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Pomegranate juice increases levels of paraoxonase1 (PON1) expression and enzymatic activity in streptozotocin-induced diabetic mice fed with a high-fat diet

G. Betanzos-Cabrera [a,*], J.A. Guerrero-Solano [a], M.M. Martínez-Pérez [a], Z.G. Calderón-Ramos [a], H. Belefant-Miller [b], J.C. Cancino-Diaz [c]

[a] Área Académica de Nutrición, Instituto de Ciencias de la Salud, Universidad Autónoma del Estado de Hidalgo, Mexico
[b] Dale Bumpers National Rice Research Center, Stuttgart, AR, USA
[c] Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional, México D.F., Mexico

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ABSTRACT

Pomegranate (*Punica granatum L.*) is a polyphenol-rich fruit. In the past decade, studies in human and murine models on the antioxidant, anticarcinogenic, antihypertensive, and anti-inflammatory properties of pomegranate constituents have been published, focusing mainly on treatment and prevention of cardiovascular disease, diabetes, bacterial infections, and antibiotic resistance. In animals, the esterase paraoxonase1 (PON1) prevents LDL oxidation *in vitro*. Decreased levels of PON1 are associated with an increased risk for cardiovascular disease. In this work, streptozotocin-induced diabetic mice fed with a high-fat diet were supplemented daily with pomegranate juice (PJ) in order to study the effect of PJ on PON1 gene expression and activity. The supplementation with PJ significantly induced PON1 gene expression and activity compared to mice that did not receive the PJ, although not to the levels reached by mice fed a non-high fat diet. Interestingly, animals supplemented with PJ showed the lowest body weight. In addition, the PJ significantly reduced blood glucose but not triacylglycerols and cholesterol levels, demonstrating that PJ has a hypoglycemic effect. Thus, the daily PJ supplementation in our unique model indicates that including this fruit juice in the diet has potential in the management of diabetes as well as cardioprotective benefits that deserve further clinical investigation.

1. Introduction

Pomegranate (*Punica granatum L.*) fruit has significantly higher antioxidant capacity than more commonly consumed fruit juices such as grape, cranberry, grapefruit, or orange (Azadzoi, Schulman, Aviram, & Siroky, 2005; Basu & Penugonda, 2009). The most important antioxidant polyphenols in pomegranate juice (PJ) include the ellagitannins and anthocyanins concentrated in the peel, membranes, and piths of the fruit (Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000). Punicalagins are the major ellagitannins in the whole fruit and can be hydrolyzed to ellagic acid (EA) and other smaller polyphenols *in vivo*. Current research indicates that the most therapeutically beneficial pomegranate constituents are ellagitannins, ellagic acid, punicic acid, flavonoids, anthocyanidins, anthocyanins, estrogenic flavonols and flavones (Jurenka, 2008; Lansky & Newman, 2007). The potential therapeutic properties of pomegranate components have been investigated and vary widely including treatment and prevention of cancer (Lansky & Newman, 2007), cardiovascular disease (Aviram et al., 2002) and diabetes (Rosenblat, Hayek, & Aviram, 2006). Thus, the mechanisms of action of PJ consumption deserve further clinical investigation.

Paraoxnase1 (PON1) belongs to the family of serum paraoxonases consisting of PON1, PON2 and PON3. The PON genes coding for these enzymes are located as a cluster on human chromosome 7 (Primo-Parmo, Sorenson, Teiber, & La Du, 1996). Human PON1 has a molecular mass of 43 kDa (355 amino acids) and is a glycoprotein with calcium-dependent esterase activity. PON1 and PON3 are expressed in the liver and excreted in the blood where they are associated with high-density lipoprotein (HDL) particles (Mackness, Hallam, Peard, Warner, & Walker, 1985; Reddy et al., 2001). PON2 is not present in blood, but is expressed widely in a number of tissues including the liver, lungs, brain and heart (Mochizuki et al., 1998). Unlike PON2 and PON3, PON1 has efficient esterase activity towards many organophosphates (OPs) including paraoxon, parathion and chlorpyriphos as well as the nerve agents sarin and soman (van Himbergen, van Tits, Roest, & Stalenhoef, 2006).

There is growing evidence that PON1 plays an important role in lipid metabolism and the onset of cardiovascular disease. PON1 has been proposed to play an important role in protecting LDL and HDL from oxidation *in vitro*, thus lowering the risk of developing atherosclerosis (Mackness et al., 2000; Mackness, Mackness, & Durrington, 2002). PON1 knockout mice exhibit about a two-fold increase in atherosclerosis (Rozenberg, Rosenblat, Coleman, Shih & Aviram, 2003), whereas

* Corresponding author at: Abasolo 600, Pachuca de Soto, 42000, México. Tel.: +52 771 7172000x5116; fax: +52 7717172000x5114.
E-mail address: gbetanzo@uaeh.edu.mx (G. Betanzos-Cabrera).

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transgenic mice overexpressing human PON1 were more resistant to atherosclerosis (Tward et al., 2002). PON1 expression and activity can be modulated by dietary polyphenols i.e. LDL receptor deficient mice supplemented with quercitine (a polyphenol contained in pomegranate) and moderate ethanol inhibited the progression of atherosclerosis by upregulating the hepatic expression with concomitant increased serum PON1 activity (Leckey et al., 2010; Gouédard, Barouki, & Morel, 2004). Similarly, pomegranate polyphenols seem to have a specific transcriptional role in hepatocyte PON1 expression upregulation (Khateeb, Gantman, Kreitenberg, Aviram, & Fuhrman, 2010). Although it is known that diabetes is associated with increased oxidative stress and the development of atherosclerosis (Mooradian, 2009), no expression studies have been examined in a diabetic model that is fed with high fat. Therefore, it is of interest to know if PJ induces PON1 gene expression and activity, especially in conditions known for affecting PON1 enzymatic function.

2. Material and methods

2.1. Pomegranate juice

Pomegranates from Valle de Mezquital, Hidalgo, Mexico were handpicked, washed, wrapped, and stored at 4 °C. Aril juice was used to ensure that the results would be valid for juice as consumed by humans. The fruit was peeled and the seeds were crushed and then squeezed with a squeezing machine. The PJ was filtered to remove any water-insoluble materials and 12.5 mL was immediately diluted in water and placed in dark bottles wrapped with aluminum foil.

2.2. Diets

Two diets were used: (i) a control diet for rodents (Harlan Tekla® Madison, WI, USA) and (ii) a high fat diet designed by a nutritionist utilizing Mexican foods such as butter and lard that contained 32% carbohydrates, 18% proteins and 50% saturated fat (Betanzos-Cabrera et al., 2009). The diet (i) was used for the control group, whereas diet (ii) was used for the nonsupplemented and JP supplemented groups.

2.3. Mouse study

Animal management was supervised by a veterinarian in accordance with the principles set forth in the NIH guide for the care and use of laboratory animals and approved by the Animal Care Committee of the Instituto de Ciencias de la Salud, UAEH. Male CD-1 mice of approximately 35–40 g were provided by the animal facility of the Instituto de Ciencias de la Salud (Universidad Autónoma del Estado de Hidalgo). To analyze the effect of PJ on the expression and activity of PON1, 60 animals CD1 mice were divided into three groups of 20: control (1), nonsupplemented with JP (2), and supplemented with JP (3).

2.3.1. Induction of diabetes

Groups 2 and 3 were diabetized with a single dose of streptozotocin (Sigma, St. Louis, MO) 180 mg/Kg *ip*. Immediately post-injection and 1, 2, 3 and 4 months after, four mice from each group were weighed and killed by decapitation and their blood was collected.

2.4. Group supplemented with JP

Fresh PJ prepared daily as described above was diluted in water (12.5 mL/L of juice in 1 L of water) equivalent to 0.35 mmol total polyphenols according to Kaplan et al. (2001). The diluted PJ was given to the mice in their drinking water from the first day until the end of the experiment. Control and nonsupplemented groups received *ad libitum* drinking water without adding PJ.

2.5. RNA isolation and RT-PCR analysis

Livers were washed in D-PBS to eliminate blood contamination. Total RNA extraction was performed with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was supplemented with RNAse-free DNAse I and RNA were re-extracted with TRIzol reagent. For the reverse transcriptase (RT) reaction, total RNA (3 μg) with 0.5 μg of oligo-$(dT)_{15-18}$ (Invitrogen) was denatured at 70 °C for 10 min. Then, 1X single strand buffer, 0.5 mM DTT, 500 μM of each dNTP and 200 U of MMLV reverse transcriptase (Invitrogen) were added. The RT reactions were performed at 42 °C for 1 h. The polymerase chain reactions (PCR) were performed with 1 μL of the cDNA, 1X buffer, 1 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each PON1 and GAPDH specific primer (Table 1) and 2 U of TaqDNA polymerase (Invitrogen). Optimal PCR conditions were 30 cycles of 30 s at 92 °C, 30 s at 60 °C, and 30 s at 72 °C.

2.6. Semiquantitative PCR

The intensity of the amplified bands was analyzed with the Alpha Imager software. The band intensities were normalized with the corresponding GAPDH signal (PON1/GAPDH rate). The results were analyzed by a one-way ANOVA with a Tukey test.

2.7. Measurement of PON1 activity

The activity was assayed at pH 8.0 because other non-esterase paraoxon hydrolytic activities occur at pH >8.5 (Furlong, Richter, Seidel, & Motulsky, 1988). The PON1 activity was measured with some modifications by a semiautomated method using paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenylphosphate) as a substrate as described Charlton-Menys, Liu, and Durrington (2006). Briefly, the spectrophotometer (Biotek® microplate reader, USA) settings were as follows: kinetic mode; filter, 405 nm; lag time, 0.42 min; interval time, 0.5 min; and total time, 3 min. 10 μL of the sample was added to a microtiter plate (Nunc Immunoplate Maxisorp; Scientific Laboratory Supplies Ltd, Nottingham, United Kingdom) with a multitip pipettor, 0.2 mL of fresh 3.3 M paraoxon in buffer containing 2 mmol/L $CaCl_2$ and 100 mmol/L Tris pH 8.0 was added to all eight wells to start the assay. As a blank, 0.210 mL of paraoxon substrate was used. Results were printed out in sequence with the *A*/min calculated as nmol min^{-1} mL^{-1}.

2.8. Measurement of blood glucose, triacylglycerols and cholesterol levels

The blood was allowed to stand for 30 min at 4 °C and then centrifuged to obtain serum. Glucose, triacylglycerols and cholesterol were measured by a medical diagnostic kit (Boehringer-Mannheim, FRG).

2.9. Statistical analysis

A one-way ANOVA with a Tukey test was used to analyze the results of all experimental groups. Results were expressed as mean ± SD. Differences were considered significant at $p<0.05$.

Table 1
Oligonucleotide sequences used to amplify PON1 mRNA.

Oligonucleotide name	Sequence 5′ to 3′	PCR product size (bp)
PON1-R	GCAGCTATATCGTTGTAGCTAG	335
PON1-F	GGACTAACTTTCTTTAGCACTG	
GAPDH-R	GGTCATCCATGACAACTTTGG	350
GAPDH-F	GTCATACCAGGAAATGAGCTTGAC	

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3. Results

3.1. Body weight of high-fat diet diabetic-mice supplemented with pomegranate juice

CD1 mice injected with streptozotocin three days later had blood glucose levels higher than uninjected mice, confirming the diabetic state (data not shown). The group 2 (diabetized animals fed with a high-fat diet) mice showed an increased body weight at the end of the experiment when compared to group 1 (control group) and group 3 (PJ supplemented group) (Fig. 1). The result suggests that the high-fat diet increased body weight, regardless of the presence of diabetes in the animals. In contrast, PJ resulted in body weights lower than the control group.

3.2. Expression of PON1 mRNA in high-fat diet diabetic-mice supplemented with pomegranate juice

As shown in Fig. 2, PON1 gene expression is increased throughout the test, regardless of treatment. However, only at the fourth month, the PJ supplemented group 3 had significant expression levels of PON1 mRNA compared to group 2 ($p<0.05$). This expression was higher throughout the test, although the expression levels did not reach those of the control group.

3.3. Activity of PON1 in high-fat diet diabetic-mice supplemented with pomegranate juice

As expected, PON1 activity was significantly decreased in groups of diabetic animals (group 2 and 3) compared to control group 1 (Fig. 3). However, supplementation with PJ (group 3) resulted in higher activity than unsupplemented (group 2) ($p<0.05$). Similar to the expression analysis in Fig. 2, PON1 activity was enhanced by PJ, but not to the levels reached by control mice.

3.4. Blood glucose, cholesterol, and triacylglycerols levels in high-fat diet diabetic-mice supplemented with pomegranate juice

As expected, the diabetic mice of groups 2 and 3 had high blood glucose levels (Fig. 4). However, supplementation with PJ in group 3 had a significant hypoglycemic effect by month 4 ($p<0.05$ compared to group 2), suggesting that long-term pomegranate supplementation can decrease blood glucose levels. In contrast, we did not find significant changes in cholesterol and triacylglycerols in groups 2 and 3 relative to group 1 (data not shown).

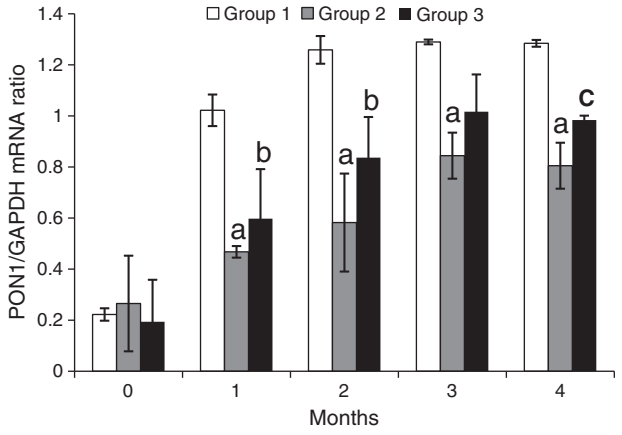

Fig. 2. Expression of PON1 mRNA in high-fat diet fed diabetic-mice supplemented with pomegranate juice. Group 1 is control (untreated). Groups 2 and 3 are diabetized. The diet of group 3 is supplemented with PJ. Significant differences among the groups are described: a = comparison between group 1 and group 2; b = comparison between group 1 and group 3; and c = comparison between group 2 and group 3.

4. Discussion

In human and murine models, PJ has been shown to exert significant anti-atherosclerotic (Aviram et al., 2000, 2002, 2008; Rosenblat et al., 2006), anti-hypertensive (Aviram et al., 2004; Aviram & Dornfeld, 2001; Sumner et al., 2005) antioxidant (Azadzoi et al., 2005; Gil et al., 2000; Mertens-Talcott, Jilma-Stohlawetz, Rios, Hingorani, & Derendorf, 2006; Rosenblat & Aviram, 2006; Seeram et al., 2008), and anti-inflammatory (de Nigris et al., 2007; Ignarro, Byrns, Sumi, de Nigris, & Napoli, 2006; Shukla, Gupta, Rasheed, Khan, & Haqqi, 2008) effects.

PON1 activity is reduced in several chronic diseases, including type 1 and 2 diabetes, and hypercholesterolemia (Ikeda et al., 2009). PON1 expression was induced by dietary polyphenolic compounds such as quercetin (present in pomegranate) (Gouédard et al., 2004), as well as by PJ polyphenols (Khateeb et al., 2010) in HuH7 hepatocytes. PON1 activity was also enhanced in atherosclerotic apolipoprotein E-deficient mice (Aviram et al., 2000; Kaplan et al., 2001) and humans (Rock et al., 2008) supplemented with PJ. In our model, diabetized mice

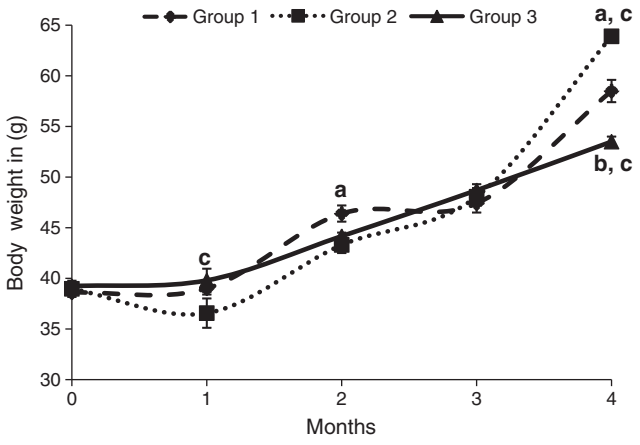

Fig. 1. Changes in body weight in CD-1 mice. Group 1 is control (untreated). Groups 2 and 3 are diabetized. The diet of group 3 is supplemented with PJ. Significant differences among the groups are described: a = comparison between group 1 and group 2; b = comparison between group 1 and group 3; and c = comparison between group 2 and group 3.

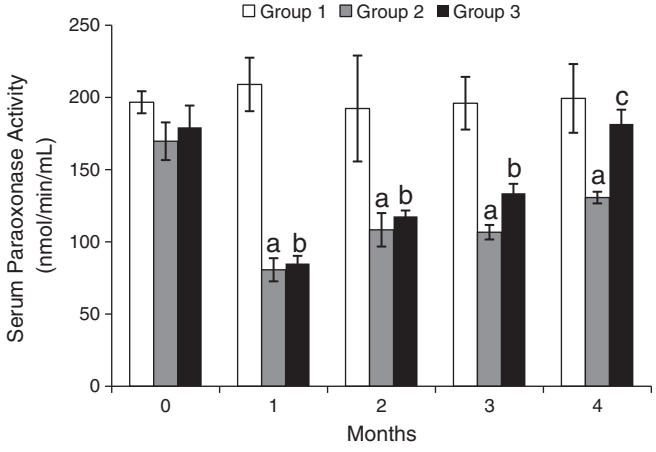

Fig. 3. Activity of PON1 in high-fat diet fed diabetic-mice supplemented with pomegranate juice. Group 1 is control (untreated). Groups 2 and 3 are diabetized. The diet of group 3 is supplemented with PJ. Significant differences among the groups are described: a = comparison between group 1 and group 2; b = comparison between group 1 and group 3; and c = comparison between group 2 and group 3.

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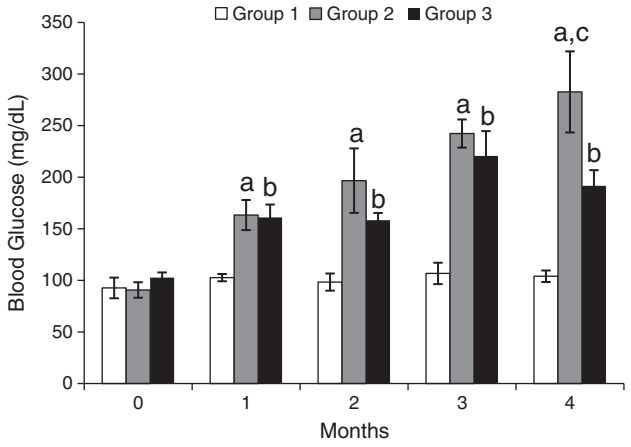

Fig. 4. Blood glucose levels in high-fat diet fed diabetic-mice supplemented with pomegranate juice. Group 1 is control (untreated). Groups 2 and 3 are diabetized. The diet of group 3 is supplemented with PJ. Significant differences among the groups are described: a = comparison between group 1 and group 2; b = comparison between group 1 and group 3; and c = comparison between group 2 and group 3.

were fed with a high-fat diet to investigate if PJ was able to induce PON1 expression and activity, even in these unfavorable metabolic conditions.

As expected, PON1 expression and activity were significantly reduced in diabetic animals fed a high-fat diet when compared to the control group. In contrast, the animals supplemented with PJ showed significantly higher levels of PON1, though not to the levels of the control group. These results demonstrate that the constituents of PJ were able to induce PON1 expression and activity, which is inactivated by oxidative stress and high-fat diets. Gouédard et al. (2004) demonstrated that dietary polyphenols such as flavone or quercetin (phenol compounds identified in fresh PJ) increased PON1 gene expression and arylesterase activity in HuH7 human hepatoma cell line. *In vivo* studies also have revealed an increase of PON1 serum activity following treatment with flavonoids (Fuhrman & Aviram, 2002). Thus, PJ polyphenols are thought to modulate the expression level of PON-1 and enhance its activity. The increase in PON1 mRNA expression and enzymatic activity, could be a possible mechanism of action of PJ in the inhibition of atherosclerosis as PON1 has been shown to protect against cardiovascular disease by: (i) preventing the formation of oxidized HDLs and low-density lipoproteins (LDLs) (Aviram et al., 1998); (ii) hydrolyzing the thiolactone form of homocysteine, which alters proteins in the arterial wall (Jakubowski, 2000); and (iii) hydrolyzing platelet-activating factor, a bioactive phospholipid which is involved in vascular disease development (Rodrigo, Mackness, Durrington, Hernandez, & Mackness, 2001).

Several studies suggest that a low-level plasma PON1 activity is associated with an increased prevalence of atherosclerosis and could be an independent risk factor for coronary events (Mackness et al., 2003). PON1-deficient mice are more susceptible to lipoprotein oxidation and atherosclerosis (Shih et al., 1996), while transgenic mice overexpressing PON1 display decreased atherosclerotic lesions (Tward et al., 2002). Supporting this, Rock et al. (2008), reported that PJ consumption by diabetic patients increased PON-1 association with high-density lipoprotein which stimulated its catalytic activities. Likewise, Khateeb et al. (2010) demonstrated that PJ polyphenols have a specific transcriptional role in hepatocyte PON1 expression upregulation, so that the anti-atherogenic characteristics of PJ polyphenols can be modulated (Khateeb et al., 2010).

Several extracts or concentrates of PJ have shown variable therapeutic benefits (Aviram et al., 2008; Mertens-Talcott et al., 2006; Seeram et al., 2008). Ground pomegranate flower extract has among the highest therapeutic effects in diabetes and atherosclerosis (Aviram et al., 2008; Jurenka, 2008); nevertheless, preparation of the extract is complex and the product is not very palatable. For that reason, we used PJ that was easily made and palatable in order to promote its routine consumption.

While PJ significantly reduced blood glucose levels at the last month of treatment, no significant effects on triacylglycerols and cholesterol levels were observed. This was particularly interesting because it appears that the sugars contained in PJ, which are similar in content to those found in other fruit juices, did not worsen blood sugar levels. Thus, constant PJ supplementation in the form tested here may exert beneficial effects on serum PON1 stability and activity and could lead to retardation of atherosclerosis development in the presence of diabetes.

Based on these findings, this study reports the induction by an easily produced PJ of PON-1 gene expression and activity in a diabetic mouse model fed with high fat. PJ constituents may induce molecular changes which result in the observed protective effects of pomegranate with respect to atherosclerosis such that a daily PJ supplementation may exert beneficial effects in patients with diabetes and atherosclerosis. And, the PON-1 gene and enzyme may serve as potential therapeutic targets for prevention of atherosclerosis and in the management of diabetes.

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