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High intrinsic aerobic capacity and pomegranate juice are protective against macrophage atherogenicity: Studies in high vs. low capacity runner (HCR vs. LCR) rats

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Abstract

We studied the rat model system of high vs. low capacity runner (HCR vs. LCR) rats to question the atherogenic properties (oxidative stress, triglycerides and cholesterol metabolism) in the rat macrophages, serum, liver, and heart. Half of the LCR or HCR rats consumed pomegranate juice (PJ, 15μmol of gallic acid equivalents / rat / day) for 3 weeks, and were compared to placebo – treated rats. At the end of the study blood samples, peritoneal macrophages (RPM), livers, and hearts were harvested from the rats. RPM harvested from HCR vs. LCR demonstrated reduced cellular oxidation (21%), increased paraoxonase2 (PON2) activity (28%) and decreased triglycerides mass (44%). Macrophage uptake rates of FITC- labeled LDL or oxidized LDL were significantly lower, by 37% or by 18% respectively, in HCR vs. LCR RPM. PJ consumption significantly decreased all the above atherogenic parameters with more substantial beneficial effects observed in the LCR vs. the HCR rats (~80% vs. ~40% improvement, respectively). Similar hypo-triglyceridemic pattern was noted in serum from HCR vs. LCR. In contrast to the above results, liver oxidation and triglycerides mass were both minimally increased in HCR vs. LCR rats by 31% and 28% respectively. In the heart, lipids content was very low and interestingly, an absence of any significant oxidative stress, along with modest triglyceride accumulation.

We conclude that HCR vs. LCR rats demonstrate reduced atherogenicity, mostly in their macrophages. PJ exerts a further improvement, mostly in macrophages from LCR rats.

Abstract

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Keywords

HCR rats; LCR rats; Rat Peritoneal Macrophages; Oxidative Stress; liver; heart; Pomegranate Juice

1. Introduction

Cardiovascular patients are characterized by high levels of lipids (cholesterol, triglycerides) and increased oxidative stress in their blood and tissues [1-4]. Life style, including exercise, is an important feature for cardiovascular protection and according to the *aerobic hypothesis*, variations in capacity for oxygen metabolism is an important determinant of heart and blood vessels health, and for attenuation of atherosclerosis development [5]. Macrophages play a major role in the pathogenesis of atherosclerosis as they can take up oxidized LDL at enhanced rate, leading to cholesterol and oxidized lipids accumulation and foam cell formation, the hallmark of early atherogenesis [6, 7]. Arterial macrophage foam

cells are characterized by increased cellular levels of oxidative stress, triglycerides, and cholesterol [8]. A similar pattern is shared by blood monocytes, the precursors of macrophages. In contrast, to arterial macrophages, the liver was shown to accumulate lipids and oxidized lipids by their removal from the circulation [9] which decreases their atherogenicity in the blood. As for the heart, it is known to accumulate only small amount of lipids, mainly for the purpose of energy metabolism.

Protection from oxidative stress may be achieved by endogenous antioxidants, including the paraoxonases (PON) enzymes [10] and exogenous antioxidants, mainly from dietary origin. The most nutritionally potent antioxidant and anti-atherosclerotic agent is the pomegranate juice (PJ) which is rich in ellagitannin compounds, such as punicalagin [11]. Previous studies have demonstrated that PJ consumption by healthy volunteers significantly decreased their LDL, HDL, macrophages, and atherosclerotic lesion oxidative status [12]. Moreover, consumption of PJ by patients with carotid artery stenosis significantly increased serum PON1 activity, decreased serum oxidative stress, lesion oxidative stress, and lesion cholesterol content, and even attenuated atherosclerotic plaque development in the carotid arteries [13]. Similarly, daily consumption of PJ improved stress-induced myocardial ischemia in patients with coronary artery disease [14].

The strong clinical association between low aerobic capacity and all-cause morbidity and mortality [15] led us to formulate the idea that variation in capacity for aerobic metabolism is a central mechanistic determinant of the divide between complex diseases and health which we termed the *aerobic hypothesis* [16]. By the early 1990s we proposed that the aerobic hypothesis could be tested by applying two-way artificial selection to develop rat lines of Low Capacity Runner (LCR) and High Capacity Runner (HCR) rats [17]. That is, if the aerobic hypothesis is true, we expected that susceptibility to disease would segregate with LCR rats, and resistance to disease would segregate in HCR rats, and simultaneously provide uniquely contrasting models for study. Consistent with the hypothesis, the LCR harbor and the HCR are relatively free from numerous disease risks including metabolic syndrome, hepatic steatosis, body weight gain on a high fat diet, intra-cerebral hemorrhage, inducible cancer, unsuccessful aging, and diminished longevity [18]. These contrasting rat lines are currently at 35 generations of selection and differ by over 10-fold for treadmill running capacity [19]. Conceptually, exercise capacity can be considered to derive from two parts: 1) an intrinsic component that operates in the sedentary (non-trained state) and 2) an extrinsic component that follows as an adaptive response that accrues from all activity above the sedentary state [20]. The LCR/HCR model system mostly, but not purely, represents intrinsic capacity.

Here we used this rat model system to evaluate the possible role of intrinsic aerobic running capacity in atherogenesis, in the absence or presence of the antioxidant agent – PJ. Our results clearly demonstrate reduced atherogenicity in HCR vs. LCR rats, mostly in their macrophages. In addition, PJ retrieves atherogenesis in both groups, but more strongly in LCR compared to HCR rats.

2. Experimental Procedures

2.1 Materials

Fluorescein-isothiocyanate (FITC), 2', 7'-dichlorofluorescein diacetate (DCFH-DA), dihydrocoumarin (DHC), phenyl acetate, and thiobarbituric acid, were all purchased from Sigma-Aldrich (St. Louis, MO, USA). BBL™ thioglycolate medium brewer modified was purchased from Becton, Dickinson and Company Sparks (MD, USA). Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, nystatin, L-glutamine, and sodium pyruvate were all purchased from Biological Industries (Beth Haemek, Israel). PJ was obtained from PomWonderful, Los Angeles, CA, USA, PJ concentrate was diluted x5 with double distilled water (DDW) to get the single strength PJ.

2.2 The LCR/HCR rat model system

The development of LCR and HCR rats has been described previously [17]. Briefly, starting in 1996, two-way artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity. The selection criterion was the distance run to exhaustion on a graded treadmill test performed on Monday, Wednesday, and Friday at 11 weeks of age. The best run of the three trials was considered the trial most closely associated with the heritable component of endurance running capacity. The founder population was the genetically heterogeneous N: NIH out crossed stock of rats that we obtained from Carl Hansen [21]. The 13 lowest and 13 highest running capacity rats of each sex were selected from the founder population and randomly paired for mating. At each subsequent generation, within-family selection from 13 mating pairs was practiced for each line: this number of families maintains a relatively low coefficient of inbreeding (<0.01/generation), and maximizes the retention of genetic variation. Run-tested rats from generation 34 were shipped via overnight flight from the University of Michigan to Technion- Israel Institute of Technology for further study.

The study was conducted on male LCR (weight 405±30 gr) and HCR (weight 332±25 gr) rats. When tested at 11 weeks of age the LCR ran to exhaustion at 157±28 m and the HCR exhausted at 2103±235 m. The rats were 5 months old at the time of study and were on a chow diet. Ten LCR rats and nine HCR were included in the study. Four LCR rats and four HCR rats consumed for 3 weeks, in their drinking water, 15µmol of gallic acid equivalents (GAE)/ rat/day of PJ, and compared to placebo – treated rats. At the end of the study the rats were anesthetized with isoflurane, injected intraperitoneal (IP) with thioglycolate to induce inflammatory reaction and the differentiation of blood monocytes into macrophages in the peritoneum. Four days post-injection blood samples, peritoneal macrophages, livers, and hearts were harvested from the rats. This protocol was approved by the Committee for the Supervision of Animal Experiments and complied with the Guidance for Care and Use of Laboratory Animals at the Technion-Israel Institute of Technology, Haifa, Israel.

2.3 Lipoprotein isolation

LDL and HDL were isolated from fresh plasma derived from healthy normolipidemic volunteers (Rambam Hospital Helsinki Committee number 30572-10 - RBM), by

discontinuous density gradient ultracentrifugation [22]. The LDL was separated at $d=1.063\text{g/mL}$, and the HDL at $d=1.210\text{g/mL}$. Both lipoproteins were dialyzed against 150 mmol/L NaCl , $1\text{ mmol/L Na}_2\text{EDTA}$ (pH 7.4) at 4°C , and then sterilized by filtration ($0.45\mu\text{M}$), kept under nitrogen in the dark at 4°C , and used within 2 weeks. The LDL and HDL protein concentration was determined by the Lowry method [23].

2.3.1 Copper ion-induced LDL oxidation—Prior to oxidation, the LDL was dialyzed against EDTA-free, PBS solution, pH 7.4, at 4°C . LDL (1mg of protein/ mL) was incubated with $5\mu\text{mol/L}$ of CuSO_4 for 20 hours at 37°C . At the end of the incubation, the extent of LDL oxidation was determined by measuring the generated amount of thiobarbituric acid reactive substances (TBARS) [24] and the amount of lipid peroxides [25].

2.4 Serum analyzes

2.4.1 Serum lipids and glucose concentrations—Serum cholesterol, triglyceride and glucose concentrations were determined using commercially available diagnostic kits.

2.4.2 Serum paraoxonase 1 (PON1) arylesterase activity—Arylesterase activity was measured using phenyl acetate as the substrate [26]. Initial rates of hydrolysis were determined spectrophotometric ally at 270 nm . The assay mixture included $5\mu\text{L}$ serum (diluted 1:10), 1.0 mmol/L phenyl acetate, and 1 mmol/L CaCl_2 in $50\text{ mmol/L Tris HCL}$, pH 8.0. The E_{270} for the reaction is $1,310\text{ M}^{-1}\text{ cm}^{-1}$. One unit of arylesterase activity is equal to $1\mu\text{mol}$ of phenyl acetate hydrolyzed/ min/mL .

2.4.3 Serum lipid peroxidation—Serum samples were diluted $\times 4$ in PBS, and were incubated with or without 100mol/L of 2,2'-azobis, 2-amidinopropane hydrochloride (AAPH, Wako, Japan) for 2h at 37°C [27]. The extent of lipid peroxidation was measured by the TBARS assay [24].

2.5 Rat Peritoneal Macrophages (RPM) analyses

2.5.1 RPM harvesting—RPM were harvested from the peritoneal fluid of the rats, 4 days after IP injection into each rat of 10mL of aged thioglycolate (40g/L) in saline. The cells ($20\text{--}30\times 10^6/\text{rat}$) were washed and centrifuged three times with PBS at $1000\times g$ for 10 min, then re-suspended at 10^9 cells/L in DMEM containing 5% FCS, $1\times 10^5\text{ units penicillin/L}$, $100\text{mg streptomycin/L}$ and 2mmol/L glutamine . The dishes were incubated in a humidified incubator ($5\%\text{CO}_2$, $95\%\text{ air}$) for 2h, washed with DMEM to remove non adherent cells, and the analyses to measure cellular oxidative stress were immediately performed.

2.5.2 RPM cholesterol mass—Lipids from RPM (2×10^6) were extracted with hexane: isopropanol (3:2, v: v) and the hexane phase was evaporated under nitrogen. The amount of cellular total cholesterol was determined using a commercial kit (CHOL, Roche Diagnostics GmbH, Mannheim, Germany). The remaining cells in the dish were dissolved in 0.1 mol/L NaOH , and an aliquot was taken for measurement of cellular protein by the Lowry assay [23]. Results are expressed as $\mu\text{g/mg}$ cell protein.

2.5.2 RPM triglyceride mass—Lipids from RPM (2×10^6 cells) were extracted with hexane: isopropanol (3; 2, v: v). The hexane phase was evaporated under nitrogen, and the amount of triglyceride was then determined, after the addition of 50 μ L DMSO, using a triglyceride assay commercial kit (Sigma Co., Cat no. TR0100). The remaining cells in the dish were dissolved in 0.1 mol/L NaOH, and an aliquot was taken for measurement of cellular protein, by the Lowry assay [23]. Results are expressed as μ g/mg cell protein.

2.5.3 RPM uptake of FITC-labeled LDL or Ox-LDL—LDL or Ox-LDL were conjugated to FITC for cellular lipoprotein uptake studies [28]. RPM were incubated at 37°C for 3h with FITC-conjugated LDL at a final concentration of 25 μ g of protein/mL. The uptake of the lipoprotein was determined by flow cytometry. Measurements of cellular fluorescence determined by FACS were done at 510 nm to 540 nm after excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was measured in terms of mean fluorescence intensity (MFI).

2.5.4 Cholesterol efflux from RPM—RPM were incubated with [3 H]-labeled cholesterol (2 μ Ci /mL) in DMEM with no addition for 1h at 37°C, followed by cell wash in ice-cold PBS ($\times 3$), and a further incubation in the absence [basal cholesterol efflux, non-specific loss of cholesterol from the cells to the DMEM media with no addition], or in the presence of 100 μ g of HDL protein/mL for 3h at 37°C in DMEM medium [29]. Cellular and medium [3 H]-labels were quantified, and basal or HDL-mediated cholesterol efflux were calculated as the ratio of [3 H]-label in the medium / ([3 H]-label in the medium+ [3 H]- label in the cells). Net HDL-mediated cholesterol efflux from macrophages data were corrected by subtraction of the basal, non-specific loss of cholesterol from the cells to the media from the whole HDL – mediated cholesterol efflux data.

2.5.5 RPM cholesterol biosynthesis rate—Cellular cholesterol biosynthesis was assayed after incubation of macrophages (2×10^6 /well) for 3 hours of incubation at 37°C with [3 H]-acetate (3.3 μ Ci/mL, 30) . Cellular lipids were extracted with hexane: isopropanol (3:2, v: v), and the upper phase was dried under nitrogen. The lipids were then separated by thin layer chromatography (TLC) on silica gel 60 F₂₅₄ plates, 20 $\times$ 20 cm (Merck; Darmstadt, Germany) and developed in hexane: ether: acetic acid (130:30:1.5, v:v:v). Unesterified cholesterol spots were visualized by iodine vapor (by using an appropriate standard for identification) and counted in a β -counter. Results are expressed as cpm/mg cell protein

2.5.6 RPM oxidative status-DCFH assay—RPM were washed ($\times 1$) with PBS and further incubated with 10 μ mol/L of DCFHDA, for 30 minutes at 37°C [31]. The cells were washed ($\times 2$) with PBS, and then the adherent cells were detached by gentle scraping. Measurements of cellular fluorescence determined by FACS were performed at 510 nm to 540 nm after excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was measured in terms of MFI.

2.5.7 RPM PON2 lactonase activity—Lactonase activity was measured in intact cells using DHC as a substrate. DHC (1mmol/L) was added to the cells in 1mL of 1 mmol/L CaCl₂ in 50 mmol/L of Tris HCL, pH 8.0. One unit of lactonase activity = 1 μ mol of DHC hydrolyzed/min. DHC was incubated under similar conditions in a cell-free system in order

to measure non enzymatic hydrolysis. These values were subtracted from the values observed with the cells. The remaining cells in the dish were dissolved in 0.1 mol/L NaOH, and aliquots were taken for measurement of cellular protein by the Lowry assay [23]. Results are given as units/mg cell protein.

2.6 Tissues (Liver, Heart) analyses

The rat livers and hearts were rinsed with PBS and homogenized in 2 mL of PBS on ice. After centrifugation at 1000×g, the supernatant was collected and analyzed for protein concentration by the Lowry method [23].

2.6.1 Tissue Content of lipid peroxides—Lipids were extracted from the tissues' homogenate (100µL) with hexane: isopropanol (3; 2, v: v). The hexane phase was evaporated under nitrogen, and the amount of lipid peroxides was determined by the El-Saadani method [25]. Results are expressed as nmol peroxides /mg protein.

2.6.2 Tissue cholesterol content—Lipids were extracted from the tissues' homogenate (100µL) with hexane: isopropanol (3; 2, v: v). The hexane phase was evaporated under nitrogen, and the amount of cholesterol was determined using a commercial kit (CHOL, Roche Diagnostics GmbH, Mannheim, Germany). Results are expressed as µg/mg protein.

2.6.3 Tissue triglyceride content—Lipids were extracted from the tissues' homogenate (100µL) with hexane: isopropanol (3; 2, v: v). The hexane phase was evaporated under nitrogen, and the amount of triglyceride was determined (after addition of 50µL DMSO) using a triglyceride assay commercial kit (Sigma, Cat no. TR0100). Results are expressed as µg/mg protein.

2.6.4 Tissue PON lactonase activity—The assay was performed in 96 well UV plates, in a total volume of 200µL per well. Lactonase activity was measured using DHC as the substrate. Initial rates of hydrolysis were determined spectrophotometric ally at 270nm, for 10 minutes (every 15 seconds). The assay mixture included 1mmol/L CaCl₂ in 50 mmol/L Tris HCl, pH 8.0, containing 3µL of heart homogenate, or 3µL of 1×10 diluted liver homogenate and 1mmol/L DHC. Nonenzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. One unit of lactonase activity equals 1µmol of DHC hydrolyzed/min/mL. Activity is expressed as units/mg protein.

2.7 Statistics

Each individual experiment measure was replicated 3 times. Statistical analyses used the Students't test for comparing differences between the 2 groups, and 1-way ANOVA, followed by the Student- Newman- Keuls test for comparing differences between multiple groups. Results are given as mean ±SD.

3. Results

3.1 Atherogenic properties of RPM from HCR vs. LCR rats: effect of PJ consumption

Macrophage oxidative stress, as measured by ROS formation, was significantly ($p<0.05$) decreased, by 21%, in HCR vs. LCR RPM (Fig. 1A and Fig. 1B). In addition, the macrophage antioxidant paraoxonase 2 (PON2) lactonase activity was significantly increased by 28% in HCR vs. LCR RPM (0.855 ± 0.085 vs. 0.665 ± 0.060 units/mg cell protein, respectively). Consumption of PJ, a most potent antioxidant resulted in a significant decrement in RPM oxidative stress in both groups, but whereas in HCR RPM, it was reduced by only 44%, in LCR RPM it was decreased by as much as 81% (Fig. 1A and Fig. 1B). Macrophage cholesterol content was similar in both groups of rat RPMs (Fig. 1C and Fig. 1D). PJ consumption decreased RPM cholesterol mass by 47% in HCR RPM, and by 44% in LCR RPM (Fig. 1C and Fig. 1D). Finally, we measured RPM triglyceride content, and found that it was increased by 37% in LCR vs HCR (Fig. 1E and Fig. 1F). PJ consumption attenuated macrophage triglyceride content in LCR (by 45%), but had no significant effect in HCR (Fig. 1E and Fig. 1F). We next analyzed the extent of macrophage cholesterol fluxes and cholesterol biosynthesis rate (Fig. 2). Cellular uptake of FITC- labeled LDL was significantly decreased by 37% in HCR vs. LCR RPM (Fig. 2A). PJ consumption significantly decreased the extent of LDL uptake by the cells by 44% in HCR RPM, and by 48% in LCR RPM (Fig. 2A). Similarly, the uptake of Ox-LDL by HCR vs. LCR RPM was decreased by 18% (Fig. 2B). Upon PJ consumption, only a slight (6%) decrease was noted in Ox-LDL uptake by HCR RPM, whereas in LCR RPM it was significantly reduced by 29% (Fig. 2B). Macrophage cholesterol biosynthesis rate was increased by 33% in HCR vs. LCR RPM (Fig. 2C). After PJ consumption, a significant decrement in the cells ability to synthesize cholesterol, by 77% or by 61%, was noted in HCR RPM or in LCR RPM, respectively (Fig. 2C). Finally, we determined the extent of HDL – mediated cholesterol efflux from the cells and found out that it was similar in HCR vs. LCR RPM ($29.8\pm3.3\%$ vs. $28.3\pm2.6\%$, respectively).

3.2 Atherogenic properties of Serum from HCR vs. LCR rats: effect of PJ consumption

Serum liver or kidney function tests (AST or creatinine respectively) were similar in both rat group, and PJ consumption had no significant effect (Table 1). Serum glucose concentrations were decreased by 16% in the HCR vs. LCR rats and PJ consumption had no significant effect on serum glucose level in the HCR rats, but it decreased it in the LCR mice by 19% (Table 1). Similarly, serum triglycerides level was somewhat lower in the HCR vs. LCR rats and PJ consumption increased serum triglyceride level in the HCR rats by 26%, but decreased it in the LCR rats by 18% (Table 1). Serum cholesterol levels were similar in both rat groups, and PJ consumption had no significant effect. PON1 arylesterase activity was lower by 19% in the HCR vs. LCR rats, and PJ consumption had no significant effect in both groups (Table 1). Finally, the susceptibility of the rats serum to oxidation by the free radical generator AAPH as measured by the TBARS and lipid peroxides assay were both similar in both rat groups, and there was no significant effect of PJ consumption (Table 1).

3.3 Lipid and oxidized lipids levels and PON activity in tissues (Liver, Heart) from HCR vs. LCR rats: effect of PJ consumption

3.3.1 Liver—Both groups of rats had similar liver weight, i.e. 11.3 ± 2.7 in the LCR rats vs. 11.2 ± 1.9 grams in the HCR rats. PJ consumption did not significantly affect the liver weight in either LCR or HCR rats and it was 11.6 ± 1.8 vs. 11.8 ± 1.2 grams, respectively.

In the livers from HCR vs. LCR rats the lipid peroxides content was higher by 31% (Fig. 3A). PJ consumption increased the lipid peroxides content in the HCR rats' liver by 91%, and in the LCR liver only by 45% (Fig. 3A). PON lactonase activity was similar in both rat groups, and PJ consumption had no significant effect (Fig. 3B). Liver cholesterol content was lower by 6% in HCR vs. LCR rat (Fig. 3C). PJ consumption significantly increased the cholesterol content in HCR rats liver by 49%, whereas in the LCR rats liver it was increased by only 12% (Fig. 3C). The liver triglyceride content was significantly higher by 28% in HCR vs. LCR rats (Fig. 3D). PJ consumption increased the aortic triglyceride content in both rat groups with an increment by 23% in HCR rats and by 6% in LCR rats (Fig. 3D).

3.3.2 Heart—Both groups of rats had similar heart weight, 1.00 ± 0.11 in the LCR rats vs. 1.00 ± 0.13 grams in the HCR rats. PJ consumption did not significantly affect the heart weight in either group and it was 1.20 ± 0.18 in LCR and 1.00 ± 0.14 gram in HCR.

Rat hearts contain small amount of lipids in comparison to rat livers or macrophages. A most interesting finding in the rat's heart was that no measurable lipid peroxides could be determined in hearts harvested from either rat group. PON lactonase activity was similar in the hearts from both rat groups, and PJ consumption had no significant effects on PON2 (Fig. 4A). Cholesterol content was increased by 43% in hearts from HCR vs. LCR rats (Fig. 4B), and PJ consumption increased the heart cholesterol mass only in the LCR rats, by 27% (Fig. 4B). Triglyceride mass was increased by 64% in hearts from HCR vs. LCR rats (Fig. 4C). PJ consumption increased the heart triglycerides content in both rat groups (Fig. 4C).

4. Discussion

The present study demonstrates, for the first time, increased anti-atherogenic properties of macrophages harvested from high vs. low capacity runners (HCR vs. LCR) rats as determined by a reduction in macrophage oxidative stress, in cellular triglyceride content, and in the extent of LDL or Ox-LDL cellular uptake. In addition, in the HCR vs. LCR rats there was a substantial increment in macrophage PON2 activity. PJ consumption by both rat groups further resulted in improvement in macrophage atherogenicity, and these effects were more pronounced in the more atherogenic animals – the LCR rats. However, no similar effects to those observed in macrophages could be found in the rats' liver or heart. As the liver is responsible for the removal of excess circulatory lipids [5, 32, 33], HCR rats' liver could be more active than LCR rats' liver in this respect, and this represents an anti-atherosclerotic phenomenon. Consistent with this hypothesis, recent work reveals that HCR have a greater resistance for dietary-induced hepatic steatosis, which is associated with a higher energy expenditure and increased whole body and hepatic mitochondrial lipid oxidation relative to LCR rats [34].

The concentration of PJ used in the current study (15µmol GAE/rat/day) was chosen based on our previous studies with apolipoprotein E knockout atherosclerotic mice [12], and was adjusted to the rat weight. This concentration is similar to that which is reached after the intake of a “normal” food containing ellagitannins, and is in the nanomolar (nM) range [35].

We have measured macrophage PON2 lactonase activity and PON tissue lactonase activity since healthy liver and muscle contain measurable amounts of PON1, while is absent in macrophages. PON1, just like PON2 also hydrolyzes dihydrocoumarin [36], and thus lactonase activity in tissues cannot be exclusively attributed only to PON2. The LCR vs. HCR rats demonstrated higher body weight, along with increased serum triglyceride and glucose levels. Serum PON1 activity however was significantly higher in the LCR vs. HCR rats. In contrast, in human runner's serum PON1 activity was slightly increased [37]. Both serum and peritoneal macrophages originated from the blood, and although we studied peritoneal macrophages and not arterial cells (which can be harvested only to a limited extent), their biological characteristics are known to be similar [8]. The results obtained in the heart showed very low lipids content, thus the changes in the tissue quantity of lipids are rather small. The most impressive effects of PJ consumption on reducing macrophage oxidative stress and lipids were shown in both rat groups, but it was much more potent in macrophages from LCR vs. HCR, probably due to the increased levels of lipids and oxidation which were shown in the present study in the LCR vs. HCR rats. In searching for the selective beneficial characteristics of PJ in LCR vs. HCR rats, we further analyzed the above hypolipidemic effect of PJ on macrophage lipids fluxes and observed a significant reduction in cellular uptake of LDL and Ox-LDL, as well as, a decrement in macrophage cholesterol biosynthesis rates, but with no significant effect on HDL –mediated cholesterol efflux from macrophages. Similar effects were observed in-vitro using a macrophage cell line [38, 39]. The stimulatory effect of PJ consumption on liver and heart lipids content, unlike the inhibitory effect of PJ on macrophage lipids mass, could be related to the liver lipids removal and to the heart energy requirements. The beneficial effects of PJ consumption in serum or macrophages could be related to the potent antioxidant punicalagin and/or also to phytosterols present in PJ [40]. An important issue regarding the macrophage model system, as related to the beneficial anti-atherogenic effects observed in HCR vs. LCR, as well as that of PJ consumption, could be also related to the fact that whereas macrophages represent an homogenic cell population, the liver and also the heart are composed of several different cell types with different properties. Still, macrophages represent an appropriate cell line model system for atherogenesis research as affected by the environment in relation to fitness and diet. Although the number of rats we used was small, based on studies like Lessard et al. [20], the calculated sample size was estimated to be appropriate for the current research.

In conclusion, HCR vs. LCR rats demonstrate reduced atherogenicity in their macrophages. PJ contributes to a further anti-atherogenic pattern in both rat groups, but mainly in LCR rats. Taken together, the current study demonstrates that intrinsic aerobic capacity along with nutritional antioxidant consumption (Pomegranate) provide health benefits against major atherogenesis mechanisms of action (oxidative stress and lipids metabolism) and their consequent cardiovascular events.

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Abbreviations

HCR	high capacity runner
LCR	low capacity runner
RPM	rat peritoneal macrophages
HDL	high density lipoprotein
LDL	low density lipoprotein
Ox-LDL	oxidized LDL
PON1	paraoxonase 1
PON2	paraoxonase 2
PJ	pomegranate juice
GAE	gallic acid equivalents
DCFH	2', 7'-dichlorofluorescein diacetate
DHC	dihydrocoumarin
AAPH	2,2'-azobis, 2-amidinopropane hydrochloride
TBARS	thiobarbituric acid reactive substances
IP	intraperitoneal
PBS	phosphate-buffered saline
DMEM	dulbecco's modified eagle's medium
FCS	fetal calf serum
MFI	mean fluorescence intensity

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Fig.1.

Oxidative stress and lipids content in RPM harvested from HCR vs. LCR rats: effect of PJ consumption. The LCR rats or HCR rats consumed for 3 weeks either water (Placebo), or PJ (15µmol GAE/rat/day). At the end of the study, RPM were harvested from the rats. The following analyses were performed: (A&B) RPM ROS formation- by the DCFH assay, (C&D) RPM cholesterol content, and (E&F) RPM triglyceride content. Results are expressed as mean ±SD for each group of rats.*p< 0.05 vs. LCR placebo, #p< 0.01 after PJ vs. Placebo.

Fig. 2.

Cholesterol fluxes and metabolism in RPM from HCR vs. LCR rats: effect of PJ consumption. The LCR rats or HCR rats consumed for 3 weeks either water, (Placebo), or PJ (15 μ mol GAE/rat/day). At the end of the study, RPM were harvested from the rats. The following analyses were performed: (A) RPM uptake of FITC-labeled LDL (B) RPM uptake of FITC-labeled Ox-LDL, and (C) RPM cholesterol biosynthesis rate. Results are expressed as mean $\pm$ SD for each group of rats. ** $p < 0.01$ vs. LCR Placebo, # $p < 0.01$ after PJ vs. Placebo.

Fig. 3.

Oxidative stress and lipids mass in livers from HCR vs. LCR rats: effect of PJ consumption. The LCR rats or HCR rats consumed for 3 weeks either water, (Placebo), or PJ (15 μ mol GAE/rat/day). At the end of the study, livers were harvested from the rats. The following analyses were performed: (A) Liver lipid peroxides levels (B) Liver PON lactonase activity, (C) Liver cholesterol content, and (D) Liver triglyceride content. Results are expressed as mean $\pm$ SD for each group of rats. * $p < 0.05$ vs. LCR Placebo, # $p < 0.01$ after PJ vs. Placebo.

Fig. 4.

Oxidative stress and lipids in hearts from HCR vs. LCR rats: effect of PJ consumption. The LCR rats or HCR rats consumed for 3 weeks either water, (Placebo), or PJ (15µmol GAE/rat/day). At the end of the study, hearts were harvested from the rats. The following analyses were performed: (A) Heart PON lactonase activity, (B) Heart cholesterol content, and (C) Heart triglyceride content. Results are expressed as mean $\pm$ SD for each group of rats. ^{*}p< 0.05 vs. LCR Placebo, [#]p< 0.01 after PJ vs. Placebo.

Table 1

Serum biochemical, lipids, and oxidative stress parameters in HCR vs. LCR rats: effect of pomegranate Juice (PJ) consumption

Parameter	LCR Rats		HCR Rats	
	Placebo	+PJ	Placebo	+PJ
AST (units/L)	128±9	125±8	127±21	124±5
Creatinine (units/L)	0.34±0.02	0.50±0.04	0.40±0.0	0.50±0.04
Glucose (mg/dL)	182±13	147±5	153±8 *	162±19
Cholesterol (mg/dL)	82±15	84±14	79±5	80±7
Triglycerides (mg/dL)	102±13	84±24	76±8 *	96±9
PON1 Arylesterase Activity (units/mL)	258±18	245±14	208±9 *	215±16
AAPH-Induced Lipid Peroxidation TBARS (nmol/ml)	17.4±0.4	17.1±1.6	17.4±0.8	17.3±1.3
AAPH-Induced Lipid Peroxidation Lipid Peroxides (nmol/ml)	699±35	726±19	760±13 *	768±17

Results are expressed as mean ±SD, LCR rats Placebo - n=6, HCR rats Placebo - n=5, LCR rats PJ n=4, HCR rats PJ n=4

* p<0.05 (vs. LCR Placebo)