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The effect of pomegranate extract on coronary artery atherosclerosis in SR-BI/APOE double knockout mice

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ABSTRACT

Objectives: To examine the effects of pomegranate extract on inflammation and oxidative stress and the development of spontaneous occlusive coronary artery atherosclerosis in the SR-BI/apoE double knockout mouse model of coronary heart disease.
Methods and results: SR-BI/apoE double KO mice were treated for two weeks with pomegranate extract via drinking water, beginning at three weeks of age. Treatment with pomegranate extract increased cholesterol ester content and reduced the abnormally high unesterified/esterified cholesterol ratio of VLDL-sized lipoproteins. Despite the increase in cholesterol levels associated with VLDL-sized particles, pomegranate extract treatment reduced the size of atherosclerotic plaques in the aortic sinus and reduced the proportion of coronary arteries with occlusive atherosclerotic plaques. Treatment with pomegranate extract resulted in substantial reductions in levels of oxidative stress and monocyte chemotactic protein-1 in atherosclerotic plaques in the aortic sinus and coronary arteries. In addition, treatment with pomegranate extract reduced lipid accumulation, macrophage infiltration, levels of monocyte chemotactic protein-1 and fibrosis in the myocardium, attenuated cardiac enlargement and the development of ECG abnormalities in SR-BI/apoE double KO mice.
Conclusion: Pomegranate extract reduced aortic sinus and coronary artery atherosclerosis in SR-BI/apoE dKO mice. The atheroprotective effects of pomegranate extract appear to involve reduced oxidative stress and inflammation in the vessel wall despite unaltered systemic markers of inflammation and increased lipoprotein cholesterol in these mice.

1. Introduction

Increased inflammation, dysregulated lipid metabolism and increased oxidative stress are key elements in the development of atherosclerosis [1]. Complex networks with complex interactions exist between these factors [1]. Oxidative stress may act as an initiator of the process, resulting in lipid peroxidation and increased inflammation [2], and as an important player in plaque rupture leading to atherothrombosis and myocardial infarction [3].

Multiple experimental studies and clinical trials have used anti-oxidants as therapeutic agents [4]. However, anti-oxidant therapy trials in coronary heart disease (CHD) patients have generally shown little or no benefit [5–10]. Many of these studies involved treatment with single anti-oxidants (reviewed in Ref. [4]). However, patients with CHD may be deficient in multiple antioxidants or micronutrients affecting different pathways [11]. Moreover, the properties of polyphenolic anti-oxidants have been shown to be enhanced when present in combination as in natural fruit extracts [12].

Pomegranate (*Punica granatum*) fruit is rich in polyphenolics, substances characterized by the presence of more than one phenol unit per molecule, and have been shown to have multiple beneficial effects in the cardiovascular system (reviewed in Ref. [13]). Several studies focused on the ability of different components of the fruit, including the juice, seed oil, peel, flower extract or their derivatives,

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to kill bacteria and viruses, fight vascular disease, diabetes and cancer (reviewed in Ref. [13]). Other studies however, elaborated on the antioxidant properties of pomegranate polyphenolics in vitro, ex vivo and in vivo (reviewed in Ref. [13]). Polyphenolic compounds are capable of reducing and preventing the oxidation of essential biomolecules directly by quenching reactive oxygen species whereby phenol groups accept free electrons to form relatively stable phenoxyl radicals thus terminating the oxidative chain reaction (reviewed in Ref. [14]). In addition, pomegranate polyphenolic compounds were shown to protect against oxidative stress by activating the endogenous anti-oxidant defense systems [15]. Studies in patients with carotid artery stenosis showed that the consumption of pomegranate juice for a period of three years increased serum paraoxonase-1 activity, reduced serum oxidative stress and reduced intima-media thickness [16]. Other studies demonstrated that pomegranate juice or the liquid extract of the pomegranate fruit (POMx) reduce development of aortic atherosclerosis in apolipoprotein (apo) E deficient mice [17–20]. Recently, pomegranate juice administration to mice was shown to reduce macrophage total cholesterol and triglyceride contents by inhibiting their synthesis and increasing cholesterol efflux to high density lipoprotein (HDL) [21] as well as reducing low density lipoprotein (LDL) and oxidized (ox) LDL uptake [19]. However, the effects of pomegranate extract consumption on coronary artery (CA) atherosclerosis or myocardial infarction (MI) have not been tested directly.

Despite developing spontaneous and diet accelerated atherosclerosis in a number of arteries including the aorta and branching arteries, apoE knockout (KO) mice do not reproducibly develop coronary artery atherosclerosis with high frequency [22]. In contrast, apoE KO mice that also lack the scavenger receptor, class B type I (SR-BI) spontaneously develop occlusive CA atherosclerosis, MI, cardiac enlargement and severe cardiac dysfunction [23]. SR-BI is a multiligand cell surface receptor that mediates the exchange of lipids, including cholesterol, between cells and lipoproteins and appears to play a key role in the metabolism of HDL [24]. SR-BI is expressed in a variety of tissues, with highest expression in hepatic and steroidogenic tissues, and somewhat lower levels of expression in macrophages, endothelial cells, intestinal enterocytes and many other cell types [24]. Hepatic SR-BI plays a key role in driving reverse cholesterol transport via HDL [24]. SR-BI expressed in endothelial cells appears to mediate atheroprotective HDL dependent signaling (reviewed in Ref. [25]). The CA disease and accompanying cardiac pathology in SR-BI/apoE double KO (dKO) mice exhibit features reminiscent of human CHD, suggesting that the SR-BI/apoE dKO mouse may be a useful mouse model of this disease [22,23,26,27]. These mice exhibit premature death between 5 and 8 weeks of age [23]. Inhibition of intestinal cholesterol absorption by ezetimibe, or reduction of bile acid recirculation by the small molecule, SC-435, have been shown to reduce LDL cholesterol levels, delay disease development and prolong survival of these mice [26]. Treatment of SR-BI/apoE dKO mice with probucol, an anti-oxidant drug that has been used clinically in humans [28], has also been demonstrated to reduce CA atherosclerosis, cardiac pathology and prolong the survival of these mice [27]. Probucol has anti-oxidant properties but also appears to reduce cholesterol associated with very low density lipoprotein (VLDL) sized particles and restore HDL cholesterol in SR-BI/apoE dKO mice, to levels similar to those in apoE single KO mice that do not develop occlusive CA atherosclerosis or MI [27]. The role of vascular inflammation and/or oxidative stress in the development of CA atherosclerosis and MI in SR-BI/apoE dKO mice have not, however, been explored directly.

In this study, we demonstrate that atherosclerotic plaques are absent from the aortic sinus and CAs of SR-BI/apoE dKO mice at 3 weeks of age. However, aortic and CA atherosclerosis develop by 5 weeks of age, accompanied by significant myocardial fibrosis and cardiac enlargement. ECG abnormalities were observed in the majority of these mice by 6 weeks of age. Administration of pomegranate extract in drinking water for 2 wks to SR-BI/apoE dKO mice reduced oxidative stress and inflammation in atherosclerotic vessels and in myocardial tissue, attenuated aortic sinus and CA atherosclerosis, and reduced the degree of cardiac fibrosis and enlargement. Finally, fewer SR-BI/apoE dKO mice developed ECG abnormalities by 6 weeks of age when administered pomegranate extract.

2. Materials and methods

2.1. Materials

Pomegranate liquid extract (POMx) or juice (POM Wonderful, CA, USA) was purchased from local grocery stores. All other materials were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2. Mice

All procedures involving mice were approved by the McMaster University Animal Research Ethics Board and were in accordance with the guidelines of the Canadian Council on Animal Care. C57BL/6J (Jackson Labs, Bar Harbor ME, USA) or SR-BI$^{+/-}$ apoE KO mice (mixed C57BL/6J and 129Sv genetic background, originally provided by Monty Krieger, Massachusetts Institute of Technology, Cambridge, USA) were bred and housed in micro-isolator cages at McMaster University or the TaARI animal facilities and had free access to normal chow (Teklad 18% protein rodent diet, Harlan Laboratories, Mississauga ON, Canada) and water. SR-BI/apoE dKO and apoE single KO littermates received either water alone (control group) or water containing pomegranate extract (307.5 μl/L) beginning at three weeks of age. Wild type, control untreated and pomegranate extract-treated mice were euthanized at 5 or 6 weeks of age. Additional control SR-BI/apoE dKO and apoE single KO mice were euthanized at 3 weeks of age. Male and female mice were used and data were pooled since preliminary analysis revealed no differences between males and females in the parameters measured. All pomegranate extract-treated and untreated mice were fasted from 4 to 16 h prior to euthanasia. Blood was collected by cardiac puncture and serum was prepared using serum collection tubes (Sarstedt Inc., Montreal, PQ, Canada) and frozen at −80 °C for later analysis. Mice were perfused with phosphate buffered saline containing 10 U of heparin/ml and the harvested tissues were frozen in Shandon Cyromatrix (Thermo Fisher Scientific, Ottawa, ON, Canada) and stored at −80 °C for further analysis.

2.3. ECG analysis

Wild type C57BL/6J, or untreated or pomegranate extract supplemented SR-BI/apoE dKO mice or littermate control apoE KO mice aged 5 or 6 weeks, were anesthetized with isoflurane gas (0.8% O_2, induction with 5%, maintenance with 2% isofluorane). ECG's were determined using one needle electrodes in each of the forearms and left hind limb. ECG's were recorded using a Dataq Model DI-205 instrument and Windaq software (DATAQ Instruments, Inc. Akron, Ohio, USA).

2.4. Histology

Cryosections (10 μm) were stained for lipids with oil red O [23], for oxidative stress markers with dihydroethidium (DHE) [29] or for