Pomegranate juice consumption increases GSH levels and reduces lipid and protein oxidation in human blood

Chrysoula M. Matthaiou b, Nikolaos Goutzourelas a, Dimitrios Stagos a, Eleni Sarafoglou a, Athanasios Jamurtas c, Sofia D. Koulocheri b, Serkos A. Haroutounian b, Aristidis M. Tsatsakis d, Dimitrios Kouretas a,⇑

a Department of Biochemistry and Biotechnology, University of Thessaly, Ploutonos 26 & Aiolou, Larissa 41221, Greece
b Agricultural University of Athens, Athens, Laboratory of Nutritional Physiology and Feeding, Iera Odos 75, Athens 11855, Greece
c Department of Exercise and Sport Sciences, University of Thessaly, Trikala 42100, Greece
d Department of Forensic and Toxicology, Medical School, University of Crete, Heraklion 71409, Greece

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ABSTRACT

The aim of the present study was the assessment of the antioxidant effects of pomegranate juice (PJ) consumption in humans. Thus, 14 healthy volunteers consumed PJ daily for a period of 15 days and the changes of oxidative stress markers in their blood were assessed at four different time points, immediately before the experiment (T1), after 15 days of juice administration (T2), one (T3) and three weeks (T4) after the interruption of PJ administration. The markers studied were total antioxidant capacity (TAC), levels of malondialdehyde (MDA), and protein carbonyls (CARB) measured in plasma, as well as reduced glutathione (GSH), and catalase activity (CAT) measured in erythrocytes. The MDA was reduced by 24.4% at T3 and CARB were reduced by 19.6% and 17.7% at T2 and T3, respectively, supporting the evidence that PJ consumption enhances the antioxidant status in humans by decreasing lipid peroxidation and protein oxidation. Moreover, GSH levels were significantly increased (22.6%) at T2, indicating that PJ consumption improves the antioxidant mechanisms in erythrocytes by increasing GSH levels. Finally, it was shown that even a week after stopping PJ consumption some of its beneficial effects on antioxidant status still remained in the organism.

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1. Introduction

Pomegranate (Punica granatum L.) constitutes one of the first recorded cultivated trees by humans. This tough, native plant of Himalayas (northern India), is currently cultivated in many regions including the Mediterranean basin countries, Iran, Afghanistan, India, China, Japan, Russia and some parts of the United States. The pomegranate fruit has been used in folk medicine from ancient times as antimicrobial (Gurib-Fakim, 2006) and as natural astringent for the treatment of diarrhea and harmful internal parasites (Das et al., 1999). Nowadays, the research interest on pomegranate fruit is increased as a consequence of reports establishing its benefits on human health (Faria and Calhau, 2011). In this respect, pomegranates have been studied as protective means of the cardiovascular system, the treatment of the acquired immune deficiency syndrome, in hormone replacement therapy, in oral hygiene (Faria and Calhau, 2011), in chemoprevention (Lansky et al., 2005), as microbicidal (Neurath et al., 2004) and as antihyperlipidemic (Fuhrman et al., 2005).

Pomegranate juice (PJ) is the greatest contributor for pomegranate ingestion which contains 85% water, 10% total sugars, 1.5% pectin, ascorbic acid, and polyphenols (Aviram et al., 2000). Several studies have reported a series of results for its clinical benefits, such as reduction of systolic blood pressure in hypertensive patients, decrease of common carotid artery intima-media thickness (IMT) (Aviram et al., 2000), attenuation of myocardial ischemia and the lipid profile improvement of diabetic patients.
and evaporated under vacuum. A 0.3 mL portion of 10% AlCl₃ in 50% H₂O was added, the mixture was allowed to stand at room temperature for 3 min. A 1.5 mL portion of 20% Na₂CO₃ was added and the solution was diluted to the desire volume (10 mL) with deionized water. Absorbance was measured at 725 nm versus a blank after 2 h at room temperature. The results are expressed as gallic acid equivalents using the standard curve (absorbance versus concentration) prepared from authentic gallic acid.

2.4. Determination of total flavonoids
The total flavonoid content of juice was determined using a modified colorimetric method developed by Jia et al. (1999). In particular, 1 mL of solid sample was added into a 10 mL flask containing 4 mL of deionized water. A 0.5 mL portion of 5% NaNO₂ was added to this mixture and allowed to stand for 5 min at room temperature. Then, 0.3 mL of 10% AlCl₃H₂O was added, the mixture was allowed to stand for 1 min at room temperature and 2 mL of 1 M NaOH was added. The solution was diluted to 10 mL with the addition of deionized water and the absorbance of the solution versus a blank at 510 nm was measured immediately. The results are expressed as catechin equivalents using a standard curve (absorbance versus concentration) prepared from authentic catechin samples.

2.5. Participants
Fourteen volunteers (men: 8; women: 6; age, 33.5 ± 3.2 yr; height, 172 ± 2.2 cm; weight, 73.3 ± 4.3 kg; body fat, 22.3 ± 2.7%; body mass index, 25.4 ± 1.3 kg/m²) participated in the present study. All were nonsmokers, were not receiving any medication or nutritional supplements and did not perform any special physical effort either before or after juice administration. A written informed consent to participate in the study was provided to all subjects involved in the study after they were informed of all risks, discomforts and benefits. The procedures were in accordance with the Helsinki declaration of 1975 and an approval was received by the human subjects committee of the University of Thessaly. The subjects visited the laboratory for the first time for a screening of their anthropometric parameters and commenced a healthy and activity questionnaire. Each participant reported to the laboratory in the morning after an overnight fast and abstained from alcohol and caffeine for 24 h. Body mass was measured to the nearest 0.5 kg (Beam Balance 710, Seca, UK) with the subjects lightly dressed and barefoot. Standing height was measured to the nearest 0.5 cm (Stadiometer 208, Seca), while the percentage of their body fat was calculated from seven skinfold measures (an average of two measurements of each site) using a Harpenden caliper (John Bull, UK), in accordance to published guidelines (American College of Sports Medicine, 2000). Their body mass index was calculated as the ratio of body weight (kg)/height (m²).

2.6. Diet and activity before the experiment
The subjects were instructed to follow their usual eating habits during the days prior to the experiment. They were also asked to record their diet for 3 days before the blood collections on a dietary record sheet. The subjects received a copy of their dietary record sheets and were asked to exactly follow the same food intake patterns (as recorded in their dietary record sheets) before all blood collections.

2.7. Study design
The subjects visited the laboratory (08:00–09:00 in the morning) for the first day for anthropometry measurements. Each subject received 30 packs of 250 mL of PJ. The PJ was kindly provided by VITOM Christodoulou Bros SA, Greece. Blood samples were obtained prior to the experiment (T1). Then, for the next 15 days the subjects consumed 0.5 L (2 packs of 250 mL a day) of pomegranate juice. After 15 days injected by means of a Rhodyne injection valve (model 7725i). The gradient eluted consisted of solvent A (obtained by the addition of 3% v/v acetic acid in 2 mM sodium acetate aqueous solution) and solvent B (acetonitrile, CH₃CN). Run time was set at 70 min with a constant flow rate at 1.0 mL/min in accordance with the following gradient time table: at zero time, 95% A and 5% B; after 45 min, the pumps were adjusted to 85% A and 15% B; at 60 min, 65% A and 35% B; at 65 min, 50% A and 50% B; and finally at 70 min, 100% B. This routine was followed by a 30 min equilibration period with the zero time mixture prior to injection of the next sample. The column effluent was monitored at 280, 320, and 360 nm simultaneously. Peaks were identified by comparing their retention time and UV–vis spectra against the corresponding commercial polyphenols (obtained from Sigma–Aldrich) which were used as reference standards. Data were quantified using the corresponding curves of the reference compounds as standards. All standards were dissolved in methanol.

2.3. Assessment of the Total Phenolic Content (TPC)
The TPC of the extracts was determined in accordance with a modified version of the Folin–Ciocalteu method (Singleton et al., 1999). In particular, 100 μL of the solid sample of the juice was added to a 10 mL flask containing 6 mL of deionized water. One milliliter of Folin–Ciocalteu reagent was added to the mixture, and the flask was stoppered and allowed to stand at room temperature for 3 min. A 1.5 mL portion of 20% Na₂CO₃ was added and the solution was diluted to the desire volume (10 mL) with deionized water. Absorbance was measured at 725 nm versus a blank after 2 h at room temperature. The results are expressed as gallic acid equivalents using the standard curve (absorbance versus concentration) prepared from authentic gallic acid.
the subjects visited the laboratory again at the same time (08:00–09:00 h in the morning) and blood samples were collected (T2). The same procedure was repeated after 7 (T3) and 21 (T4) days following the end of the Pj consumption.

2.8. Blood collection and handling

Blood samples (10 mL) were drawn from a forearm vein with subjects in a seated position. Blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes, centrifuged immediately at 1370g for 10 min at 4 °C and the plasma was collected and used for the measurement of TAC and the determination of MDA and protein carbonyl concentrations. The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4020g for 15 min at 4 °C and the erythrocyte lysate was collected for measurement of catalase activity. A portion of erythrocyte lysate (500 µL) was treated with 5% trichloroacetic acid (TCA) (1:1 v/v), vortexed vigorously, and centrifuged at 28,000g for 5 min at 4 °C. The supernatants were removed, treated again with 5% TCA (1:3:1 v/v) and centrifuged again at 28,000g for 5 min at 4 °C. The clear supernatants were transferred to eppendorf tubes and were used for the determination of GSH concentration. A blood aliquot (1 mL) was immediately mixed with EDTA to prevent clotting for hematology. Plasma and erythrocyte lysate were then stored at −80 °C until biochemical analyses.

2.9. Assessment of MDA, protein carbonyl levels, GSH levels, CAT activity, and TAC

For MDA determination, a slightly modified assay of Porter et al. (1976) was used. According to this method, 40 µL of plasma was mixed with 934 µL of 5% TCA and 576 µL of 0.8% BHT (0.8 g BHT in 100 mL of water). The samples were centrifuged at 3000g for 3 min at 4 °C. Then the hexane was removed, which is the upper layer of supernatant and 900 µL TCA 5% was added. Then 625 µL was mixed with 375 µL of TBA 0.8%. The samples were placed in a water bath at 70 °C for 30 min and then transferred on ice for 10 min and centrifuged at 3000g for 3 min at 4 °C. The absorbance of the supernatant was read at 521 nm. A baseline absorbance was taken into account by running a blank (containing 40 µL water instead of plasma) along with all samples during the measurement. Calculation of MDA concentration was based on the molar extinction coefficient of malondialdehyde.

The intra- and inter-assay coefficients of variation (CV) for TBARS were 3.9% and 5.9%, respectively.

Protein carbonyls were determined based on the method of Patsouki et al. (2004). In this assay, 50 µL of 20% TCA was added to 50 µL of plasma and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000g for 5 min at 4 °C. The supernatant was discarded and 500 µL of 10 mM 2,4-dinitrophenylhydrazine-N,N-dihydrochloride (DNPHHCl) [in 2.5 N hydrochloric acid (HCl) for the sample, or 500 µL of 2.5 N HCl for the blank, was added to the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min and were centrifuged at 15,000g for 5 min at 4 °C. The supernatant was discarded and 1 mL of ethanol–ethyl acetate (1:3 v/v) was added, vortexed and centrifuged at 15,000g for 5 min at 4 °C. This washing step was repeated twice. The supernatant was discarded and 1 mL of M urea (pH 2.3) was added, vortexed and incubated at 37 °C for 15 min. The samples were centrifuged at 15,000g for 3 min at 4 °C and the absorbance was read at 375 nm. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH. The intra- and inter-assay CV for protein carbonyls were 4.3% and 7.0%, respectively. Total plasma protein was assayed using a Bradford reagent from Sigma–Aldrich.

GSH was measured according to Reddy et al. (2004). Twenty microliters of erythrocyte lysate treated with 5% TCA were mixed with 660 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) and 28,000g for 5 min at 4 °C. The supernatants of erythrocyte lysate treated with 5% TCA were mixed with 660 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) and 28,000g for 5 min at 4 °C. The supernatants of erythrocyte lysate treated with 5% TCA were mixed with 660 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) and 28,000g for 5 min at 4 °C. The supernatants of erythrocyte lysate treated with 5% TCA were mixed with 660 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) and 28,000g for 5 min at 4 °C. The clear supernatants were transferred to eppendorf tubes and were used for the determination of GSH concentration. A blood aliquot (1 mL) was immediately mixed with EDTA to prevent clotting for hematology. Plasma and erythrocyte lysate were then stored at −80 °C until biochemical analyses.

The intra- and inter-assay coefficients of variation (CV) for GSH were 3.5% and 5.9%, respectively.

2.10. Statistical analysis

The distribution of all dependent variables was examined by Shapiro–Wilk test and was found not to differ significantly from normal. Thus, oxidative stress, hematological and anthropometric data were analyzed by one-way analysis of variance (ANOVA) with repeated measures on time. Pairwise comparisons were performed through simple main-effect analysis. The level of statistical significance was set at p < 0.05. For all statistical analyses SPSS, version 13.0 (SPSS Inc., Chicago, IL) was used. Data are presented as mean ± SEM.

3. Results

3.1. Polyphenolic composition of the pomegranate juice

Since, the antioxidant properties of pomegranate juice is mainly attributed to its polyphenolic content (Gil et al., 2000; Seeram et al., 2005), the polyphenolic composition, the TPC and the total amount of flavonoids were estimated. The results showed that the TPC was 405.00 mg/g of equivalent gallic acid and the total amount of flavonoid was 12.67 mg/L of equivalent quercetin. Moreover, the analysis of the polyphenolic composition exhibited that the pomegranate juice contained different classes of polyphenols as flavonoids, phenolic acids, and stilbenes. In particular, the phenolic acids gallic acid, chlorogenic acid, p-coumaric acid, ellagic acid, protocatechuic acid and ferulic acid were identified with values ranging from 0.63 to 2.00 mg/g of lyophilized juice (p-coumaric acid, and ellagic acid were identified but it was not possible their separate quantification and their amount was estimated as a whole) (Table 1). In addition, the flavon quercetin (0.50 mg/g of lyophilized juice) and its glycosylated form rutin (1.37 mg/g of lyophilized juice) were identified (Table 1). The flavon (−)–epicatechin (0.62 mg/g of lyophilized juice) and the glycosylated dihydrochalcone phlorizin (0.48 mg/g of lyophilized juice) were also contained in the juice (Table 1). Finally, one of the most bioactive polyphenol, the stilbene trans-resveratrol was found at a concentration of 0.66 mg/g of lyophilized juice (Table 1).

3.2. Oxidative stress markers

The levels of CARB were significantly reduced by 19.6% (p = 0.024) and 17.7% (p = 0.045) at T2 and T3, respectively compared to T1 (Fig. 1A). TAC did not change significantly at any time point following PJ consumption (Fig. 1B). The MDA levels appeared significantly lower at T3 (p = 0.040) compared to T1 (Fig. 1C). On the other hand, the GSH levels were significantly increased by 22.6% (p = 0.001) at T2 compared to T1. Even though the GSH levels remained higher from the control levels for 7 (T3) and 14 (T4) days after stopping the PJ consumption, that difference was not

Table 1 Polyphenolic composition of the pomegranate juice used for the experiment.

<table>
<thead>
<tr>
<th>Polyphenol</th>
<th>Pomegranate juice contenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>0.69</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2.00</td>
</tr>
<tr>
<td>p-Coumaric acid–ellagic acid</td>
<td>7.13</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>0.63</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.67</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.50</td>
</tr>
<tr>
<td>Rutin</td>
<td>1.37</td>
</tr>
<tr>
<td>(−)-Epicatechin</td>
<td>0.62</td>
</tr>
<tr>
<td>trans-Resveratrol</td>
<td>0.66</td>
</tr>
<tr>
<td>Phlorizin</td>
<td>0.48</td>
</tr>
<tr>
<td>TPC</td>
<td>405.00</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>12.67</td>
</tr>
</tbody>
</table>

a Determined as mg/g of lyophilized juice.

b Inseparable mixture.

c Total polyphenolic content: determined as mg/L of equivalent gallic acid.

d Determined as mg/L of equivalent quercetin.
significantly different (Fig. 2A). Finally, the catalase activity did not change significantly at any time point following PJ consumption (Fig. 2B).

4. Discussion

The results from this study showed that pomegranate juice consumption enhanced the antioxidant mechanisms of the subjects participated in the study and more importantly this effect lasted for a week after stopping its intake.

In particular, the levels of protein carbonyls, a marker of protein oxidation, decreased significantly both immediately and one week after stopping PJ consumption by 19.6% and 17.7% respectively as compared to those before starting PJ consumption. Even two weeks after stopping the PJ consumption, the protein carbonyl levels were lower (although not statistically significant) than those before starting juice administration. Similar to our results, other studies have shown that pomegranate juice consumption for four weeks (Guo et al., 2008) or three times a week for 1 year (Shema-Didi et al., 2012) decreased the levels of protein carbonyls in plasma. The observed herein protection of PJ against ROS-induced protein oxidation is critical, since the oxidative modification of proteins by ROS has been implicated in the etiology and/or progression of numerous diseases (Stadtman and Levine, 2000), since the oxidatively modified proteins are not repaired and must be removed by proteolytic degradation. Thus, a decrease in the efficiency of proteolysis results in a respective increase in the cellular content of the oxidatively modified proteins, which in turn may disrupt the cellular function either through the loss of catalytic and structural integrity or by interrupting the regulatory pathways.

Apart from protein oxidation, PJ consumption decreased lipid peroxidation. Specifically, the MDA levels that constitute a biomarker of lipid peroxidation, were statistically significantly lower one week after stopping the PJ consumption as compared to those before starting PJ consumption. MDA levels were also lower – but not in a statistically significant manner – immediately and two weeks after stopping the PJ consumption. Other studies have also shown that PJ consumption for two weeks (Aviram et al., 2000), four weeks (Guo et al., 2008), three months (Rosenblat et al., 2006) or three times a week for 1 year (Shema-Didi et al., 2012) decreased lipid peroxidation in plasma. Two of these studies have also demonstrated that pomegranate consumption increase the activity of serum paraoxonase, a high density lipoprotein (HDL)-associated esterase that can protect against lipid peroxidation (Aviram and Dornfeld, 2001; Rosenblat et al., 2006). Lipid peroxidation is a process generated naturally, when reactive oxygen species (ROS) readily attack the polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction (Mylonas and Kouretas, 1999). The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are especially dangerous for the viability of cells, and even tissues. Moreover, lipid peroxidation in plasma is considered one of the main etiological factors for atherogenesis. Thus, the protective effect of pomegranate juice against lipid peroxidation in plasma may prevent the pathogenesis of several diseases such as cardiovascular diseases (Aviram et al., 2000).
Furthermore, PJ consumption significantly increased the GSH levels in erythrocytes immediately after stopping PJ consumption. GSH levels were also higher but not statistically significant one and two weeks after stopping PJ consumption compared to those before starting juice administration. Another study has also reported that PJ consumption for three months increased GSH levels in serum (Rosenblat et al., 2006). Moreover, the same study demonstrated that PJ consumption increased GSH in monocytes-derived macrophages (Rosenblat et al., 2006). However, Guo et al. (2008) reported that PJ consumption does not affect GSH levels in plasma. The difference between the results of that study and ours may be explained by the fact that most of the plasma GSH comes from liver and not from erythrocytes (Abbas et al., 2011). The GSH increase induced by PJ consumption is important for the antioxidant status of the human organism, since GSH, a tripeptide consisting of cysteine, glycine and glutamate, is one of the most important intracellular antioxidant molecules, that protects cellular components from damage caused by ROS. Specifically, the sulfhydryl group (SH) of cysteine serves as a proton donor and is responsible for the antioxidant activity of GSH (Schulz et al., 2000). The observed increase in erythrocyte GSH levels may be due to the induction of expression or the catalytic activity of enzymes involved in GSH biosynthesis such as glutamate cysteine ligase (GCL) and GSH synthetase (GS). For example, it has been shown that plant polyphenols increase expression of GCL (Moskaug et al., 2005). Moreover, compounds such as polyphenols contained in PJ may exert an antioxidant activity per se resulting in the rescue of GSH and thus increasing its concentration.

Furthermore, the observed increase in the antioxidant status of subjects receiving PJ is not attributed to the increase of catalase activity in erythrocytes, one of the most important antioxidant enzymes. This may be rationalized considering that the results indicated that the PJ intake caused only a non-significant increase in catalase activity immediately after stopping consumption compared to those before starting PJ administration. However, Guo et al. (2008) found that PJ consumption for four weeks increased the catalase activity, but they assessed the enzyme activity in plasma.

In a similar line as the catalase activity, the PJ consumption did not affect the TAC levels in plasma at any time point of blood collection. This was an intriguing result, since PJ administration seems to improve the antioxidant status in plasma as shown by the decrease in levels of both lipid and protein oxidation. Other studies have also shown an increase in plasma TAC after PJ consumption for two weeks (Aviram et al., 2000) or four weeks (Guo et al., 2008). The difference between these studies and our study may be due to the different methods used for TAC assessment. Additionally, Aviram et al. (2004) have shown that PJ consumption increased TAC activity in serum by 130% but in their experiment the length of PJ administration (one year) was much longer as compared to our study.

The antioxidant activity of pomegranate juice is attributed to a large extent to its high polyphenolic content (Gil et al., 2000; Seeram et al., 2005; Lansky, 2006). Thus, the polyphenolic composition of the pomegranate juice was assessed. Different classes of polyphenols as flavonoids, phenolic acids, and stilbenes were identified in the pomegranate juice and their amounts were comparable to those found in other studies (Elfallahi et al., 2011). All of these polyphenols identified in the pomegranate juice exhibit antioxidant activity (Li et al., 2014; Rice-Evans et al., 1996). However, it has been reported that the major polyphenolic compounds accounting for the antioxidant capacity of pomegranate juice are the hydroxybenzoic acids such as gallic acid and ellagic acid identified at high concentrations in the used pomegranate juice. Moreover, it has been shown that the derivatives of the gallic acid and ellagic acid such as gallotannins and ellagitannins (e.g. punicalagin) respectively, and the anthocyanins (3-glucosides and 3.5-glucosides of delphinidin, cyanidin, and pelargonidin), are also responsible for the high antioxidant activity of pomegranate juice (Gil et al., 2000; Seeram et al., 2005; Lansky, 2006; Elfallahi et al., 2011).

In conclusion, the results herein support further the evidence that the consumption of PJ may enhance the antioxidant status in humans by decreasing the oxidative damage in lipids and proteins, while it was shown for the first time that it may improve the antioxidant mechanisms in erythrocytes by increasing GSH levels. In addition, this study demonstrates for the first time that even after a week of stopping PJ consumption some of the beneficial effects on antioxidant status may still remain in the organism. Thus, PJ may be used for the prevention of diseases associated with oxidative stress.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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