Effects of a Pomegranate Fruit Extract rich in punicalagin on oxidation-sensitive genes and eNOS activity at sites of perturbed shear stress and atherogenesis

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Abstract

Background: Atherosclerosis is enhanced in arterial segments exposed to disturbed flow. Perturbed shear stress increases the expression of oxidation-sensitive responsive genes (such as ELK-1 and p-CREB). Polyphenolic antioxidants contained in the juice derived from the pomegranate contribute to the reduction of oxidative stress and atherogenesis during disturbed shear stress.

Aim of the study: To evaluate the effects of intervention with the Pomegranate Fruit Extract (PFE) rich in polyphones (punicalagin, which is a potent antioxidant) on ELK-1, p-CREB, and endothelial nitric oxide synthase (eNOS) expression induced by high shear stress in vitro and in vivo.

Results: At the doses used in the study, both the PFE and the regular pomegranate juice concentrate reduced the activation of ELK-1 and p-CREB and increased eNOS expression (which was decreased by perturbed shear stress) in cultured human endothelial cells and in atherosclerosis-prone areas of hypercholesterolemic mice. PFE and pomegranate juice increased cyclic GMP levels while there was no significant effect of both compounds on the conversion of L-arginine to L-citrulline. Administration of these compounds to hypercholesterolemic mice significantly reduced the progression of atherosclerosis and isoprostane levels and increased nitrates. This protective effect was relevant with PFE. Vasomotor reactivity was improved and EC25 values in response to Ach and NONOate were significantly increased in treated mice in comparison to controls.

Conclusion: This study indicates that the proatherogenic effects induced by perturbed shear stress can be also reversed by chronic administration of PFE.

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Keywords: Shear stress; eNOS; ELK-1; p-CREB; Pomegranate; Nitric oxide; Polyphenols; Antioxidants

1. Introduction

Vascular endothelial cells are exposed physiologically to hemodynamic forces (regular laminar shear stress), which
stimulates the release of nitric oxide (NO) by constitutively expressed endothelial NO synthase (eNOS) [1]. Perturbed shear stress alone, or turbulent shear stress associated with other classical risk factors of atherosclerosis, may trigger signal transduction events that in turn may lead to endothelial dysfunction and enhanced atherogenesis [2–4]. It has been demonstrated that areas highly prone to atherogenesis have unique patterns of disturbed flow, characterized by regions of flow separation, recirculation, and spatial gradients of shear stress [4]. Furthermore, vascular endothelial cells under turbulent blood flow increase their production of reactive oxygen species (ROS) and other free radicals that are capable of inducing oxidative stress [5–12]. NO controls vascular oxidative stress and the expression of redox-regulated genes [13–15]. Evidence exists that eNOS activity is reduced at sites of perturbed shear stress [2,5–8,11].

There is an abundant mythological history regarding the pomegranate fruit which is detailed elsewhere [16–21]. The fruit of the pomegranate (about 50% of total pomegranate weight) comprises 80% juice and 20% seeds; the fresh juice contains 85% water, 10% total sugars, and 1.5% pectin, ascobic acid, and polyphenolic flavonoids [21]. The soluble polyphenol content varies within the limits of 0.2–1.0%, depending on variety, and includes mainly anthocyanins, catechins, ellagic tannins, and gallic and ellagic acids [17,18,21]. More importantly, pomegranate juice possesses potent antioxidant activity that are associated to its antiatherogenic properties in mice [21–23], and inhibition of cyclooxygenases and lipooxygenases [19]. Recently, a novel Pomegranate Fruit Extract (PFE) rich in polyphenols (punicalagin) was developed [24,25].

We have previously shown that intervention with antioxidants and L-arginine reverses perturbed shear stress-related redox gene activation and increases eNOS expression both in cultured endothelial cells and in hypercholesterolemic mice [11]. The PFE, because of its robust content of polyphenolic flavonoid antioxidants, is expected to enhance the biological actions of naturally produced NO in vivo. Regular pomegranate juice administered to hypertensive patients caused a significant drop in blood pressure [26], a reduction in carotid plaque development [27] and an improvement of stress-induced myocardial ischemia in patients who have coronary heart disease [28]. From a pathogenic point of view, we have shown that regular pomegranate juice reduced the expression of oxidation-sensitive genes at the sites of perturbed shear stress and protected against high-prone atherosclerotic areas in hypercholesterolememic mice [29].

Antioxidants are well known to enhance the biological actions of NO by virtue of their capacity to stabilize NO by protecting against the oxidative destruction of NO by ROS and other radicals [13–15]. This antioxidant effect results in much higher and more prolonged cellular concentrations of NO, leading to markedly increased biological actions of NO. In order to determine whether proatherogenic conditions induced by turbulent shear stress can be attenuated also by the PFE, we first studied cultured human coronary artery endothelial cells subjected to high shear stress. The experimental protocols for this aim will be essentially the same as those which we described [11,29]. We then analyzed high-prone and low-prone atherosclerotic aortic areas and vascular function of hypercholesterolemic mice after chronic oral administration of PFE in comparison to regular pomegranate juice.

2. Materials and methods

2.1. Pomegranate juice processing

Pomegranate juice concentrate (Wonderful variety, POM Wonderful, LLC, Los Angeles, CA) and Pomegranate Fruit Extract (Trademark POMx) were used in this study. Pomegranates were handpicked, washed, chilled and stored in tanks. The fruit was then crushed, squeezed, and treated enzymatically to yield the juice and the Pomegranate Fruit Extract. Pomegranate Fruit Extract includes not only juice but also the inner and outer peels and the seeds of the pomegranate. Flavonoids constitute 40% (anthocyanins, catechins, and phenols) of total polyphenols in pomegranate juice [20–22]. Both juices were filtered, pasteurized, concentrated, and stored at ~20 °C until use. More details on these compounds and preparation should be addressed to www.pomwonderful.com.

2.2. Endothelial cell culture

Human coronary artery endothelial cells were cultured as described previously [11,29–31]. Investigation was conform with the principles outlined in the Declaration of Helsinki for use of human tissue. Cells were incubated at 37 °C for 4 days in a humidified atmosphere of 95% air and 5% CO₂. The incubation medium (delipidated DMEM) was supplemented with 10 ng/ml human epidermal growth factor, penicillin/streptomycin, amphotericin B, and glutamine [11,29–31]. All experiments were conducted between passages 3 and 5.

2.3. Flow apparatus for shear stress

Endothelial cells were subjected to laminar shear stress by constant angular velocity in a cone-and-plate viscometer at a relatively low level of 1 dyn/cm³ (mean shear stress in veins), at 5 dyn/cm³, and at a higher level of 15 dyn/cm³ (shear stress in arteries) one day after reaching confluence, as described previously [11,29]. The viscometer consists of a cone at a 0.5° angle rotating on top of a 94×16-mm cell culture dish. Flow conditions achieved are considered laminar because the parameter $R (r_{200/2})$ was smaller than 4 (R1 and R15: 0.006 and 0.03 dyn/cm², respectively) [11,29]. On the basis of previous studies [21–23,25,29], endothelial cells were exposed to different flows for 24 h in the absence or presence of 7 or 14 µL both of regular pomegranate juice
and PFE (from 0.2 to 0.4 μmol total polyphenols). Both compounds did not affect cell viability.

2.4. Determination of cGMP and NOSIII bioactivity measurements

Endothelial cells (10⁶ cells) were incubated with 0.5 mmol/L isobutyl-1-methylxanthine and incubated with different treatments for 24 h. The content of cGMP was measured using a specific immunoreactivity kit (Amersham) according to the manufacturer’s recommendations, as described [32]. The effect of different treatments on NOSIII metabolism of [3H]-arginine to [3H]-citrulline was determined using standard techniques, as described [32]. Cell lysates (150 to 250 μg of protein) were incubated with NADPH (2 mM), CaCl2 (230 μM), tetrahydrobiopterin (3 μM), and [3H]-arginine (0.2 μCi, 10 μM) for 20 min at 37 °C.

2.5. Treatment of mice

This study was conducted according to the Guidelines for Animal Experiments of the American Heart Association and in accordance with the guidelines published by the National Institutes of Health (NIH publication No. 85-23, revised 1985) and was approved by the institutional care of experimental committees. All efforts were made to minimize the number of animals used and their suffering. Quality standards of Laboratories at the University of Naples (Italy) are in accordance with rules established from the Italian Ministry of Health and the European College of Laboratory Animal Medicine, while Laboratories of the Mayo Clinic at Rochester (USA) and Universities of California at Los Angeles are in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). We used male hypercholesterolemic low-density-lipoprotein receptor-deficient (LDLR−/−) mice (n=70, mean weight 42.3±10.8 g, mean ±SD) receiving a cholate-free, high cholesterol diet (21% milk fat, 1.5% cholesterol and 19.5% casein; #8137, Harlan/Teklad, Madison, WI), as described [11,29,32,33]. This diet raised the mean plasma cholesterol levels to about 900 mg/dL and plasma triglyceride levels to 300 mg/dL. Some groups of mice will be given the PFE in the drinking water for 4 months before the disease is accelerated by a fatty diet, and then continued on the juices of an additional 6-months. Of course, an appropriate controls will be run simultaneously with pomegranate juice. To assess whether pomegranate juice influenced stress-responsive genes, mice were randomly divided into 5 groups (n=14 each). Two groups of 3 month old mice received regular pomegranate juice (high-dose and regular dose) in the drinking water for 4 weeks before the disease was accelerated by superimposing a fatty diet, and then continued on the juice for an additional 24 weeks. The chosen doses of regular pomegranate juice were based on previous studies conducted in mice [21,22,29]. Concentrated pomegranate juice or PFE were diluted in water (6.25 mL of concentrated juice in 1 L of water). This solution was given to the treated groups of mice, whereas only water was given to the placebo control group of mice (Group 1). One group effectively drank a mean of 30 μL of pomegranate juice/day (Group 2, regular dose group), which is equivalent to 0.8 μmol of total polyphenols/day [21,22,29] while the second group effectively drank a mean of 50 μL of pomegranate juice/day (Group 3, high-dose group). Similarly, another group effectively drank a mean of 30 μL of PFE (Group 4, regular dose group) while the second group effectively drank a mean of 50 μL of pomegranate juice/day (Group 5, high-dose group) [24,25]. More details on the polyphenol contents of these juices should be addressed to www.pomwonderful.com.

2.6. Blood determinations, preparation of arterial samples and Western blot analysis

At the end of the treatment period, mice were euthanized by CO₂ asphyxia. Blood was drawn from the inferior vena cava into heparinized tubes [11,29,33,34]. Plasma cholesterol was determined enzymatically [29,33,34] and plasma isoprostanate 8-epi-PGF2α was determined using a commercially available immunoassay (Cayman Chemical, Ann Arbor, MI), and nitrates (NOx levels) were measured with Griess reagent (Calbiochem), as described previously [29,35–38]. The aorta was continuously immersed in PBS containing 10 μg/mL aprotinin and 0.1 mmol/L PMSF from the time of the dissection until the computerized determination of the atherosclerotic lesion area was completed [11,29,33–37]. Immunostaining of macrophage-derived foam cells in paraffin-embedded arterial sections was made using the F4/80 monoclonal antibody (Serotec, dilution 1:250) against mouse foam cells [11,29,33–37], while oxidation-specific epitopes were identified by using the MDA-2 monoclonal antibody [29,35,36]. Intercellular junctions were stained with 0.25% silver nitrate to visualize the endothelium in the high-prone and low-prone atherosclerotic aortic regions (located mainly in the proximal aorta), as described [11,29,36,39]. Tissue sections (5 μm) from the different arterial regions were homogenized in 250 μL of protein extraction buffer for determination of protein content by Western blot as described in detail [20,22,29,36]. We used the following monoclonal antibodies: ELK-1 (I-20, goat antibody), p-CREB (Sc:7978), and eNOS III (N-20, epitope corresponding to an amino acid sequence mapping at the amino terminus of NOS-III, no cross reactivity with NOS-I or NOS-II, neuronal and inducible forms, respectively) purchased from Santa Cruz Biotechnology (San Diego, CA) [11,29]. Semiquantitative densitometry of Western blots was performed using a Scan LKB (Pharmacia-Sweden) [11,29–37].

2.7. Vasomotor reactivity

Endothelial function was studied by using endothelial rings (a term usually referred to pieces of arteries placed in a bath) [40]. In a subset of hypercholesterolemic mice, carotid
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control untreated EC or EC treated with PFE or PJ diluted in the cellular medium were exposed to shear stress for 24 h. Data are representative of the mean±SD of 3 different experiments.</th>
<th>PFE 7 µL</th>
<th>PJE 14 µL</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0 1 dyn 5 dyn 15 dyn</td>
<td>0 1 dyn 5 dyn 15 dyn</td>
</tr>
<tr>
<td>eNOS</td>
<td>2.1±0.3 3.0±0.4* 2.5±0.5 2.3±0.4</td>
<td>2.4±0.5 3.4±0.5# 2.9±0.5# 2.5±0.5 4.4±0.5** 3.6±0.6** 3.6±0.5** 3.6±0.5**</td>
<td></td>
</tr>
<tr>
<td>ELK-1</td>
<td>2.0±0.3 3.2±0.4 4.0±0.5§ 5.0±0.5§</td>
<td>2.1±0.3 2.6±0.4# 3.0±0.5# 3.9±0.4# 1.9±0.4 2.4±0.4** 2.8±0.5** 3.3±0.5**</td>
<td></td>
</tr>
<tr>
<td>p-CREB</td>
<td>2.0±0.4 2.4±0.3 2.8±0.3* 3.0±0.4*</td>
<td>2.1±0.2 2.1±0.2 1.8±0.4# 2.2±0.3# 1.7±0.3 1.9±0.4# 1.6±0.4# 1.8±0.3#</td>
<td></td>
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</tbody>
</table>

Legend: Control untreated EC or EC treated with PFE or PJ diluted in the cellular medium were exposed to shear stress for 24 h. Densitometric analysis of blots normalized with γ-tubulin are representative of the mean±SD of 6 different experiments. *p, significance by Bonferroni’s corrected t test.*p<0.05 vs. 0 dyn; °p<0.05 vs. respective force in Control untreated cells; **p<0.01 vs. respective force in Control untreated cells; °p<0.001 vs. 0 dyn; °p<0.05 vs. respective force of PJ.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Cyclic guanosine monophosphate (cGMP) levels and endothelial nitric oxide synthase (eNOS) activity measured by monitoring the conversion of L-arginine to L-citrulline in human endothelial cells exposed to shear stress and treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 dyn 5 dyn 15 dyn</td>
</tr>
<tr>
<td>cyclic GMP (mol/µg)</td>
<td>6.1±1.2 8.2±2.1* 6.5±1.4 6.4±1.6 6.2±1.3 9.8±2.3* 8.0±2.1* 7.6±1.5* 6.5±1.8 10.2±2.6* 8.4±1.6* 8.2±1.5*</td>
</tr>
<tr>
<td>NOS activity (% of control)</td>
<td>95±36 92±28 90±25 88±35 92±34 90±29 90±27 87±30 93±30 91±25 89±28 90±31</td>
</tr>
</tbody>
</table>

Legend: Control untreated EC or EC treated with PFE or PJ diluted in the cellular medium were exposed to shear stress for 24 h. Data are representative of the mean±SD of 3 different experiments.*p<0.05 vs. 0 dyn; °p<0.05 vs. respective force in Control untreated cells.
increase at all levels of shear stress (Table 1). These effects were concentration-dependent since cell exposure to 14 μL of both juices induced further increments of eNOS activity (Table 1). However, at this higher dose, PFE was more potent than regular pomegranate juice. Comparable results were obtained for p-CREB (Table 1). PFE and pomegranate juice increased cyclic GMP while there was no significant effects of both compounds on eNOS activity measured as the conversion of L-arginine to L-citrulline (Table 2).

### 3.2. Effects of PFE and pomegranate juices in hypercholesterolemic mice

Plasma cholesterol levels were not significantly affected by pomegranate juice or PFE consumption (882±66, 875±69, 885±68 μmol/L in placebo, regular and high-doses pomegranate juice groups, respectively, vs. 884±70 μmol/L in PFE group). The differences between placebo and PFE-treated groups were not significant (Fig. 1). Markers of atherosclerosis, such as atherosclerotic lesion area, macrophage accumulation, and oxidation-specific epitopes increased in hypercholesterolemic mice compared to healthy controls. Treatment with PFE and pomegranate juice significantly reduced these markers in a dose-dependent manner (Fig. 1).

Fig. 1. Effects of regular (rd) and high-doses (hd) of PFE and pomegranate juice (PJ) or placebo on atherosclerosis progression (Panel A), macrophage accumulation (F4/80 antibody, Panel B), and oxidation-specific epitopes (MDA-2 antibody, Panel C) in hypercholesterolemic mice fed high-fat diet. HV, healthy vessel areas; LP, low-prone atherosclerotic areas; HP, high-prone atherosclerotic areas; rD, regular dose; hD, high-dose. Atherosclerotic lesion area and both the number of F4/80 and MDA-2 positive arterial sections were assessed by computer-assisted imaging analysis as described in methods. Mean±SD; p, significance by Bonferroni’s corrected t test. *p<0.05 vs. placebo control group of mice; **p<0.01 vs. placebo control group of mice; §p<0.05 vs. respective PJ-treated group; °p<0.01 vs. respective PJ-treated group.

ELK-1 protein levels compared to the untreated control at the same flow or shear stress (Table 1). Again, treatment with 14 μL of both juices further decreased ELK-1 expression (Table 1). Also in this case, PFE was more potent than regular pomegranate juice. Comparable results were obtained for p-CREB (Table 1). PFE and pomegranate juice increased cyclic GMP while there was no significant effects of both compounds on eNOS activity measured as the conversion of L-arginine to L-citrulline (Table 2).

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Fig. 2. Effects of pomegranate juice (PJ rd in b; PJ hd in c) and Pomegranate Fruit Extract (PFE rd in d; PJ hd in e) on aortic atherosclerotic lesion areas in hypercholesterolemic mice. Comparison of the extent of lipid staining by oil red O in representative sections of the aorta of a placebo-treated control mouse (a). Cryosections 10 μm thick were stained with oil red O, and counterstained with hematoxylin. Immunohistochemical staining of aortic sections for macrophages of a placebo-treated control mouse (f), in a PJ-rd-treated mouse (g) or PJ-hd-treated mouse (h), PFE-rd-treated mouse (i) or PFE-hd-treated mouse (l). Finally, immunohistochemical staining of aortic sections for oxidation-specific epitopes (MDA-2 monoclonal antibody). Placebo-treated control mouse (m), PJ-rd-treated mouse (n), PJ-hd-treated mouse (o), PFE-rd-treated mouse (p) or PFE-hd-treated mouse (q). See Methods for more technical details. All figures are 125×.
and 865±70 mg/dL in the placebo-treated, high-dose pomegranate juice-treated mice, and high-dose PFE, respectively. Plasma isoprostanes in mice treated with both juices were reduced compared to placebo-treated mice (109±18⁎, 102±20⁎ vs. 145±32 pg/ml, high-dose pomegranate juice, high-dose PFE and placebo-treated mice, respectively, ⁎p < 0.03 vs. placebo group). Note that isoprostanes were also reduced in the regular dose pomegranate juice-treated groups of mice (119 ± 21⁎) as well as in the regular dose PFE-treated group (115 ± 24⁎; ⁎p < 0.05 vs. placebo group). Accordingly, plasma NOx increased in mice treated with both juices in comparison to placebo-treated mice (26±3⁎, 29 ± 4⁎ vs. 18±2 μm, high-dose pomegranate juice, high-dose PFE and placebo-treated mice, respectively, ⁎p < 0.05 vs. placebo group). As expected [29], atherosclerotic lesions in low-prone and high-prone areas of placebo groups of mice were larger than those observed in treated groups of mice in both protocol doses, and consisted of many more lipid-laden macrophage foam cells (F4/80 immunostaining) and oxidation-specific epitopes (MDA-2 immunostaining) (Figs. 1 and 3).

Table 3
Effects of PFE or regular pomegranate juice (PJ) on eNOS expression and shear stress-responsive genes (ELK-1 and p-CREB) in hypercholesterolemic mice fed high-fat diet in the study protocol

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n=14)</th>
<th>PJ rD group (n=14)</th>
<th>PFE rD group (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HV</td>
<td>LP</td>
<td>HP</td>
</tr>
<tr>
<td>eNOS</td>
<td>3.3±0.4</td>
<td>2.5±0.5</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>ELK-1</td>
<td>1.1±0.3</td>
<td>2.0±0.4</td>
<td>3.1±0.5</td>
</tr>
<tr>
<td>p-CREB</td>
<td>1.5±0.4</td>
<td>2.2±0.3</td>
<td>3.0±0.4</td>
</tr>
<tr>
<td>PJ hD group(n=14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>3.5±0.4</td>
<td>3.3±0.4**</td>
<td>2.7±0.2**</td>
</tr>
<tr>
<td>ELK-1</td>
<td>1.0±0.2</td>
<td>1.5±0.4*</td>
<td>2.2±0.4**</td>
</tr>
<tr>
<td>p-CREB</td>
<td>1.4±0.4</td>
<td>1.5±0.4*</td>
<td>2.0±0.3*</td>
</tr>
<tr>
<td>PFE hD group (n=14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>3.4±0.4</td>
<td>4.0±0.5**,§</td>
<td>3.0±0.4**</td>
</tr>
<tr>
<td>ELK-1</td>
<td>1.0±0.2</td>
<td>1.3±0.4**</td>
<td>2.0±0.4**</td>
</tr>
<tr>
<td>p-CREB</td>
<td>1.4±0.4</td>
<td>1.3±0.3**</td>
<td>1.8±0.3**</td>
</tr>
</tbody>
</table>

Legend: HV, healthy vessel areas; LP, low-prone atherosclerotic areas; HP, high-prone atherosclerotic areas; rD, regular dose; hD, high-dose. Densitometric analysis of Western blots normalized with γ-tubulin are representative of the mean ± SD of each mouse in study groups. p, significance by Bonferroni’s corrected t test.*p < 0.05 vs. placebo control group of mice; **p < 0.01 vs. placebo control group of mice; §p < 0.05 vs. respective PJ-treated group.

Table 4
Vasomotor reactivity parameters in carotid arteries segments

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PJ</th>
<th>PFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Diameter(μM)</td>
<td>412±10.4</td>
<td>413±8.2*</td>
<td>412±10.2</td>
</tr>
<tr>
<td>Diameter of contraction to U46619 (μM)</td>
<td>175±12</td>
<td>173±14*</td>
<td>176±15*</td>
</tr>
<tr>
<td>Maximum Relaxation</td>
<td>To Ach (%)</td>
<td>57±12</td>
<td>72±11*</td>
</tr>
<tr>
<td>To DEA-NONOate (%)</td>
<td>58±11</td>
<td>84±12*</td>
<td>87±13*</td>
</tr>
<tr>
<td>EC25-Log M</td>
<td>To Ach</td>
<td>6.85±0.15</td>
<td>7.56±0.16*</td>
</tr>
<tr>
<td>To DEA-NONOate (%)</td>
<td>6.66±0.14</td>
<td>7.22±0.09*</td>
<td>7.27±0.12*</td>
</tr>
</tbody>
</table>

Legend: Data are representative of the mean±SE; n=6 mice per group. *p<0.05 vs. Control untreated cells. U-46619, 9,11-dideoxy-11-9-epoxymethanoprostaglandin F2; Ach, acetylcholine; DEA-NONOate, diethylammonium-(Z)-1-(N,N-diethylamino)diazen-1-1-2-diolate.
and 2). There was approximately a 25% decrease in both lesion area and foam cell formation in pomegranate-treated mice. These antiatherogenic properties of regular pomegranate juice were consistent with previous findings from the Aviram’s group [21–23]. More importantly, the treatment with PFE induced a more significant reduction of atherosclerotic lesion development (around 40%) than that achieved with regular pomegranate juice (Fig. 1, Panel A).

When considering perturbed shear stress-related areas, the artery can be visualized as possessing healthy vessel areas, and low-prone and high-prone-atherosclerotic areas [11,29]. Densitometric analysis of Western blots showed that in comparison to healthy vessel areas, basal eNOS activity before treatment was decreased by about 25% and 45% in low-prone and high-prone areas, respectively (Fig. 3 and Table 3). After regular pomegranate juice administration, eNOS activity increased significantly both in low-prone and high-prone areas; this phenomenon was more evident when PFE was used (Table 3). As expected [11,29], ELK-1 was increased significantly in low-prone and in high-prone areas in control mice; comparable results were obtained for p-CREB (Table 2). After regular pomegranate juice treatment, ELK-1 and p-CREB protein levels were significantly decreased in low-prone and high-prone areas (Table 3). This beneficial effect was more pronounced when PFE was administered to mice (Table 3). Thus, therapeutic beneficial effects (e.g., increasing eNOS and decreasing redox gene expression in perturbed shear stress areas) were elicited both by PFE and pomegranate juices during chronic intervention.

When considered vasomotor reactivity, there was a significant maximum relaxation to Ach and DEA-NONOate in arteries exposed to PFE (Table 4). Moreover, the EC_{25} values of arteries in response to both Ach and DEA-NONOate were significantly increased with both pomegranate juice and PFE (Table 4). There was no difference observed between groups when considered diameter of contraction to U46619 (Table 4).

4. Discussion

The present study shows that prolonged supplementation with PFE or regular pomegranate juice can largely correct the perturbed shear stress-induced proatherogenic disequilibrium by increasing eNOS activity (and cGMP) and decreasing redox-sensitive transcription factors both in vitro and in vivo in cultured human coronary endothelial cells and in vivo in hypercholesterolemic mice. The degree of potency of PFE was significant higher than that of pomegranate juice. The hypercholesterolemic mice spontaneously develop atherosclerosis, which can be accelerated by feeding the mice a diet rich in fats [11,29,33–37]. Accordingly, the purpose of this study was to determine whether PFE, like other natural sources of antioxidants and pomegranate juice, reduces oxidative stress, oxidation-specific epitopes and atherogenesis in arteries from hypercholesterolemic mice. Vascular disorders such as atherosclerosis cause disturbed blood flow in the affected regions and this leads to perturbed shear stress that, in turn, causes endothelial damage. Moreover, endothelial dysfunction is attributed to increased oxidative stress and decreased NO production and action [12–15]. Thus, the protective effects of NO in the vasculature are lost and atherosclerosis develops as a result. Administration of antioxidants in experimental models has been shown to reduce the severity of atherosclerosis by reducing oxidative stress and increasing NO production and action [14,42]. In the present study, PFE and pomegranate juice supplementation to LDLR m/s mice, which are under oxidative stress, resulted in substantially lower plasma lipid peroxidation (assessed by plasma isoprostanes) and higher plasma nitrates than in control mice. Reductions in macrophage foam cell formation, oxidation-specific epitopes and lesion area in atherosclerotic prone lesion regions (low-prone and high-prone areas) both in PFE and regular pomegranate juice-treated mice clearly confirm the correlation between antioxidative effects and their antiatherogenic properties, as was observed in other studies [21–23,25]. Moreover, we have shown that vasomotor reactivity (EC_{25} of relaxation in response to Ach and NONOate) was improved with both treatments. Thus, it was improved both endothelium-dependent (Ach) and -independent relaxation induced by NONOate.

The second aim of the present study was to determine the possible beneficial effects of PFE and pomegranate juice on oxidation-sensitive gene expression, and eNOS at sites of disturbed shear stress. The present study confirmed that modulation of ELK-1, p-CREB and eNOS expression (and cGMP levels in cells) is associated with antiatherogenic activity in such areas. These effects are similar to those elicited by antioxidants (vitamin E and C) and L-arginine [11]. However, PFE and pomegranate juice did not appreciably inhibit eNOS activity at dilutions that exhibited vascular protective effects. Thus, these compounds did not influence eNOS catalytic activity. Polyphenols are the most abundant antioxidants in our diets. The main classes of polyphenols are phenolic acids (mainly caffeic acid) and flavonoids (the most abundant in the diet are flavanols (catechins plus proanthocyanidins), anthocyanins and their oxidation products), which account for one- and two-thirds, respectively [43]. Polyphenols are reducing agents that may protect the body’s tissues against oxidative stress and associated pathologies such as cancer, cardiovascular diseases and inflammation [43,44]. It is important to understand the nature of the main polyphenols ingested, their dietary origin, the amounts consumed in different diets, their bioavailability and the factors controlling their bioavailability. Pomegranate juice and PFE are rich in antioxidants of the polyphenolic class, which includes tannins and anthocyanins [19,22–24]. These antioxidants are more potent, on a molar basis, than many other antioxidants including vitamin C, vitamin E, coenzyme Q-10 and alpha-lipoic acid [19–22,24]. The antioxidant level in pomegranate juice was found to be higher than that in other natural juices such as blueberry, cranberry and orange, as well as red wine [24,45]. The
development of PFE further increases the content of punicalagin [24,25].

Polyphenols from red wine can reduce LDL aggregation in vitro and in vivo and caused alone an upregulation of eNOS expression and NO formation [45–47], and regular pomegranate juice administered to cardiovascular patients ameliorate blood pressure and myocardial perfusion [26–28]. Moreover, regular pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduced common carotid intima-media thickness, blood pressure and LDL oxidation [27]. Accordingly, an early study showed that tea-pigment (and possibly polyphenols) exerted some anti-atherosclerotic effects [48]. Short- and long-term black tea consumption reverses endothelial dysfunction in patients with chronic heart disease [49]. Similarly, the ingestion of polyphenols contained in purple grape juice had beneficial effects on endothelial function in patients with coronary heart disease [50]. Taken together, these data suggest that polyphenols can protect arteries from vascular damage via antioxidant effects and NO restoration. However, certain large clinical trials employing different antioxidants have failed to show any beneficial effects in terms of prevention of major cardiovascular events [5,12,14,42]. One possible explanation of this divergence is that the models employed in experimental studies, although very useful to study pathophysiological mechanisms, may not precisely reflect the disease in humans [5,12,14,42]. Alternatively, the doses of antioxidants used in those few studies may not have been appropriate, and/or the progression of disease may have been too severe. Finally, it was proposed that pomegranate juice can be used also in the chemoprevention of prostate cancer [51] and exerted beneficial effects during chronic obstructive pulmonary disease [52].

The data in the present study show that elevation of the redox factors ELK-1 (a ternary complex factor of the Ets family) and p-CREB, which are associated with perturbed shear stress [11,29], can be reversed by the intervention with PFE or regular pomegranate juice in cultured human coronary artery endothelial cells and in hypercholesterolemic mice. Indeed, eNOS, p-CREB and ELK-1 are “primed” to respond to systemic activation stimuli in high-prone versus low-prone areas that represent areas with most and least atherosclerosis in mice and pigs. Using PFE or pomegranate juice therapeutic intervention, we demonstrate that it is possible to attenuate this proatherogenic scenario. Antioxidant protection elicited decreased cellular production and release of oxygen radicals in the vascular wall, inhibits endothelial activation of oxidation-sensitive genes, improves the biologic activity of NO through a cell- or tissue-specific antioxidant action [14,53] and upregulates eNOS expression in the presence of oxidized low-density-lipoprotein [54]. Very recent data shows that pomegranate juice protects NO against oxidative destruction in bovine pulmonary artery endothelial cells [55].

In summary, therapeutic intervention with antioxidant polyphenols contained in PFE and regular pomegranate juice may promote a sustained correction of the perturbed shear stress-induced proatherogenic profile in vitro and in vivo with atherosclerosis reduction and improvement of vascular function. These findings may have nutritional implications for the prevention of atherosclerosis.

4.1. Financial disclosure

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