ administration to hypertensive patients.[49] As reactive oxygen and nitrogen species contribute to endothelium-dependent contraction, anti-oxidants can possibly restore endothelial function, and hence may decrease blood pressure.[49,50] The decrement in systolic blood pressure upon PJ consumption may thus be associated with reduced IMT,[51] as was also observed in the present study.

Finally, the findings of reduced oxidative stress in carotid lesions obtained from patients that consumed PJ, in comparison with lesions from control CAS patients (that did not consume PJ), could possibly be related to the effect of PJ-induced increased paraoxonase 1 hydrolytic activity on the lesions' oxidized lipids.[9] This phenomenon shows that, in addition to the regression of carotid lesion size, the lesion itself may be considered less atherogenic after PJ consumption, as its cholesterol and oxidized lipid content decreased, and since its ability to oxidize LDL was significantly reduced. We thus conclude that, as previously shown in atherosclerotic mice,[13,25,26,52] also in humans pomegranate juice consumption (by patients with carotid artery stenosis) possess anti-atherosclerotic properties, as it substantially decreased serum oxidative stress and, in parallel, reduced common carotid intima-media thickness. Although the use of a small number of patients could cause a statistical error, the study was performed over a 3-year period using a power calculations ANOVA tests. We also compared each patient to himself along the PJ treatment period. Clinical trials are now needed to further prove the beneficial effect of dietary antioxidants in general and of flavonoid-rich antioxidants in particular in patients with cardiovascular diseases. The latter include potent free radical scavengers, such as red wine and pomegranate juice.

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