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Brief Communication

The influence of pomegranate fruit extract in comparison to regular pomegranate juice and seed oil on nitric oxide and arterial function in obese Zucker rats

Filomena de Nigris [a], Maria Luisa Balestrieri [b], Sharon Williams-Ignarro [a], Francesco P. D'Armiento [c], Carmela Fiorito [a], Louis J. Ignarro [d], Claudio Napoli [a,*]

[a] *Department of General Pathology and Excellence Research Center on Cardiovascular Diseases, Complesso S. Andrea delle Dame, Via de Crecchio 7, 1st School of Medicine, II University of Naples, Naples 80138, Italy*
[b] *Department of Chemical Biology and Physics, 1st School of Medicine, II University of Naples, Naples 80138, Italy*
[c] *Department of Human Pathology, Federico II University of Naples, Naples 80131, Italy*
[d] *Division of Anesthesiology and the Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA*

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Abstract

Metabolic syndrome includes most widely distributed clinical conditions such as obesity, hypertension, dislipidemia, and diabetes. Pomegranate fruit extract (PFE), rich in polyphenolic antioxidants, reduces the expression of oxidation-sensitive genes at the sites of perturbed shear-stress. The aim of this study was to evaluate the effect of PFE in comparison to regular pomegranate juice (PJ) and seed oil on the biological actions of nitric oxide (NO) and the arterial function in obese Zucker rats, a model of metabolic syndrome. Our results indicated that supplementation with PFE or PJ significantly decreased the expression of vascular inflammation markers, thrombospondin (TSP), and cytokine TGFβ1 ($P < 0.05$), whereas seed oil supplementation had a significant effect only on TSP-1 expression ($P < 0.05$). Plasma nitrate and nitrite (NO_x) levels were significantly increased by PFE and PJ ($P < 0.05$). Furthermore, the effect of PFE in increasing endothelial NO synthase (eNOS) expression was comparable to that of PJ. These data highlight possible clinical applications of PFE in metabolic syndrome.

Keywords: Pomegranate; Arterial function; Nitric oxide; Metabolic syndrome

Pomegranate juice (PJ) is rich in antioxidants of the polyphenolic class, which includes tannins and anthocyanins [1]. These antioxidants are more potent, on a molar basis, than many other antioxidants including vitamin C, vitamin E, coenzyme Q-10, and α-lipoic acid against low density lipoprotein (LDL) oxidation and atherosclerosis [1]. The antioxidant level in PJ was found to be higher than that in other natural juices such as blueberry, cranberry, and orange, as well as in red wine [1]. A novel pomegranate fruit extract (PFE) was recently developed [2].

Antioxidants possess numerous important biological properties including anti-inflammatory and anti-aging effects, and protection against cholesterol oxidation, atherogenesis, and Alzheimer's disease [3–5]. These antioxidant therapeutic actions are identical to many of the actions of NO [6]. Many investigators in the fields of oxidative stress, reactive oxygen species (ROS), and NO believe that many of the above actions of antioxidants are, in fact, mediated by NO [6]. The reason is that antioxidants protect NO against oxidative destruction and, thereby, enhance NO actions. ROS like superoxide anion react chemically with NO to form peroxynitrite [7]. Antioxidants react with and destroy superoxide or prevent the production of

* Corresponding author.
E-mail address: claunap@tin.it (C. Napoli).

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superoxide, thereby, protecting NO against oxidation-mediated destruction. Not only do antioxidants enhance the actions of NO, but combinations of antioxidants and NO donor agents provide markedly increased protective effects against cardiovascular diseases including hypercholesterolemia and atherosclerosis [8].

PFE, because of its robust content of polyphenolic antioxidants, is expected to enhance the biological actions of *in vivo* naturally produced NO. Previous studies established that administration of regular PJ to animals and humans afflicted with atherosclerosis produced a significant protective effect [1,9]. In addition, PJ administered to hypertensive patients caused a significant drop in blood pressure [10].

More importantly, PJ possesses potent antioxidant activity that is associated to its anti-atherogenic properties in mice [6,11]. This effect was recently extended to mice treated with the PFE [2]. Clinically, a widely distributed condition is called the ''metabolic syndrome'' which includes obesity, hypertension, dislipidemia, and diabetes [12].

The goal of this study was to determine the influence of the PFE in comparison to PJ and seed oil on NO and arterial function in an animal model of metabolic syndrome represented by the obese Zucker rat [14,15]. Moreover, the activity of thrombospondin (TSP) and cytokine TGFβ1, both increased in this model, will be evaluated as markers of vascular inflammation.

Methods

Animals

Female obese Zucker rats were used. Rats, matched for blood pressure and serum cholesterol, were divided into groups to receive different diets (1.0 g/kg body/day; estimated as plenary supplementation diet, see Ref. [16]) containing the PFE, regular PJ, or seed oil. The doses of PJ and PFE were as described in previous studies [2,11]. Concentrated PJ or PFE were diluted in water (6.25 ml of concentrated juice in 1 L of water). Seed oil was administered in the diet (1 ml/die in 1 L of water). This solution was given to the treated groups of rats, whereas only water was given to the placebo control group. The procedures and protocols of the study were in accord to our institutional guidelines and approved by the Animal Experimentation Committee of the University of Naples and of the University of California at Los Angeles (UCLA). Quality standards of laboratories at the University of Naples are in accordance with rules established from the Italian Ministry of Health and the European College of Laboratory Animal Medicine, while Laboratory of the UCLA is in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The telemetry system was used for measurements of systolic, diastolic and mean arterial pressure, heart rate and motor activity, as described [13,14]. Briefly, telemetry transmitters were implanted 1 week before the experiment into the abdominal cavity. Blood pressure of each rat was monitored every 5 min as a waveform curve for 10 s. Peaks and troughs in the blood pressure curve were detected. Systolic blood pressure, diastolic blood pressure, and heart rate sample values were calculated by the dedicated software. BPM and motor activity were calculated by using three measurements at 10, 18, and 24 AM and was monitored as changes in transmitter signal strength due to transmitter locomotion and was collected for each 5-min interval. For further evaluation, mean values were calculated for intervals of 30 min.

The rats were unrestrained and free to move within their cages at all times. The data were sampled every 5 min for 10 s for each rat. The rats were housed individually in metabolic cages. Food intake was measured and urine collected for 24 h.

Mesenteric arterial responses in vitro

Groups of six rats each fed with the same diet as in the telemetry experiment but did not undergo surgery so as not to interfere with the mesenteric artery, as described [2]. After the experiment, these rats were killed by decapitation, and 3-mm standard sections of the mesenteric artery, 3 mm distal from the artery–aorta junction, were cut. The rings were placed between stainless steel hooks and mounted in an organ bath chamber in the following physiological salt solution (pH 7.4) (mmol/L): NaCl, 119.0 mmol/L; $NaHCO_3$, 25.0 mmol/L; glucose, 11.1 mmol/L; $CaCl_2$, 1.6 mmol/L; KCl, 4.7 mmol/L; KH_2PO_4, 1.2 mmol/L; $MgSO_4$, 1.2 mmol/L. The organ bath chamber was aerated with 95% O_2 and 5% CO_2. The rings were equilibrated for 20 min at 37 °C with a resting tension of 1.0 g. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph. The relaxation concentration curves to acetylcholine (ACh) (to test endothelium-dependent relaxation) and sodium nitroprusside (for endothelium-independent relaxation) were determined [2].

Immunohistochemistry

Arterial eNOS expression was detected by immunohistochemistry (monoclonal anti-eNOS from Transduction Laboratories, Lexington, KY, USA) as described [2,11]. TSP-1 arterial immunoreactivity was stained with an anti-TSP-1 antibody (Neomarkers, clone A6.1, Fremont, CA, USA) [14]. Rabbit anti-TGFβ1 (sc-146, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used to detect tissue TGFβ1 expression [16].

Biochemical determinations

Nitrate and nitrite (NO_x) levels were measured using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA). Plasma glucose was determined enzymatically. Plasma insulin levels were determined by RIA (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or ELISA using rat insulin as standard (Linco Research, Inc, St. Charles, MI, USA).

Statistics

Statistical analysis was carried out by one-way ANOVA followed by Tukey's test. Data for multiple observations over time were analyzed by two-way ANOVA with repeated measures for overall treatment effect, and Tukey's test used for multiple pair-wise comparisons of treatment groups at different times.

Results

Cumulative vascular relaxation response

Endothelium-dependent relaxation was determined in isolated endothelium-intact mesenteric arterial rings (Fig. 1a) from Zucker rats after 5 weeks of consuming an atherogenic diet (ATH), or an atherogenic diet supplemented with regular PJ (ATH + PJ), pomegranate fruit extract (ATH + PFE), or seed oil (ATH + seed oil). Results indicated that ACh-induced relaxation responses were significantly increased by PJ and PFE supplementation ($P < 0.01$) and, to a less extent, by seed oil supplementation ($P < 0.05$). There were no significant differences

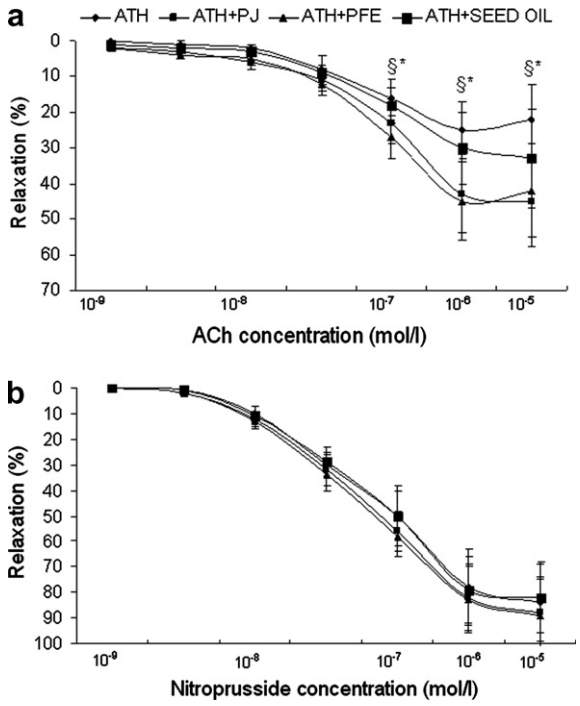

Fig. 1. Cumulative relaxation response to (a) acetylcholine (ACh) and (b) sodium nitroprusside expressed as percentage of 1 μmol/l noradrenaline-induced precontraction of isolated endothelium-intact mesenteric arterial rings from Zucker rats after 5 weeks of consuming an atherogenic diet (ATH) or ATH supplemented with regular PJ (ATH + PJ), PFE (ATH + PFE) or seed oil (ATH + seed oil). Values are means ± SEM, $n = 6$ for each group. Repeated measures ANOVA for ACh between lanes $^{*}P < 0.05$ vs. ATH, $^{\S}P < 0.01$ vs. ATH (for ATH + PFE) within lane effect $P < 0.001$.

among groups in sodium nitroprusside-induced relaxation responses (Fig. 1b). Control experiments with untreated mesenteric arterial rings showed that arterial wall tension did not change with time (data not shown).

Lipid profile, mean arterial pressure, heart rate, motor activity

Lipid profile was similar among groups. Supplementation with PFE oil had no significant effect on total cholesterol and LDL cholesterol. Seed oil supplementation had no effects on total and LDL cholesterol whereas determined an increase in triglyceride levels (Table 1). Mean arterial pressure, motor activity, and heart rate of rats fed with atherogenic diet supplemented with PFE were not significantly reduced (Table 1).

Evaluation of vascular inflammation markers, eNOS expression, and glucose/insuline levels

Immunostaining for arterial markers of vascular inflammation are increased in this model of metabolic syndrome [13,14]. Our data indicated that either supplementation with PFE and PJ significantly decreased both TSP-1 and TGFβ1 expression ($P < 0.05$) whereas seed oil supplementation had a significant effect only on TSP-1 expression ($P < 0.05$) (Fig. 2). Moreover, the effect of PFE in increasing vascular eNOS expression was comparable to that of PJ. Plasma NO_x levels were significantly increased only by PJ and PFE supplementation ($P < 0.05$) whereas seed oil supplementation had no effect (Table 2). Analysis of plasma insulin by two different sensitive methods and glucose levels revealed that all three different supplemented regimens did not significantly affect these parameters (Table 2).

Discussion

The present study illustrates that supplementation of an atherogenic diet with PFE can exert beneficial effects on vascular function and inflammation in obese Zucker rats. These rats fed with atherogenic diet had pronounced endothelial dysfunction, high blood pressure and high serum LDL cholesterol and represent a suitable model of metabolic syndrome [14]. Previous reports showed that plant sterols and minerals [14] or L-arginine [17] can reduce the effect of atherogenic diet in this animal model by lowering total and LDL cholesterol and by reducing fat mass.

The PFE, because of its high content of polyphenolic antioxidants, is expected to possess beneficial effects similar to those exerted by PJ. Administration of regular PJ to animals and humans had a significant protective effect on atherosclerosis or hypertension [1,9,10]. Chronic

Table 1
Lipid profile and mean arterial pressure and heart rate and motor activity in obese Zucker rats

	ATH	ATH + PJ	ATH + PFE	ATH + seed oil
Total chol (mmol/l)	11.5 ± 2.3	11.0 ± 2.6	10.8 ± 2.7	11.7 ± 3.0
LDL chol (mmol/l)	3.5 ± 0.6	3.4 ± 0.7	3.2 ± 0.5	3.6 ± 0.8
HDL chol (mmol/l)	0.5 ± 0.2	0.6 ± 0.3	0.6 ± 0.3	0.5 ± 0.3
Triglycerides (mmol/l)	13.4 ± 3.6	13.7 ± 4.0	13.8 ± 4.2	14.0 ± 4.2*
Daytime MAP (mmHg)	126 ± 12	118 ± 15	115 ± 18	118 ± 19
BPM (beats/min)	340 ± 25	350 ± 24	335 ± 26	346 ± 23
Motor activity (counts)	10 ± 4	11 ± 5	10 ± 5	11 ± 5
Energy intake (kJ/24 h)	31.2 ± 1.4	33.7 ± 2.0	34.5 ± 2.1	36.3 ± 2.6*
Urine volume (ml/24 h)	26 ± 8	22 ± 6	23 ± 7	25 ± 7

Rats received atherogenic diet (ATH) alone or ATH supplemented with regular PJ (ATH + PJ), PFE (ATH + PFE) or seed oil (ATH + seed oil) (means ± SD). Measurements of daytime MAP were the mean values reached during the last week of treatment. Mean BPM and motor activity were calculated by using three measurements at 10, 18, and 24 AM during the last week of the study.

* $P < 0.05$ vs. ATH diet alone.

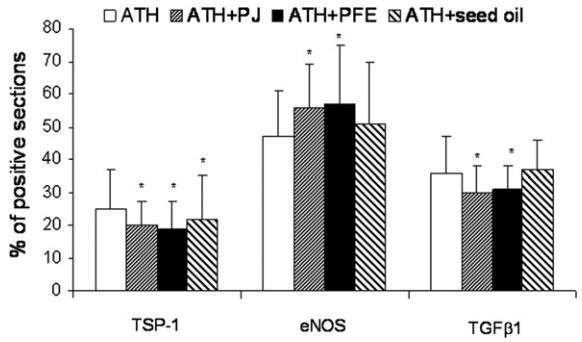

Fig. 2. Arterial eNOS, TSP-1, and TGFβ1 expression. Rats fed with atherogenic diet (ATH) or ATH supplemented with regular PJ (ATH + PJ), PFE (ATH + PFE) or seed oil (ATH + seed oil) for 5 weeks (means ± SD). $^{*}P < 0.05$ vs. ATH.

Table 2
NO_x, glucose, and insulin levels in obese Zucker rats

	ATH	ATH + PJ	ATH + PFE	ATH + seed oil
NO_x (μmol/L)	30 ± 2.1	37 ± 2.7*	40 ± 3.1*	32.4 ± 3.0
Insulin (pmol/L)	251 ± 34	246 ± 34	248 ± 36	238 ± 39
Insulin (ng/ml)	29.5 ± 7.3	30.4 ± 6.5	30.8 ± 7.2	28.7 ± 3.9
Glucose (mg/dl)	158 ± 4.6	161 ± 3.4	156 ± 3.8	155 ± 4.2

Biochemical parameters were determined in Zucker rats after 5 weeks of atherogenic diet (ATH) or ATH supplemented with regular PJ (ATH + PJ), PFE (ATH + PFE) or seed oil (ATH + seed oil) (means ± SD). Insulin levels were determined by two different methods to achieve more sensitivity.
* $P < 0.05$ vs. ATH.

administration of PJ and, recently, of PFE have been shown to reduce the proatherogenic effects induced by perturbed shear-stress [2,11]. Here, we show evidences that PFE supplementation to Zucker rats may positively influence arterial reactivity, vascular expression of eNOS and NO_x levels, most likely by both increasing NO production and preventing its degradation.

The PFE-supplementation prevented the rise of total and LDL cholesterol induced by atherogenic diet and reduced mean arterial pressure and heart rate. When we compared the effects of PFE, regular PJ, and seed oil on NO and arterial function, we found that PFE and PJ were the most effective in increasing vascular eNOS expression and plasma NO_x levels. Moreover, both PFE and PJ supplementation significantly increased the relaxation response to acetylcholine in a resistance artery *in vitro* with no significant effects on the relaxation response to nitroprusside. The TSP-1 and TGFβ1 expression, both increased in this animal model, were significantly decreased by supplementation with PFE and PJ whereas seed oil supplementation had a significant effect only on TSP-1 expression. This effect might be due to the different polyphenol bioavailability and content in seed oil compared to PFE and PJ [2,18,19]. These data, consistent with the evidence that PFE increases the biological actions of NO and ameliorate arterial function in obese Zucker rats, suggest potential clinical applications in metabolic syndrome.

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