Anti-oxidative effects of pomegranate juice (PJ) consumption by diabetic patients on serum and on macrophages

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Abstract

Diabetes is associated with increased oxidative stress and atherosclerosis development. In the present study, we investigated the effects of pomegranate juice (PJ; which contains sugars and potent anti-oxidants) consumption by diabetic patients on blood diabetic parameters, and on oxidative stress in their serum and macrophages. Ten healthy subjects (controls) and 10 non-insulin dependent diabetes mellitus (NIDDM) patients who consumed PJ (50 ml per day for 3 months) participated in the study. In the patients versus controls serum levels of lipid peroxides and thiobarbituric acid reactive substances (TBARS) were both increased, by 350% and 51%, respectively, whereas serum SH groups content and paraoxonase 1 (PON1) activity, were both decreased (by 23%). PJ consumption did not affect serum glucose, cholesterol and triglyceride levels, but it resulted in a significant reduction in serum lipid peroxides and TBARS levels by 56% and 28%, whereas serum SH groups and PON1 activity significantly increased by 12% and 24%, respectively. In the patients versus controls monocytes-derived macrophages (HMDM), we observed increased level of cellular peroxides (by 36%), and decreased glutathione content (by 64%). PJ consumption significantly reduced cellular peroxides (by 71%), and increased glutathione levels (by 141%) in the patients’ HMDM. The patients’ versus control HMDM took up oxidized LDL (Ox-LDL) at enhanced rate (by 37%) and PJ consumption significantly decreased the extent of Ox-LDL cellular uptake (by 39%). We thus conclude that PJ consumption by diabetic patients did not worsen the diabetic parameters, but rather resulted in anti-oxidative effects on serum and macrophages, which could contribute to attenuation of atherosclerosis development in these patients.

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1. Introduction

Diabetes mellitus is increasing worldwide, resulting from the interaction of obesity, inflammation and hyperglycemia. Both type I and type II diabetes are powerful and independent risk factors for coronary artery disease, stroke and peripheral arterial disease [1,2], and atherosclerosis accounts for 80% of all deaths among diabetic patients. Prolonged exposure to hyperglycemia is now recognized as a major risk factor in the pathogenesis of atherosclerosis in diabetes [3]. Animal and human studies elucidated three major mechanisms for the pathological alterations observed in diabetic vasculature, i.e. non-enzymatic glycosylation of proteins and lipids which can interfere with their normal function, cellular protein kinase C (PKC) activation and oxidative stress [3,4]. Diabetic patients may be highly prone to oxidative stress because hyperglycemia depletes natural anti-oxidants and facilitates the production of free radicals [5,6]. Thus, anti-oxidants treatment in diabetes could be beneficial [7]. Indeed, it was shown that alpha-tocopherol or red wine supplementation to diabetic patients significantly reduced serum oxidative stress [8,9]. Furthermore, tea catechins were able to protect diabetic erythrocytes from tert-buthyl hydroperoxide-induced oxidative stress [10]. Pomegranate juice (PJ) possesses impressive anti-oxidative properties due to its polyphenolics, tannins and
2. Methods

2.1. Subjects

Ten male healthy subjects (controls) and 10 male non-insulin dependent diabetes mellitus (NIDDM) patients (age 35–71 years old, mean age 50 ± 10) participated in the study. The controls were non-smokers, with no diabetes (glucose levels below 100 mg%) and hemoglobin Aic levels were in the range of 4.8–6.2%, hypertension or coronary artery disease, and they did not take any medications. The diabetes mellitus duration in the patients was 4–10 years, glucose levels above 160 mg%, hemoglobin Aic in the range of 7.5–11.3%. All the patients had no ischemic heart disease, no hypercholesterolemia and were no smokers, but 50% of the patients were hypertensive and were treated with ACE inhibitors. The patients consumed 50 ml of pomegranate juice per day (which contain 1.5 mmol of total polyphenols) for a period of 3 months. Blood was collected from controls and from the diabetic patients before and after PJ consumption for biochemical parameters analysis. Blood was also collected from two of the controls and from three diabetic patients before and after PJ consumption for biochemical analysis. Blood was also collected from two of the patients before and after PJ consumption for biochemical analysis.

2.2. Reagents

2',7'-Dichlorofluorescin diacetate (DCFH) was purchased from Sigma (St. Louis, MO, USA). FITC-conjugated antibody was purchased from Serotec IQ Products (Zernekpark, The Netherlands). PBS, DMEM, RPMI-1640 medium, FCS (heat-inactivated at 56 °C for 30 min), penicillin, streptomycin, nystatin, L-glutamine and sodium pyruvate were purchased from Biological Industries (Beth Haemek, Israel).

2.3. 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 anti-oxidant composition of the juice includes: 1979 mg/l of tannins (1561 mg/l of punicalagin and 417 mg/l of hydrolysable tannins), 384 mg/l of anthocyanins (delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, delphinidin-3-glucoside, cyanidin 3-glucoside and pelargonidin-3-glucoside) and 121 mg/l of ellagic acids derivatives. The juice contained also 3 mg of Vitamin C per 100 ml of PJ. For the extraction of PJ polyphenols fraction C18 sorbent column was used (Varian HF Bondesil C18 resin sorbent). Total polyphenols were eluted from the column with 1% acidified (food-grade acetic acid) ethanol.

2.4. Serum paraoxonase 1 activity

PON1 arylesterase activity towards phenyl acetate was determined as previously described [22].

2.5. Serum lipids peroxidation

Serum lipid peroxidation was measured before and after 3 months of PJ consumption. Serum samples were diluted x 4 with PBS, and were incubated without or with 100 mM of 2,2′-azobis-2-amidinopropane hydrochloride (AAPE, Wako, Japan) for 2 h at 37 °C [23]. The extent of lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay [24] and by the lipid peroxides assay [25].

2.6. Total thiols (SH groups) in serum

The assay procedure determines the amount of protein bound SH groups, as well as glutathione [26]. An aliquot of 50 μl serum was mixed with 1 ml of Tris–EDTA buffer, and the absorbance at 412 nm was measured. To this was added 20 μl of 10 mM DTNB, and after 15 min incubation at room temperature the absorbance was measured, together with a DTNB blank. Total SH groups are calculated as described before [26].
2.7. Human monocytes-derived macrophages (HMDM)

HMDM were separated from the blood [17] and plated at 10^6/ml in RPMI medium with 10% FCS. After 2 h of incubation at 37°C, non-adherent cells were removed, and RPMI with 10% autologous serum was added. Macrophages were analyzed 8 days after plating.

2.8. Detection of intracellular oxidative stress by the DCFH assay

Intracellular oxidative stress was assayed through the oxidation of DCFH-DA [27], and monitored by flow cytometry [28]. For flow-cytometric assay of DCFH-DA oxidation, cells were washed (×1) with PBS and incubated with 10 μM DCFH-DA, in medium for 30 min at 37°C. Adherent cells were detached by gentle scraping, and all cells were washed (×2) with PBS. Measurements of cellular fluorescence determined by FACS were done at 510–540 nm after excitation of the cells at 488 nm with an argon ion laser. Ten thousand events were registered for each experiment. Cellular fluorescence was quantitated by mean fluorescence intensity (MFI).

2.9. Macrophage reduced glutathione content

All the preparation steps were carried out on ice. The cells from triplicate dishes (1 × 10^6 per dish) were washed, scraped from the dish and sonicated in an ultrasonic processor (3 × 20 s at 80 W). The amount of protein was measured by the Lowry method [29] and reduced glutathione content by the DTNB-GSSG reductase recycling assay [30].

2.10. Oxidized LDL (Ox-LDL) uptake by macrophages

LDL was separated from plasma of normal healthy volunteers by discontinuous density-gradient ultracentrifugation [31] and dialyzed against saline with EDTA. Ox-LDL was conjugated to fluorescein isothiocyanate (FITC) for cellular uptake studies. HMDM were incubated at 37°C for 3 h with FITC-conjugated Ox-LDL at a concentration of 20 μg of protein/ml. The uptake of the lipoproteins was determined by flow cytometry. Measurements of cellular fluorescence determined by FACS were done at 510–540 nm after excitation of the cells at 488 nm with an argon ion laser. Ten thousand events were registered for each experiment. Cellular fluorescence was quantitated by mean fluorescence intensity.

2.11. Statistical analysis

Statistical analysis was performed using the Student paired t-test when comparing the mean of two groups. ANOVA was used when more than two groups were compared and results are given as mean ± S.E.M.

3. Results

3.1. Effect of PJ consumption by diabetic patients on serum biochemical parameters

Serum total cholesterol and LDL cholesterol levels in the patients were similar to that of the controls. In contrast, serum triglyceride levels were significantly higher by 2.8-fold in the patients versus controls, whereas HDL-cholesterol levels were significantly decreased by 28% (Table 1). PJ consumption by the patients did not affect these parameters (Table 1). As PJ contains sugars we first questioned the effect of PJ consumption by the patients on serum diabetic parameters: glucose, hemoglobin (Hb) A1c, insulin and C-peptide (a cleavage product of proinsulin). Blood Hb A1c levels significantly increased in the diabetic patients versus controls by 59% (8.9 ± 0.5% versus 5.6 ± 0.2%), whereas insulin and serum C-peptide levels were only slightly different in patients versus controls (82 ± 9 pmol/l for the patient’s insulin levels versus 103 ± 8 pmol/l for the control’s insulin levels, and 862 ± 119 pmol/l for the patient’s C-peptide versus 770 ± 88 pmol/l for the control’s C-peptide.

Table 1

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<th>Controls</th>
<th>Diabetic patients before PJ</th>
<th>Diabetic patients after PJ</th>
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<tr>
<td>Triglyceride (mg%)</td>
<td>115 ± 25</td>
<td>327 ± 79</td>
<td>362 ± 64</td>
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<tr>
<td>Total cholesterol (mg%)</td>
<td>190 ± 9</td>
<td>203 ± 13</td>
<td>193 ± 10</td>
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<td>LDL-cholesterol (mg%)</td>
<td>111 ± 10</td>
<td>112 ± 8</td>
<td>110 ± 8</td>
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<tr>
<td>HDL-cholesterol (mg%)</td>
<td>56 ± 3</td>
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<td>41 ± 3</td>
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NIDDM patients (n = 10) were compared to healthy subjects (controls, n = 10). The patients consumed pomegranate juice (PJ; 50 ml per day for 3 months). Serum samples were collected from the controls and from the patients before and after PJ consumption. The lipid profile in the serum samples was then determined. Results are given as mean ± S.E.M. (n = 10 in each group).

*p < 0.01 vs. controls.
Fig. 1. The effect of PJ consumption by diabetic patients on diabetic parameters in serum. Ten NIDDM patients consumed PJ (50 ml per day for 3 months). Blood samples were collected from the patients before PJ consumption and after 3 months of PJ consumption. Serum fasting glucose (A), hemoglobin A1c (B), insulin (C) and C-peptide (D) levels were determined. Results are given as the individual results, and also as the mean of the whole group,* p < 0.01 vs. before PJ consumption.

levels, respectively). PJ consumption by the patients resulted in a non-significant reduction, by 8%, in the serum glucose levels, with no significant effect on HbA1c levels (Fig. 1A and B). Similarly, a non-significant reduction in serum insulin levels (by 9%) was noted (Fig. 1C), whereas serum C-peptide levels were significantly lower, by 23%, in patients after PJ consumption versus before (Fig. 1D). These results indicate that, in spite of the presence of sugars in consumed PJ, serum diabetic parameters were not worsen, but even improved (Fig. 1).

3.2. Effect of PJ consumption by diabetic patients on serum oxidative status

Diabetes is known to be accompanied by increased oxidative stress [4–7]. Indeed, in our patients’ serum samples we observed significant high levels of lipid peroxides and TBARS by 350% and 51%, respectively, versus the controls (Fig. 2A and B). In addition serum sulfhydryl groups content (which is another marker for oxidative stress) was significantly reduced by 21% in the patients versus controls (Fig. 2C). Paraoxonase 1 is an HDL-associated lactonase/esterase which was shown to protect lipids in lipoproteins and cells from oxidation, by its ability to hydrolyze specific oxidized lipids [32]. PON1 arylesterase activity in the patients’ serum was significantly lower, by 23%, versus the controls (Fig. 2D). PJ consumption by the patients significantly reduced serum oxidative stress. The lipid peroxides and TBARS levels were decreased by 56% and 28%, respectively, as compared to the levels observed in the patients’ serum before PJ consumption (Fig. 1A and B). In parallel, serum total sulfhydryl groups content and PON1 arylesterase activity, significantly increased by 12% and 24%, respectively (Fig. 2C and D).

3.3. Direct effect of PJ consumption on serum oxidative stress: in vitro study

Diabetic patients’ serum samples (obtained from the patients before PJ consumption) were incubated for 1 h at room temperature (25 °C) with no addition (control) or with PJ (20 μM or 40 μM of total PJ polyphenols). Then, the amount of TBARS in the basal state, as well as after AAPH-induced lipid peroxidation, was measured. PJ significantly decreased the basal amount of TBARS in the patients’ serum by 17% or 24%, on using 20 μM or 40 μM of total polyphenols, respectively (Fig. 3A). Furthermore, the susceptibility of the patients’ serum to AAPH-induced
Fig. 2. The effect of PJ consumption by diabetic patients on serum oxidative status. Ten NIDDM patients were compared to 10 healthy subjects (controls). The patients consumed PJ (50 ml per day for 3 months). Blood samples were collected from the controls and from the patients before and after PJ consumption. The oxidative status of the serum samples was determined by the lipid peroxides (A) and TBARS (B) assays. Total serum thiols (SH) groups levels (C) and paraoxonase 1 arylesterase activity (D) were measured as described under Section 2. Results are given as mean ± S.E.M., *p<0.01 vs. controls; #p<0.01 after PJ consumption vs. before PJ consumption.

oxidation was also substantially decreased, by 48% and 73%, respectively (Fig. 3B). Upon adding increasing concentrations of PJ total polyphenols (0–40 μM) to diabetic patients’ serum samples, PON1 arylesterase activity significantly increased in a PJ dose-dependent manner, by up to 25% (Fig. 3C).

3.4. Effect of PJ consumption by diabetic patients on cellular oxidative status in their monocytes-derived macrophages

As diabetic patients are prone to develop accelerated atherosclerosis [1–3], and as macrophages play a major role in the early stages of atherogenesis [15,32], we next studied the oxidative status of the patients’ HMDM versus controls, and the effect of PJ consumption by the diabetic patients. The level of total cellular peroxides, as measured by the DCFH assay, was significantly higher by 36% in the patients’ HMDM versus controls’ HMDM (Fig. 4A). PJ consumption significantly reduced the cellular lipid peroxides content by 71% (to levels which are even lower than those observed in the controls’ HMDM), in comparison to the levels observed in the patients’ HMDM before PJ consumption (Fig. 4A).

Reduced glutathione (GSH) is a major cellular anti-oxidant against oxidative stress [33]. In the patients’ HMDM, reduced glutathione content was markedly lower, by 64%, versus the amount found in controls’ HMDM, and PJ consumption by the patients resulted in elevation in the HMDM glutathione levels by 141%, almost the level observed in control HMDM (Fig. 4B). These results indicate that in diabetic patients’ macrophages, and not only the serum, are under increased oxidative stress. PJ consumption by diabetic patients demonstrated beneficial effects by reducing cellular oxidative stress in the patients’ macrophages.

Oxidized LDL uptake by macrophages can lead to macrophage cholesterol accumulation and foam cell formation [32], and “oxidized macrophages” were shown to take up Ox-LDL at enhanced rate [34]. Thus, we next compared the extent of Ox-LDL uptake by the patients’ HMDM to its uptake by control HMDM. Ox-LDL uptake by the patients’ HMDM was significantly increased, by 37%, as compared to controls’ HMDM (Fig. 4C). PJ supplementation by the patients resulted in a substantial reduction, by 39%, in the uptake of Ox-LDL by the patients’ HMDM, as compared to the values obtained before PJ consumption (Fig. 4C).
Fig. 3. The effect of PJ on serum oxidative status and on paraoxonase activity: in vitro study. Serum from diabetic patients (n = 3) was incubated for 1 h without (+) or with PJ 20 μM polyphenols. Then, the serum was diluted 1:4 with PBS and (A) incubated for 2 h at 37 °C with no addition (basal) or (B) with 100 mM of the free radical generator AAPH (AAPH-induced). At the end of the incubation period, the amount of TBARS in all the samples was determined. (C) The serum from the diabetic patients was incubated with increasing (0–40 μM) PJ polyphenols concentrations for 1 h at room temperature. Then, paraoxonase 1 arylesterase activity was measured as described under Section 2. Results are given as mean ± S.E.M., *p < 0.01 vs. (–); #p < 0.01 vs. 0 concentration.

3.5. Direct effect of PJ on diabetic patient HMDM oxidative status and on Ox-LDL uptake: in vitro study

Incubation of diabetic patient HMDM with PJ (75 μM polyphenols) for 20 h at 37 °C resulted in a significant reduction, by 60%, in the level of cellular peroxides, as measured by the DCFH assay (Fig. 5A). Similarly, incubation of the cells with 75 μM of the PJ-derived polyphenols fraction, significantly decreased the cellular peroxides content in the patients' HMDM by 47% (Fig. 5A). Furthermore, PJ (75 μM) incubation with diabetic HMDM, inhibited also the uptake of Ox-LDL by 30%, compared to the extent of Ox-LDL uptake by non-treated cells (Fig. 5B).

4. Discussion

The present study demonstrated that pomegranate juice consumption by diabetic patients (as previously shown for healthy subjects and atherosclerotic patients) did not worsen the diabetic parameters, but rather resulted in anti-atherogenic effects with a significant reduction in oxidative stress in the patients’ serum and monocytes–macrophages, as well as in macrophage uptake of Ox-LDL.
Diabetic patients versus control healthy subjects have significant high serum triglyceride levels and low HDL-cholesterol levels, as previously shown [35]. Diabetic patients usually avoid sugar-containing juices which worsen their diabetic markers and atherosclerotic complications. In the present study, we showed that PJ (which contain 10% total sugars [36]) consumption by diabetic patients significantly reduced serum oxidative stress as shown in vitro (Fig. 3). PJ polyphenols fraction, significantly reduced macrophage oxidative stress in vitro as previously shown [13,14]. The increased oxidative stress in the patients' HMDDM could be related to cellular mechanisms induced by the high glucose levels such as, PKC activation followed by the production of free radicals [3,6,18]. In the present study, PJ consumption significantly reduced cellular peroxides in the patients' HMDDM, as was previously shown in carotid lesions from carotid artery stenosis (CAS) patients that consumed PJ [14]. This effect could be due to the increased serum PON1 activity, which can hydrolyze lipid peroxides on the macrophage surface [34,44], or to a direct effect of pomegranate juice component(s). Indeed, in vitro we observed that both PJ and the PJ-derived polyphenolic fraction, significantly reduced macrophage oxidative stress. The PJ polyphenolic fraction was less potent than PJ, indicating that other factors in the juice (unique sugars) contribute to cellular oxidative stress reduction. We have recently shown indeed that PJ reduced oxidative stress in J774A.1 macrophage cell line, and this effect was PJ polyphenols dose-dependent [45].

As glucose increases oxidative stress, it was expected that PJ sugars will add to the already increased oxidative stress present in diabetic patients. Surprisingly it did not, and in fact PJ significantly decreased oxidative stress in serum, as well as in their monocytes–macrophages. The uptake of Ox-LDL by the patients' HMDDM was significantly increased as compared to control HMDDM.
phenomenon could be related to the increased expression of the scavenger receptor CD36, which is induced by glucose and/or the high oxidative stress [34,46]. Similar results were observed in diabetic, streptozotocin-injected mice [21]. PJ consumption by the patients, as well as a direct in vitro incubation of PJ with the patients’ HMDM resulted in a significant reduction in Ox-LDL uptake by the patients’ HMDM. Similar results were noted upon incubating J774A.1 macrophages with PJ [45]. PJ-induced reduction in the cellular uptake of Ox-LDL could not be related to down-regulation of the scavenger receptor CD36 mRNA expression [45]. However, CD36 is not the only surface binding for Ox-LDL by macrophages. PJ component(s) also interact with other scavenger receptors such as the SR-A or LOX-1 (which are also up-regulated by glucose [20,19]), or with proteoglycans which were shown to mediate uptake of Ox-LDL by macrophages [47]. PJ polyphenols could possibly also interfere with the uptake of Ox-LDL by interaction with macrophage surface phospholipids and/or kinases [48]. In conclusion then, pomegranate juice consumption by diabetic patients does not worsen diabetic parameters, but rather act as an anti-atherogenic agent. This anti-atherogenicity is manifested by PJ anti-oxidant properties in serum and monocytes–macrophages, two major components of macrophage foam cell formation, the hallmark of early atherosclerosis.

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