

# Pomegranate juice sugar fraction reduces macrophage oxidative state, whereas white grape juice sugar fraction increases it

Orit Rozenberg<sup>a</sup>, Amy Howell<sup>b</sup>, Michael Aviram<sup>a,\*</sup>

<sup>a</sup> *The Lipid Research Laboratory, The Technion Faculty of Medicine, The Rappaport Family Institute for Research in the Medical Sciences and Rambam Medical Center, Haifa, Israel*

<sup>b</sup> *Marucci Center for Blueberry Cranberry Research, Rutgers University, Chatsworth, NJ 08019, USA*

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## Abstract

The antiatherogenic properties of pomegranate juice (PJ) were attributed to its antioxidant potency and to its capacity to decrease macrophage oxidative stress, the hallmark of early atherogenesis. PJ polyphenols and sugar-containing polyphenolic anthocyanins were shown to confer PJ its antioxidant capacity. In the present study, we questioned whether PJ simple or complex sugars contribute to the antioxidative properties of PJ in comparison to white grape juice (WGJ) sugars.

Whole PJ decreased cellular peroxide levels in J774A.1 macrophage cell-line by 23% more than PJ polyphenol fraction alone. Thus, we next determined the contribution of the PJ sugar fraction to the decrease in macrophage oxidative state. Increasing concentrations of the PJ sugar fraction resulted in a dose-dependent decrement in macrophage peroxide levels, up to 72%, compared to control cells. On the contrary, incubation of the cells with WGJ sugar fraction at the same concentrations resulted in a dose-dependent increment in peroxide levels by up to 37%. The two sugar fractions from PJ and from WGJ showed opposite effects (antioxidant for PJ and pro-oxidant for WGJ) also in mouse peritoneal macrophages (MPM) from control as well as from streptozotocin-induced diabetic Balb/C mice.

PJ sugar consumption by diabetic mice for 10 days resulted in a small but significant decrement in their peritoneal macrophage total peroxide levels and an increment in cellular glutathione content, compared to MPM harvested from control diabetic mice administrated with water. In contrast, WGJ sugar consumption by diabetic mice resulted in a 22% increment in macrophage total peroxide levels and a 45% decrement in cellular glutathione content.

Paraoxonase 2 activity in macrophages increases under oxidative stress conditions. Indeed, macrophage paraoxonase 2 activity was decreased after PJ sugars supplementation, but increased after WGJ sugars supplementation.

We conclude that PJ sugar fraction, unlike WGJ sugar fraction, decreases macrophage oxidative state under normal and under diabetic conditions. These antioxidant/antiatherogenic effects could be due to the presence of unique complex sugars and/or phenolic sugars in PJ.

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## 1. Introduction

The pomegranate tree, which is said to have flourished in the Garden of Eden, has been extensively used as a folk medicine in many cultures. However, this folk medicine found its reinforcement in today's scientific medicine. Pomegranate juice (PJ) was found to inhibit low-density lipoprotein (LDL) oxidation, macrophage foam cell forma-

tion and atherosclerosis development in atherosclerotic mice [1,2]. Moreover, PJ consumption for 3 years by patients with carotid artery stenosis reduced common carotid intima-media thickness, blood pressure and LDL oxidation [3]. Similarly, PJ consumption by diabetic patients resulted in antioxidative effects in their serum and their monocytes-macrophages [4]. These beneficial effects of the PJ were attributed to the antioxidative properties of pomegranate polyphenols [5] and sugar-containing polyphenolic tannins and anthocyanins [6].

Oxidative stress is thought to play a key role in early atherogenesis and in macrophage foam cell formation

\* Corresponding author. Tel.: +972 4 854 2970; fax: +972 4 854 2130.  
E-mail address: aviram@tx.technion.ac.il (M. Aviram).

which is the hallmark of the early atherosclerotic lesion [7,8].

Oxidative stress is associated with lipid peroxidation in lipoproteins and in arterial cells, including macrophages [9,10]. These “oxidized macrophages” are characterized by increased peroxide levels, decreased glutathione content, and increased capability to oxidize LDL [11,12]. These properties of the cells increase their capability to accumulate cholesterol and to form macrophage foam cells [13–15].

Type 2 diabetes is a major risk factor for the development of coronary artery disease (CAD) and premature atherosclerosis [16]. In diabetes, the postprandial phase is characterized by a rapid and large increase in blood glucose levels. Postprandial hyperglycemic episodes in diabetic patients are closely associated with increased oxidative and nitrosative stress, and are a most important factor in the onset and progress of vascular complications, both in Type 1 and 2 diabetes mellitus [17–19]. Fructose, however, was found to increase plasma antioxidant capacity after apple consumption, due to increment in plasma urate levels [20].

Serum paraoxonase 1 (PON1), an HDL-associated lactonase was found to possess antioxidative properties, probably due to its ability to decrease macrophage oxidative stress [21,22]. PON1 was shown to be decreased under oxidative state, as shown in atherosclerotic as well as diabetic patients [23–25]. In contrast, another member of the Paraoxonase family, paraoxonase 2, is increased in macrophages under oxidative stress [26].

As complex sugars (tannins and anthocyanins), as well as simple sugars (fructose) were shown to possess antioxidative properties, we hypothesized that the PJ sugar fraction could contribute to the juice’s antioxidativity. The aim of the present study was to characterize the effect of the sugar fraction that was purified from PJ on macrophage oxidative status, in comparison to white grape juice sugar fraction. We questioned whether this fraction would exhibit beneficial antioxidative properties, like the PJ, or will exhibit negative hyperglycemic effects under diabetic conditions.

## 2. Methods

### 2.1. Extraction of sugar and polyphenol fractions from pomegranate juice (PJ) and from white grape juice (WGJ)

For the extraction, C18 sorbent column was used (Varian HF Bondesil C18 resin sorbent). Sugar fraction was eluted with distilled water and total polyphenols were eluted from the column with 1% acidified (food-grade acetic acid) ethanol.

### 2.2. Mice and sugar supplementation

Twelve male Balb/C mice at the age of 3 months were injected intraperitoneally with streptozotocin (STZ) (200 mg/kg in 0.05 M sodium citrate, pH 4.5) within 5 min

of preparation as previously described [27]. Serum glucose levels were determined within 1 week by using a glucometer (Accu-Check Sensor, Roche Mannheim, Germany) and mice with serum glucose levels under 190 mg/dl were administered with a second injection.

Diabetic mice were randomly divided into 3 groups of 4 mice each. One group designated “Control” drank tap water, the two other groups were supplemented via their drinking water with PJ sugar fraction or WGJ sugar fraction (containing 30 mg of glucose per day per mouse) for a period of 10 days. At the end of this period, the mice were sacrificed and blood sample were collected from the retro-orbital plexus (under isoflurane anesthesia) and also peritoneal macrophages were harvested after thioglycollate injection as described below.

## 2.3. Cells

### 2.3.1. J-774A.1 murine macrophage-like cell line

This cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). J-774A.1 cells were plated at  $5 \times 10^5$  cells/well in 12-well dish in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml, and 2 mM glutamine. At the next day, the cells were washed and the media was replaced to low glucose (1.0 g/l = 5 mM) serum-free (SF) DMEM (control) or low glucose SF DMEM with PJ sugar fraction or WGJ sugar fraction containing 2.8 mM glucose for 18 h at 37 °C.

### 2.3.2. Mouse peritoneal macrophages (MPM) isolation

MPM were harvested from mice peritoneum 4 days after intraperitoneal injection of thioglycollate (3 ml, 40 g/l), as previously described [22]. Cells were plated at  $1 \times 10^6$  cells/well in 12-well dish and treated under the same conditions as described for the J774A.1 macrophages. For the in vitro experiments, the cells were washed at the next day and the media was replaced to high glucose (4.5 g/l) SF DMEM (control) or high glucose SF DMEM with PJ sugar fraction or WGJ sugar fraction containing 2.8 mM glucose for 18 h at 37 °C.

MPM from the mice that were administered with sugars were plated and washed similarly, with no further treatments.

## 2.4. Paraoxonase (PON) activities

### 2.4.1. Serum PON1 activity towards phenylacetate (arylesterase)

This activity was measured spectrophotometrically at 270 nm [28]. The  $E_{270}$  for the reaction was  $1310 \text{ M}^{-1} \text{ cm}^{-1}$ . One unit of paraoxonase activity is equal to 1  $\mu$ mol of phenylacetate hydrolyzed/ml/min.

Serum PON1 activity towards paraoxon (paraoxonase) is specific for PON1 and was assessed by measuring *p*-nitrophenol liberation at 412 nm [28]. One unit of paraoxonase activity produces 1 nmol of *p*-nitrophenol per minute.

#### 2.4.2. Macrophage PON2 Lactonase activity towards dihydrocoumarin

This activity was determined in the cells following dihydrocoumarin addition in Tris buffer (25 mM Tris-HCl, pH 7.6, 1 mM CaCl<sub>2</sub>) to the washed cells for 10 min at room temperature. Then the supernatant was collected and measured spectrophotometrically at 270 nm. One unit of lactonase activity is equal to 1 μmol of dihydrocoumarin hydrolyzed/ml/min [29]. Cellular protein content was determined using the method of Lowry et al. [30].

#### 2.5. Macrophage total peroxide levels (flow cytometric assay of DCFH-DA oxidation)

Cells ( $1 \times 10^6$ ) were incubated with  $2.5 \times 10^{-5}$  mol/l dichlorofluorescein-diacetate (DCFH-DA) for 30 min at 37 °C. Under oxidative stress, DCFH is oxidized to DCF, which is a fluorescent compound. Cellular fluorescence was determined with a flow cytometer (FACS-SCAN, Becton Dickinson, San Jose, CA, USA). Measurements were done at 510–540 nm after excitation of cells at 488 nm with an argon ion laser [22].

#### 2.6. Macrophage glutathione content

Cells ( $1 \times 10^6$ ) were washed three times with PBS. The cells were scraped from the dish into 670 μl of PBS and sonicated (three times for 20 s each time) at 80 watts. Total glutathione was measured in the supernatant after 5% sulfosalicylic acid addition to the sonicated cells (1:2, v/v), followed by centrifugation at  $10,000 \times g$ . Total and oxidized glutathione (GSSG) were analyzed by using the 5,5-dithiobis-(2-nitrobenzoic acid) DTNB-GSSG reductase recycling assay. Reduced glutathione (GSH) content was calculated by subtraction of the GSSG content from the total glutathione content [11]. Cellular protein content was determined using the method of Lowry et al. [30].

#### 2.7. Free radical scavenging capacity

The free radical scavenging capacity of PJ and of purified complex sugars (delphinidin-3-*o*-β-glucopyranoside, cyanidin-3-*o*-β-glucopyranoside) was analyzed by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay [31]. DPPH is a radical generating substance. Aliquots from the analyzed sugars or PJ (10 μg/ml) were mixed with 1 ml of 0.1 mmol DPPH/l in ethanol and the changes in optical density at 517 nm were continuously monitored after 5 min incubation.

#### 2.8. Statistical analysis

Student's *t*-test was performed for all statistical analyses. ANOVA was used when more than two groups were compared (in vivo study). Results are given as mean ± S.D.

### 3. Results

#### 3.1. Pomegranate juice (PJ) polyphenols decrease macrophage oxidative state to a lesser extent than PJ does

The antiatherogenic properties of PJ were mainly attributed to its antioxidant potency and its capacity to decrease macrophage oxidative state. These antioxidant properties of PJ were shown to be attributed to pomegranate juice polyphenols (PJ PPs).

Thus, we first examined the contribution of PJ PPs to the inhibitory effect of PJ on macrophage oxidative state, using J774A.1 macrophage-like cell-line. PJ decreased cellular total peroxide levels (measured as fluorescence intensity with dichlorofluorescein-acetate, DCFH) by 70–72%, on using PJ containing 50–100 μM of total PPs, after an 18 h incubation at 37 °C, compared to cells that were similarly incubated without PJ (Fig. 1A). Like PJ, the polyphenol fraction separated from PJ also decreased macrophage total peroxide levels in a dose-dependent manner, by 47%, 52%, and 57% on using 50 μM, 75 μM, and 100 μM of PPs, respectively, in comparison to cells that were incubated without PPs (Fig. 1A). These results clearly demonstrate the increased capacity of PJ over isolated PPs from PJ to decrease macrophage oxidative state.

#### 3.2. PJ sugar fraction decreases, whereas white grape juice sugar fraction increases oxidative state in J774A.1 macrophages

The greater capacity of PJ to decrease macrophage oxidative state, over similar concentrations of purified PJ PPs could account for the isolation procedure, but could also suggest that PJ components, other than PPs contribute to the antioxidant properties of PJ. Thus, we analyzed the PJ sugar fraction which is known to contain glucose, fructose, and other complex sugars, including phenolic sugars. We incubated J774A.1 macrophages with two concentrations of PJ sugar fraction (containing 0.28 mM or 2.8 mM glucose). This incubation (for 18 h at 37 °C) resulted in a substantial decrement in the macrophage peroxide levels, in a dose-dependent manner, by up to 72% on using fraction concentration containing 2.8 mM glucose, compared to control cells that were incubated without the sugar fraction (Fig. 1B). On the other hand, incubation of the cells with the same concentrations of sugar fraction that was purified from white grape juice (WGJ) resulted in a dose-dependent increment in peroxide levels, by up to 37%, on using 2.8 mM glucose in the fraction, compared to control cells (Fig. 1B).

#### 3.3. Glucose increases, while fructose decreased macrophage oxidative state

We next studied the effect of the simple sugars, glucose and fructose, which are present in the sugar fractions.

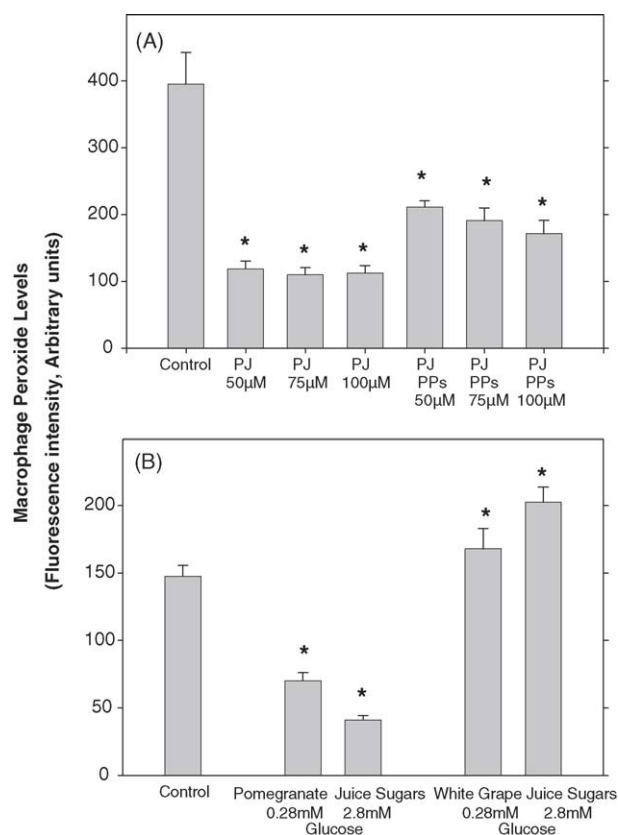


Fig. 1. The effect of pomegranate juice (PJ) and its polyphenol fraction (PJ PPs) or its sugar fraction on macrophage oxidative state. J-774A.1 macrophages ( $1 \times 10^6$ /well) were incubated in serum-free low-glucose (1.0 g/l) DMEM for 18 h at 37 °C with increasing concentrations of PJ (up to 100 µM of total polyphenol equivalents) or with PJ PP fraction (up to 100 µM) (A) or with two concentrations of sugar fractions (containing 0.28 mM or 2.8 mM glucose) purified from PJ or from white grape juice (WGJ) (B). Cellular fluorescence (indicating peroxide content) was determined after the cells were incubated for 30 min at 37 °C with  $2.5 \times 10^{-5}$  mol/l dichlorofluorescein-diacetate (DCFH-DA) by flow cytometry. Results represent mean  $\pm$  S.D. ( $n = 5$ ). \*  $p < 0.01$  (PJ or PJ PPS vs. control).

Incubation of J774A.1 macrophages for 18 h at 37 °C with increasing concentrations of glucose (up to 30 mM) resulted in an increment in macrophage peroxide levels by up to 2.2-fold (Fig. 2A) and a decrement in macrophage glutathione content by up to 41% (Fig. 2B), in comparison to cells incubated with only 5 mM of glucose. In contrast, incubation of J774A.1 macrophages with fructose for 18 h at 37 °C with a concentration as low as 7.8 mM (the same used for the cells that were incubated with the PJ sugar fraction), resulted in a decrement in macrophage peroxide levels by 16% compared to 5 mM fructose ( $117.7 \pm 2.7$  versus  $140.3 \pm 1.1$  fluorescence intensity, arbitrary units, respectively). Incubation of the macrophages with fructose also resulted in a paralleled increment in cellular glutathione content by 13% ( $9.6 \pm 1.0$  nmol/mg versus  $11.1 \pm 0.4$  nmol/mg cell protein, respectively). These findings indicate that the inhibitory effect of PJ on macrophage oxidative state could

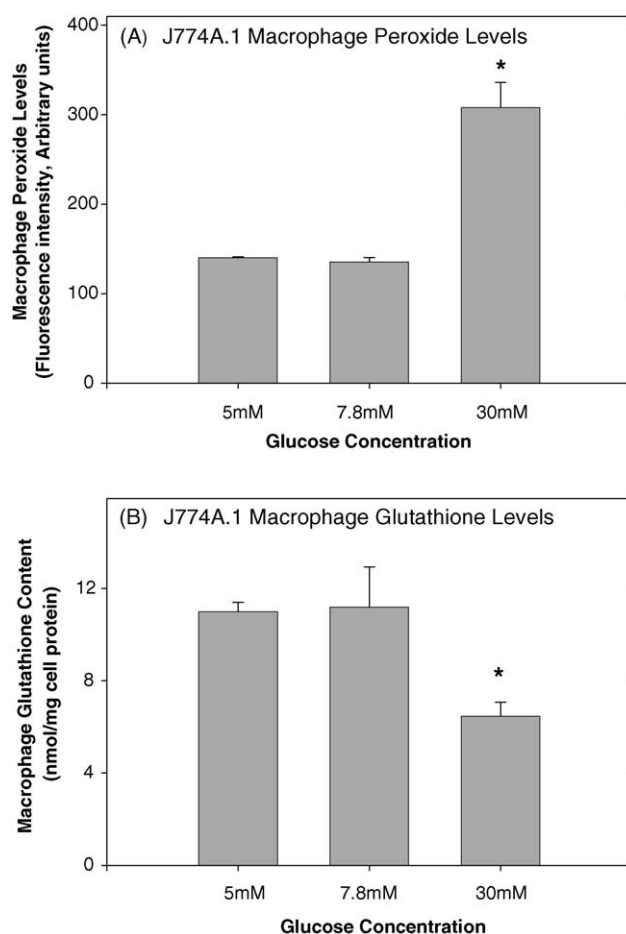


Fig. 2. The effect of increasing medium glucose concentrations on macrophage oxidative state. J-774A.1 macrophages ( $1 \times 10^6$ /well) were incubated in serum-free DMEM for 18 h at 37 °C with increasing glucose concentrations (5–30 mM). Then, cellular oxidative state was determined as follows: (A) macrophage peroxide content was determined after cell incubation for 30 min at 37 °C with  $2.5 \times 10^{-5}$  mol/l DCFH-DA. Cellular fluorescence was determined by flow cytometry. (B) Glutathione (GSH) content was measured in cell sonicate ( $1.5 \times 10^6$  cells/ml PBS) by the DTNB-GSSG recycling assay. Cellular protein content was measured by the Lowry assay. Results represent mean  $\pm$  S.D. ( $n = 5$ ).

not be attributed to the PJ glucose, and could only minimally be attributed to the PJ fructose.

#### 3.4. The PJ sugar fraction decreases, whereas the WGJ sugar fraction increases oxidative state in mouse peritoneal macrophages (MPM)

We next incubated the sugar fractions from PJ and from WGJ with MPM harvested from control Balb/C mice. As shown for the J774A.1 cell line, incubation of the control Balb/C mice MPM for 18 h at 37 °C with the PJ sugar fraction (containing 2.8 mM glucose) in low-glucose (5 mM) serum-free DMEM decreased cellular peroxide levels by 40% (Fig. 3A) and increased macrophage glutathione content by 20% (Fig. 3B), compared to control cells that

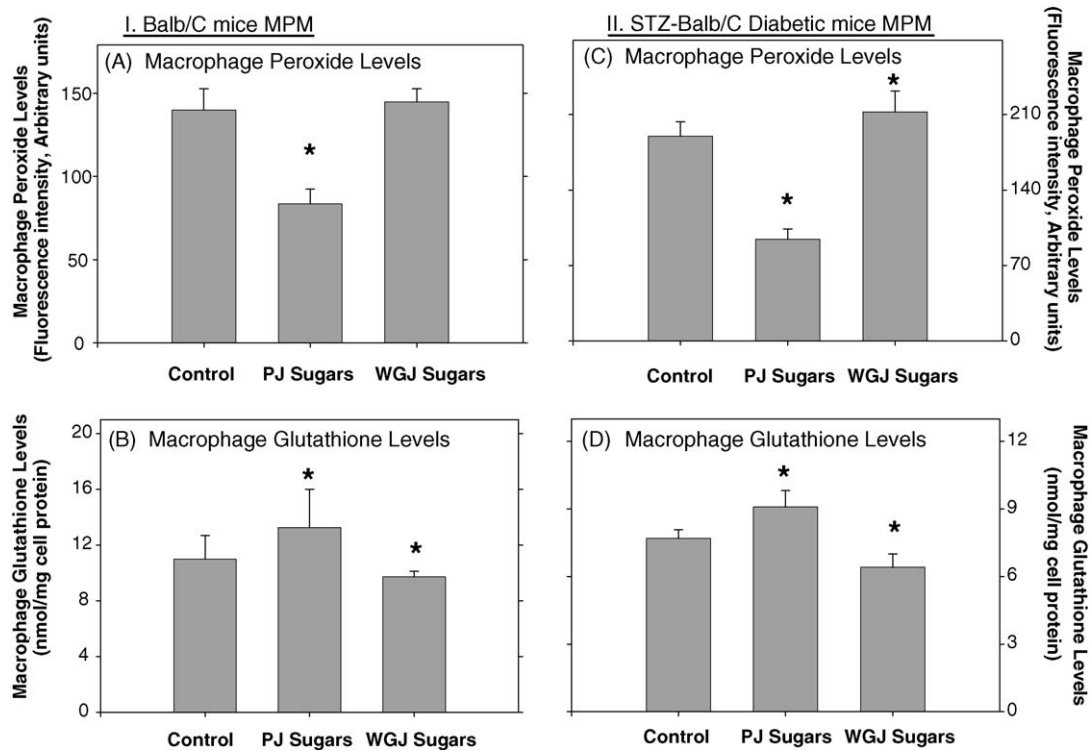


Fig. 3. The effect of sugar fractions purified from PJ or from WGJ on the oxidative state of mouse peritoneal macrophages (MPM) harvested from control mice or from STZ-induced diabetic Balb/C mice. MPM ( $1 \times 10^6$ /well) were harvested from control or diabetic Balb/C mice, plated and incubated in serum-free low-glucose (5 mM) DMEM (controls) or in serum-free high-glucose (22.5 mM) DMEM (diabetics), for 18 h at 37 °C with the sugar fractions from PJ or from WGJ (containing 2.8 mM glucose). Then, cellular oxidative state was determined as follows: (A) macrophage peroxide content was determined after cell incubation for 30 min at 37 °C with  $2.5 \times 10^{-5}$  mol/l DCFH-DA. Cellular fluorescence was determined by flow cytometry. (B) Glutathione (GSH) content was measured in cell sonicate ( $1.5 \times 10^6$  cells/ml PBS) by the DTNB-GSSG recycling assay. Cellular protein content was measured by the Lowry assay. Results represent mean  $\pm$  S.D. ( $n=3$ ). \* $p < 0.05$  (PJ sugars or WGJ sugars vs. control).

were incubated without the sugar fraction. In contrast, incubation of MPM with the WGJ sugar fraction (containing 2.8 mM glucose) did not affect peroxide levels (Fig. 3A) and decreased cellular glutathione content by 12% (Fig. 3B), compared to control cells.

We next questioned whether the beneficial effect of the PJ sugars is also evident under diabetic conditions. For this purpose we used MPM harvested from streptozotocin (STZ)-treated diabetic Balb/C mice. Incubation of MPM from the diabetic mice for 18 h at 37 °C with PJ sugars (containing 2.8 mM glucose) in high-glucose (22.5 mM) serum-free DMEM decreased macrophage total peroxide levels by 50% (Fig. 3C) and increased cellular glutathione content by 18% (Fig. 3D), compared to cells that were incubated without the sugar fraction. Again, as shown for the MPM from non-diabetic mice, incubation of the cells with the WGJ sugar fraction (containing 2.8 mM glucose) increased peroxide levels by 12% (Fig. 3C) and decreased cellular glutathione content by 17% (Fig. 3D), compared to control cells. Altogether, these findings indicate that the PJ sugar fraction, as opposed to the WGJ sugar fraction, possesses antioxidative properties against macrophage oxidation, even under diabetic conditions.

### 3.5. The PJ sugar fraction decreases, whereas WGJ sugar fraction increases macrophage oxidative stress also in vivo in diabetic mice

The effects of the sugar fractions from PJ and from WGJ on macrophage oxidative stress were further examined in vivo. Diabetic Balb/C mice were supplemented for 10 days with water (control) or with the sugar fractions (from PJ or WGJ) containing 30 mg of glucose per day per mouse. PJ sugar consumption by the diabetic mice resulted in a significant ( $p < 0.05$ ) 10% decrement in macrophage total peroxide levels (Fig. 4A) and in a significant ( $p < 0.02$ ) 7% increment in cellular glutathione content (Fig. 4B), compared to MPM harvested from the control diabetic mice administered with water only. Moreover, the WGJ sugar fraction consumption by the diabetic mice resulted in a 22% increment in macrophage total peroxide levels (Fig. 4A) and in a 45% decrement in cellular glutathione content (Fig. 4B), compared to MPM harvested from control mice. Even more significant results ( $p < 0.01$ ) were obtained upon comparing the effect of the PJ sugars to that of WGJ sugars (26% decrement in the lipid peroxide levels and 94% increment in the glutathione content). These results demonstrate that PJ sugar

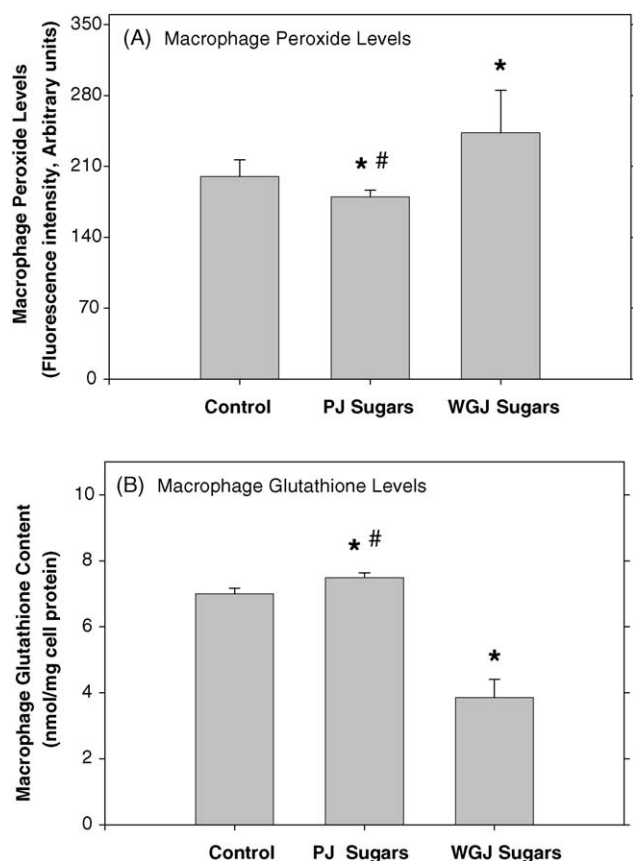


Fig. 4. The in vivo effect of sugar fraction supplementation (purified from PJ or from WGJ) to STZ-induced diabetic Balb/C mice on cellular oxidative state. STZ-induced diabetic Balb/C Mice were supplemented with the sugar fraction (containing 30 mg of glucose per day per mouse) for 10 days. Then, the mice were sacrificed and their MPM ( $1 \times 10^6$ /well) were harvested, plated and incubated in serum-free high-glucose (22.5 mM) DMEM for 18 h at 37 °C. (A) Macrophage peroxide content was determined after cell incubation for 30 min at 37 °C with  $2.5 \times 10^{-5}$  mol/l DCFH-DA. Cellular fluorescence was determined by flow cytometry. (B) Glutathione (GSH) content was measured in cell sonicate ( $1.5 \times 10^6$  cells/ml PBS) by the DTNB-GSSG recycling assay. Cellular protein content was measured by the Lowry assay. Results represent mean  $\pm$  S.D. ( $n=3$ ). \* $p < 0.05$  (PJ sugars or WGJ sugars vs. control), # $p < 0.01$  (PJ sugars vs. WGJ sugars).

fraction decreases macrophages oxidative stress not only in vitro but also in vivo, while WGJ sugar fraction increases macrophages oxidative stress in vitro and in vivo.

### 3.6. The PJ sugar fraction decreases whereas WGJ sugar fraction increases macrophage paraoxonase 2 activity

Paraoxonase 2 (PON2) activity in macrophages was shown to be increased under oxidative stress. MPM from the diabetic mice that were administrated with PJ sugar fraction exhibited a significant ( $p < 0.05$ ) 7% decrement in PON2 activity (Fig. 5A), whereas MPM from diabetic mice that were administrated with WGJ sugar fraction exhibited a 12% increment in PON2 activity (Fig. 5A), compared to mice that were supplemented with water.

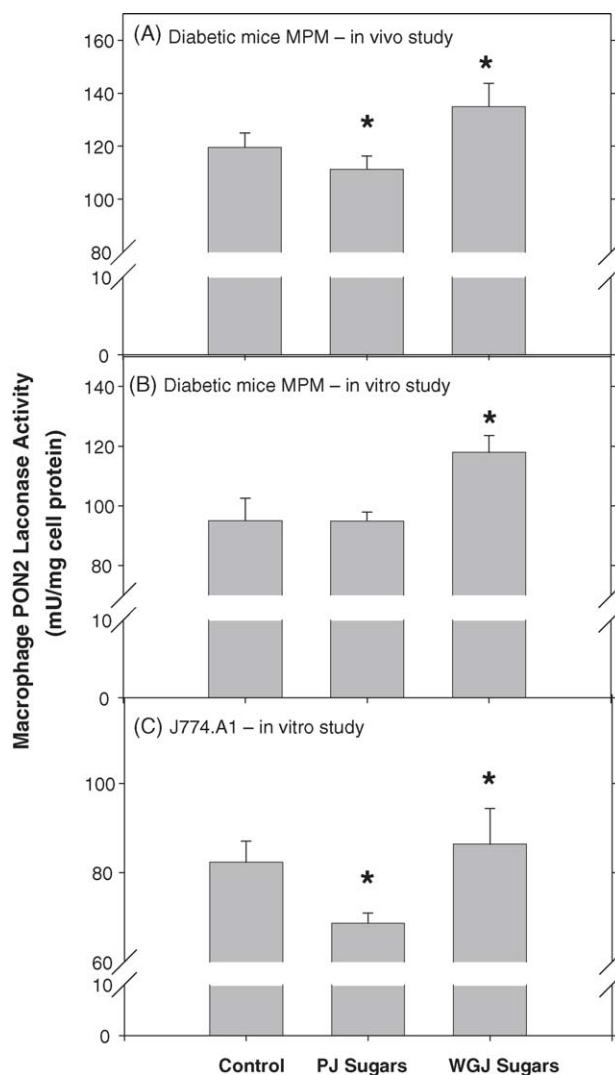


Fig. 5. The effect of sugar fractions, purified from PJ or from WGJ, on macrophage paraoxonase 2 (PON2) lactonase activity. Macrophages were plated in 12-well dishes. (A) MPM harvested from the sugar fraction-supplemented diabetic mice. (B) MPM harvested from diabetic Balb/C mice. (C) J-774A.1 macrophages. For (B) and (C) macrophages were incubated in serum-free DMEM for 18 h at 37 °C with the sugar fractions from PJ or from WGJ (containing 2.8 mM of glucose). Dihydrocoumarin was added to the reaction buffer (Tris buffer, pH 8.0) and was incubated with the cells for 10 min. Lactonase activity was measured spectrophotometrically at 270 nm. Cellular protein content was measured by the Lowry assay. Results represent mean  $\pm$  S.D. ( $n=3$ ). \* $p < 0.05$  (PJ sugars or WGJ sugars vs. control).

In MPM harvested from diabetic mice, incubation for 18 h at 37 °C with the PJ sugar fraction (containing 2.8 mM glucose) did not significantly affect PON2 activity (Fig. 5B), compared to MPM from control diabetic mice. In contrast, cell incubation with the WGJ sugar fraction (containing 2.8 mM glucose) increased PON2 activity by 24% (Fig. 5B), compared to control MPM.

In J774A.1 macrophages, cell incubation for 18 h at 37 °C with the PJ sugar fraction (containing 2.8 mM glucose) decreased PON2 activity by 16% (Fig. 5C), compared to control cells that were not incubated with the sugar fraction,

whereas cell incubation with the WGJ sugar fraction (containing 2.8 mM glucose) minimally (5%), but significantly ( $p < 0.05$ ) increased macrophage PON2 activity (Fig. 5C).

### 3.7. The antioxidative effect of the PJ sugar fraction was not associated with significant changes in serum paraoxonase 1 activity

As paraoxonase 1 (PON1) was previously shown to decrease macrophage oxidative stress, we questioned whether the administration of PJ sugars to the diabetic mice could affect their serum PON1 activity. PON1 arylesterase activity (towards phenylacetate) was not significantly changed after PJ or WGJ sugar fraction administration ( $110.6 \pm 8.8$  U/ml in control mice,  $119.8 \pm 9.7$  U/ml in mice that were administrated with PJ sugars, and  $113.7 \pm 0.1$  U/ml in mice that were administrated with WGJ sugars). Similarly, PON1 paraoxonase activity (towards paraoxon) was only slightly, but not significantly increased after the sugar fractions administration, with no differences detected between PJ and WGJ ( $35.8 \pm 3.8$  U/ml in control mice,  $41.9 \pm 2.6$  U/ml in mice that were administrated with PJ sugars, and  $42.8 \pm 3.9$  U/ml in mice that were administrated with WGJ sugars).

### 3.8. PJ Complex sugars may be the active antioxidant components in the PJ sugar fraction

Glucose and fructose are found in PJ and in WGJ at similar concentrations. We thus questioned whether other types of complex sugars that are present in relatively high levels in PJ, compared to WGJ, may be the active components in the PJ sugar fraction. Indeed, we were able to show that delphinidin-3-*o*- $\beta$ -glucopyranoside and cyanidin-3-*o*- $\beta$ -glucopyranoside exhibit potent free radical scavenging capacity. The addition of these complex phenolic sugars at a final concentration of 10  $\mu$ g/ml to a DPPH solution exhibited a remarkable free radical scavenging capacity, as demonstrated by a 53% reduction in the optical density at 517 nm with cyanidin-3-*o*- $\beta$ -glucopyranoside (from  $1.03 \pm 0.09$  to  $0.48 \pm 0.04$  OD<sub>517</sub>) and a 58% reduction obtained with delphinidin-3-*o*- $\beta$ -glucopyranoside (from  $1.03 \pm 0.10$  to  $0.43 \pm 0.04$  OD<sub>517</sub>), in comparison to a 78% reduction (from  $1.03 \pm 0.11$  to  $0.23 \pm 0.01$  OD<sub>517</sub>) obtained by a similar concentration of PJ.

## 4. Discussion

Pomegranate juice (PJ) antiatherogenicity was shown to be mainly related to its antioxidant properties. In this study, we show that PJ sugars may be partly responsible for these properties. PJ, which is one of the nutrients richest in polyphenols, was shown to reduce macrophage lipid peroxidation, cholesterol accumulation and foam cell formation, resulting in attenuation of the development of atherosclerosis in apolipoproteinE-deficient mice [32], and also in patients

with carotid artery stenosis [3] and diabetic patients [4]. Moreover, PJ reduced the activation of redox-sensitive genes (ELK-1 and p-JUN) and increased eNOS expression in cultured human coronary artery endothelial cells and in atherosclerosis-prone areas of hypercholesterolemic mice [33]. These antioxidative/antiatherogenic properties of the PJ were mainly attributed to the high content, as well as unique type of pomegranate polyphenols [5,34]. However, as shown in the present study, the capacity of PJ to decrease macrophages peroxide levels was greater than the effect of the PJ purified polyphenols. This phenomenon could be a result of the isolation procedure that may lead to properties that are suboptimal and that are fully expressed only in the whole juice. However, it led us to aim to find out whether the PJ sugar fraction could also contribute to the PJ antioxidative properties.

Type 2 diabetes, a major risk factor for the development of premature atherosclerosis [16], is characterized by increased oxidative stress. Mouse peritoneal macrophages harvested from diabetic mice that exhibited increased atherosclerotic lesion compared to controls, were recently characterized by increased lipid peroxides content and increased capability to take up oxidized LDL [35]. Moreover, glucose-enriched macrophages exhibited a dose-dependent higher peroxide content and increased Ox-LDL cellular uptake (associated with up-regulation of the scavenger receptor CD36). The present study also shows that glucose directly increases macrophages oxidative stress. Thus, the finding that the glucose-containing PJ sugar fraction decreased macrophage oxidative stress was somewhat surprising. The PJ sugar fraction exhibits impressive antioxidative effects not only on the J774 macrophage cell-line, but also on mouse peritoneal macrophages (which resemble arterial macrophages in their cholesterol and lipoprotein metabolism characteristics) [36]. Moreover, the present study demonstrates that even under diabetic conditions characterized by increased oxidative stress (using MPM from STZ-induced diabetic mice), the PJ sugar fraction still exhibited its beneficial effects, in contrast to the pro-oxidant effects of the WGJ sugar fraction.

Serum glutathione levels are decreased under oxidative stress conditions, like in atherosclerosis [37] and in diabetes [38]. In atherosclerotic mice, peritoneal macrophage glutathione content was shown to be decreased, and this phenomenon was associated with increased macrophage oxidative state. The present study shows that the PJ sugar fraction increased glutathione levels in MPM from control mice, as well as from diabetic mice. Moreover, glutathione levels were also increased in vivo as studied in MPM from diabetic mice after PJ sugar fraction consumption. The in vivo beneficial effect of the PJ sugars clearly demonstrates the physio-pathological contribution of the PJ sugar fraction to the antioxidative/antiatherogenic properties of PJ.

In contrast to glucose, fructose demonstrated beneficial effects on oxidative stress as it reduced oxidative state in macrophages. In accordance with these data, fructose was also shown to increase plasma antioxidant capacity after

apple consumption, due to increment in plasma urate levels [20].

Glucose and fructose are found in PJ and in WGJ at similar concentrations [39,40]. However, whereas PJ sugars demonstrated antioxidative effects on macrophages, WGJ sugars demonstrated pro-oxidative effects on the cells. The beneficial effect of the PJ sugars may not be attributed mainly to fructose, but to some PJ unique, not yet identified, complex sugars and also to some sugar-containing polyphenolic tannins and anthocyanins. Among PJ anthocyanins, cyanidin-3-*o*- $\beta$ -glucopyranoside and delphinidin-3-*o*- $\beta$ -glucopyranoside are the main components [6] and they were shown to possess antioxidant properties. These two compounds inhibit a Fenton reagent  $\bullet$ OH generating system, possibly by chelation of a ferrous ion. These molecules also scavenged  $O_2^{\bullet-}$  in a dose-dependent manner and inhibited  $H_2O_2$ -induced lipid peroxidation in the rat brain homogenates [41,42]. In the present study, we confirmed their capacity to scavenge free radicals. These anthocyanins are not found at all in white grape varieties [43].

HDL-associated PON1 was shown to possess antiatherogenic and antioxidative properties [44], probably due to its capacity to hydrolyze oxidized lipids in lipoproteins and in macrophages [22,23]. PJ consumption was demonstrated to increase serum PON1 activity in atherosclerotic mice and in humans with carotid artery stenosis [32,3], a phenomenon that can be the underlined mechanism by which PJ decreases atherosclerosis development. As serum PON1 was not affected by the PJ sugar fraction, a different mechanism is involved in the beneficial effects of the PJ sugars. Another member of the PON family, PON2, is expressed in cells and was shown to possess antioxidative properties [45]. Its activity was shown to be increased in macrophages under oxidative stress [26,46]. Indeed, treatment of macrophages with WGJ sugars increased PON2 activity, whilst PJ sugars decreased PON2 activity in parallel to the increment or the decrement in cellular oxidative state, respectively. These effects on macrophage PON2 demonstrate again the opposite characteristics of PJ versus WGJ sugars on macrophage oxidative state.

We conclude that PJ sugars possess an unexpected antioxidative effect on macrophage oxidative state under diabetic conditions *in vitro*, as well as *in vivo*. These effects could be related to some phenolic-sugar compounds in the PJ.

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## References

- [1] Aviram M, Dornfeld L, Kaplan M, et al. Pomegranate juice flavonoids inhibit low-density lipoprotein oxidation and cardiovascular diseases: studies in atherosclerotic mice and in humans. *Drugs Exp Clin Res* 2002;28:49–62.
- [2] Aviram M, Dornfeld L, Rosenblat M, et al. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am J Clin Nutr* 2000;71:1062–76.
- [3] Aviram M, Rosenblat M, Gaitini D, et al. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clin Nutr* 2004;23:423–33.
- [4] Rosenblat M, Hayek T, Aviram M. Anti-oxidative effects of pomegranate juice (PJ) consumption by diabetic patients on serum and on macrophages. *Atherosclerosis* 2006;187:363–71.
- [5] Cerda B, Lorach R, Ceron JJ, Espin JC, Tomas-Barberan FA. Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. *Eur J Nutr* 2003;42:18–28.
- [6] Gil MI, Tomas-Barberan FA, Hess-Pierce B, et al. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem* 2000;48:4581–9.
- [7] Stocker R, Keaney Jr JF. Role of oxidative modifications in atherosclerosis. *Physiol Rev* 2004;84:1381–478.
- [8] Aviram M. Review of human studies on oxidative damage and antioxidant protection related to cardiovascular diseases. *Free Radic Res* 2000;33(Suppl.):S85–97.
- [9] Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* 1997;272:20963–6.
- [10] Maor I, Kaplan M, Hayek T, et al. Oxidized monocyte-derived macrophages in aortic atherosclerotic lesion from apolipoprotein E-deficient mice and from human carotid artery contain lipid peroxides and oxysterols. *Biochem Biophys Res Commun* 2000;269:775–80.
- [11] Rozenberg O, Rosenblat M, Coleman R, Shih DM, Aviram M. Paraoxonase (PON1) deficiency is associated with increased macrophage oxidative stress: studies in PON1-knockout mice. *Free Radic Biol Med* 2003;34:774–84.
- [12] Fuhrman B, Oiknine J, Aviram M. Iron induces lipid peroxidation in cultured macrophages, increases their ability to oxidatively modify LDL, and affects their secretory properties. *Atherosclerosis* 1994;111:65–78.
- [13] Osterud B, Bjorklid E. Role of monocytes in atherogenesis. *Physiol Rev* 2003;83:1069–112.
- [14] Fuhrman B, Volkova N, Aviram M. Oxidative stress increases the expression of the CD36 scavenger receptor and the cellular uptake of oxidized low-density lipoprotein in macrophages from atherosclerotic mice: protective role of antioxidants and of paraoxonase. *Atherosclerosis* 2002;161:307–16.
- [15] Rosenblat M, Aviram M. Macrophage glutathione content and glutathione peroxidase activity are inversely related to cell-mediated oxidation of LDL: *in vitro* and *in vivo* studies. *Free Radic Biol Med* 1998;24:305–17.
- [16] Heller GV. Evaluation of the patient with diabetes mellitus and suspected coronary artery disease. *Am J Med* 2005;118(Suppl 2):9S–14S.
- [17] Pacher P, Obrosova IG, Mabley JG, Szabo C. Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. *Curr Med Chem* 2005;12:267–75.
- [18] Jakus V, Rietbrock N. Advanced glycation end-products and the progress of diabetic vascular complications. *Physiol Res* 2004;53:131–42.
- [19] Tanaka A. Postprandial hyperlipidemia and atherosclerosis. *J Atheroscler Thromb* 2004;11(6):322–9.
- [20] Lotito SB, Frei B. The increase in human plasma antioxidant capacity after apple consumption is due to the metabolic effect of fructose on urate, not apple-derived antioxidant flavonoids. *Free Radic Biol Med* 2004;37:251–8.

- [21] Getz GS, Reardon CA. Paraoxonase, a cardioprotective enzyme: continuing issues. *Curr Opin Lipidol* 2004;15:261–7.
- [22] Rozenberg O, Shih DM, Aviram M. Paraoxonase 1 (PON1) attenuates macrophage oxidative status: studies in PON1 transfected cells and in PON1 transgenic mice. *Atherosclerosis* 2005;181:9–18.
- [23] Aviram M, Rosenblat M. Paraoxonases and cardiovascular diseases: pharmacological and nutritional influences. *Curr Opin Lipidol* 2005;16:393–9.
- [24] Mackness M, Durrington P, Mackness B. Paraoxonase 1 activity, concentration and genotype in cardiovascular disease. *Curr Opin Lipidol* 2004;15:399–404.
- [25] Tsuzura S, Ikeda Y, Suchiro T, et al. Correlation of plasma oxidized low-density lipoprotein levels to vascular complications and human serum paraoxonase in patients with type 2 diabetes. *Metabolism* 2004;53:297–302.
- [26] Rosenblat M, Draganov D, Watson CE, et al. Mouse macrophage paraoxonase 2 activity is increased whereas cellular paraoxonase 3 activity is decreased under oxidative stress. *Arterioscler Thromb Vasc Biol* 2003;23:468–74.
- [27] Jiang Y, Nyengaard JR, Zhang JS, Jakobsen J. Selective loss of calcitonin gene-related Peptide-expressing primary sensory neurons of the  $\alpha$ -cell phenotype in early experimental diabetes. *Diabetes* 2004;53:2669–75.
- [28] Gan KN, Smolen A, Eckerson HW, La Du BN. Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. *Drug Metab Dispos* 1991;19:100–6.
- [29] Draganov DI, Stetson PL, Watson CE, Billecke SS, La Du BN. Rabbit serum paraoxonase 3 (PON3) is a lactonase and protects low density lipoprotein against oxidation. *J Biol Chem* 2000;275:33435–42.
- [30] Lowry OH, Rosebrough NJ, Farr L, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- [31] Fuhrman B, Volkova N, Suraski A, Aviram M. White wine with red wine-like properties: increased extraction of grape skin polyphenols improves the antioxidant capacity of the derived white wine. *J Agric Food Chem* 2001;49:3164–8.
- [32] Kaplan M, Hayek T, Raz A, et al. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. *J Nutr* 2001;131:2082–9.
- [33] de Nigris F, Williams-Ignarro S, Lerman LO, et al. Beneficial effects of pomegranate juice on oxidation-sensitive genes and endothelial nitric oxide synthase activity at sites of perturbed shear stress. *Proc Natl Acad Sci USA* 2005;102:4896–901.
- [34] Schubert SY, Neeman I, Resnick N. A novel mechanism for the inhibition of NF-kappaB activation in vascular endothelial cells by natural antioxidants. *FASEB J* 2002;16:1931–3.
- [35] Hayek T, Hussein K, Aviram M, et al. Macrophage-foam cell formation in streptozotocin-induced diabetic mice: Stimulatory effect of glucose. *Atherosclerosis* 2005;183:25–33.
- [36] Keller-Weibel G, Yancey PG, Jerome WG, et al. Crystallization of free cholesterol in model macrophage foam cells. *Arterioscler Thromb Vasc Biol* 1999;19:1891–8.
- [37] Rosenblat M, Coleman R, Aviram M. Increased macrophage glutathione content reduces cell-mediated oxidation of LDL and atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis* 2002;163:17–28.
- [38] Saxena R, Madhu SV, Shukla R, Prabhu KM, Gambhir JK. Postprandial hypertriglyceridemia and oxidative stress in patients of type 2 diabetes mellitus with macrovascular complications. *Clin Chim Acta* 2005;359:101–8.
- [39] Malgarejo P, Salazar DM, Artes F. Organic acids and sugars composition of harvested pomegranate fruits. *Eur Food Res Technol* 2000;211:185–90.
- [40] Duro D, Rising R, Cedillo M, Lifshitz F. Association between infantile colic and carbohydrate malabsorption from fruit juices in infancy. *Pediatrics* 2002;109:797–805.
- [41] Noda Y, Kaneyuki T, Mori A, et al. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin. *J Agric Food Chem* 2002;50:166–71.
- [42] Aviram M, Fuhrman B. Effects of flavonoids on the oxidation of LDL and atherosclerosis. In: Rice-Evans C, Packer L, editors. *Flavonoids in health and disease*. 2nd ed. 2003. p. 165–203.
- [43] Cantos E, Espin JC, Tomas-Barberan FA. Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC–DAD–MS–MS. *J Agric Food Chem* 2002;50:5691–6.
- [44] Ng CJ, Shih DM, Hama SY, et al. The paraoxonase gene family and atherosclerosis. *Free Radic Biol Med* 2005;38:153–63.
- [45] Ng CJ, Wadleigh DJ, Gangopadhyay A, et al. Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *J Biol Chem* 2001;276:44444–9.
- [46] Shiner M, Fuhrman B, Aviram M. Paraoxonase 2 (PON2) expression is upregulated via a reduced-nicotinamide-adenine-dinucleotide-phosphate (NADPH)-oxidase-dependent mechanism during monocytes differentiation into macrophages. *Free Radic Biol Med* 2004;37:2052–63.