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Journal of Toxicology and Environmental Health, Part A
Current Issues

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: http://www.tandfonline.com/loi/uteh20

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To cite this article: Roger L. dos Santos, Lais O. Dellacqua, Nathalie T. B. Delgado, Wender N. Rouver, Priscila L. Podratz, Leandro C. F. Lima, Mariela P. C. Piccin, Silvana S. Meyrelles, Helder Mauad, Jones B. Graceli & Margareth R. Moyses (2016): Pomegranate peel extract attenuates oxidative stress by decreasing coronary angiotensin-converting enzyme (ACE) activity in hypertensive female rats, Journal of Toxicology and Environmental Health, Part A, DOI: 10.1080/15287394.2016.1213690

To link to this article: http://dx.doi.org/10.1080/15287394.2016.1213690

JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH, PART A
http://dx.doi.org/10.1080/15287394.2016.1213690

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Pomegranate peel extract attenuates oxidative stress by decreasing coronary angiotensin-converting enzyme (ACE) activity in hypertensive female rats

Roger L. dos Santos[a], Lais O. Dellacqua[a], Nathalie T. B. Delgado[a], Wender N. Rouver[a], Priscila L. Podratz[a], Leandro C. F. Lima[b], Mariela P. C. Piccin[c], Silvana S. Meyrelles[a], Helder Mauad[a], Jones B. Graceli[d], and Margareth R. Moyses[a]

[a]Department of Physiological Sciences, Federal University of Espirito Santo, Vitoria, Espirito Santo, Brazil; [b]Department of Physiology and Biophysics, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; [c]Department of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil; [d]Department of Morphology, Federal University of Espirito Santo, Vitoria, Espirito Santo, Brazil

ABSTRACT

Based on the antioxidant properties of pomegranate, this study was designed to investigate the effects of pomegranate peel extract on damage associated with hypertension and aging in a spontaneously hypertensive rat (SHR) model. The influence of pomegranate consumption was examined on systolic blood pressure (SBP), angiotensin-converting enzyme (ACE) coronary activity, oxidative stress, and vascular morphology. Four- or 28-wk-old SHR model rats were treated for 30 d, with terminal experimental animal age being 8 and 32 wk, respectively, with either pomegranate extract (SHR-PG) or filtered water (SHR). Data showed significant reduction in SBP and coronary ACE activity in both age groups. The levels of superoxide anion, a measure of oxidative stress, were significantly lower in animals in the SHR-PG group compared to SHR alone. Coronary morphology demonstrated total increases in vascular wall areas were in the SHR group, and pomegranate peel extract diminished this effect. Pomegranate peel extract consumption conferred protection against hypertension in the SHR model. This finding was demonstrated by marked reduction in coronary ACE activity, oxidative stress, and vascular remodelling. In addition, treatment was able to reduce SBP in both groups. Evidence indicates that the use of pomegranate peel extract may prove beneficial in alleviating coronary heart disease.

ARTICLE HISTORY
Received 27 November 2015
Accepted 30 May 2016

Systemic arterial hypertension is considered a global public health problem with 9.4 million deaths attributed to it annually (Lima et al., 2012). As this condition is a major cardiovascular manifestation (Kearney et al.. 2005), prevention and treatment of systemic arterial hypertension should receive high priority. Age is a significant risk factor in development of cardiovascular disease; however, striking gender differences also exist in the chronological development of heart disease (Rosamond et al., 2007).

In human and experimental hypertension models, such as spontaneously hypertensive rats (SHR), endothelium-dependent relaxation may be attenuated, and the resulting endothelial dysfunction contributes to increased peripheral resistance. Endothelial dysfunction has been linked to decreases in nitric oxide (NO) bioavailability, reflecting impaired generation of NO and/or enhanced inactivation of NO by free radicals (Púzserová et al., 2010).

Free radicals are any species capable of independent existence with at least one unpaired electron, such as superoxide anions ($O_2^{\cdot -}$) (Forman et al., 2008). The relationship between free radicals and hypertension was first suggested in the 1960s (Romanowskia et al., 1960), but only in the 1990s was this association investigated extensively. The administration of heparin-bound superoxide dismutase (SOD) to SHR animals was associated with a reduction in blood pressure (Nakazono et al., 1991). This decrease in systolic blood pressure (SBP) following administration of SOD may be due to a fall in reactive oxygen species (ROS), which may contribute to elevation in blood pressure, either directly, related to vasoconstrictor effects, or indirectly, by reducing the

CONTACT Prof. Roger Lyrio dos Santos, PhD rogerlyrio@hotmail.com Department of Physiological Sciences, Biomedical Center, Federal University of Espirito Santo, Marechal Campos Avenue, 1468, 29050-755, Vitoria, ES, Brazil.

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activity of vasodilators such as NO (Reckelhoff and Romero, 2003).

Increased sympathetic nervous system activity (Prodel et al., 2015), upregulation of the renin–angiotensin–aldosterone system (Probstfield and O'Brien, 2010), and enhanced oxidative stress (Hamilton et al., 2001) are important factors that are modified in hypertension. These factors may lead to changes in the vessel structure. Angiotensin II was suggested to contribute to vascular hypertrophy and hypertension via stimulation of the NADPH oxidase system to subsequently elevate ROS levels in vascular cells (Nickenig and Harrison, 2002).

Several epidemiological studies suggested that regular consumption of foods and beverages that are rich in polyphenols, such as red wine, berries, cocoa, tea, soy, and pomegranate, is associated with a reduction in risk of a range of pathological conditions including hypertension, coronary heart disease, stroke, and dementia (Ghosh and Scheepens, 2009). Studies in rats demonstrated that pomegranates (Mohan et al., 2010) are rich in polyphenolic antioxidants, which include tannins, anthocyanin, and flavonoids (Jurenka, 2008). All parts of the fruit seem to possess considerable amounts of polyphenols, but the husk appears to contain the highest concentration of these antioxidants (Gil et al., 2000).

Antioxidants are well known to enhance the biological actions of NO by protecting against oxidative destruction mediated by ROS (Gil et al., 2000). Pomegranate is a rich source of antioxidants; however, little is known regarding the action of pomegranate on the coronary vascular bed. Therefore, the objective of this study was to examine the potential of pomegranate peel extract in protecting against damage mediated by hypertension on angiotensin-converting enzyme (ACE) activity, oxidative stress, and vascular remodelling.

Materials and Methods

Animals

Spontaneously hypertensive (SHR) Wistar female rats (4 and 28 wk old) were randomly divided into two groups: SHR and spontaneously hypertensive pomegranate extract (SHR-PG). Animals were obtained from the animal facilities at the Federal University of Espirito Santo. Pomegranate extract was dissolved in filtered water and administered orally for 30 d by gavage. The control group received filtered water. At the end of the treatment, the animals were 8 and 32 wk old, respectively. Rats were maintained in temperature-controlled rooms (22°C) under a 12-h light/dark cycle. All procedures were conducted in accordance with the institutional guidelines for animal research, and protocols were previously approved by the Institutional Ethics Committee for Use of Animals (CEUA 107/2011).

Noninvasive Arterial Blood Pressure Assessment

Noninvasive measurement of tail-cuff pressure as an estimate of systolic arterial pressure was carried out 1 d before treatment started and on the last day (d 30) of administration. Rats were warmed in a restraining chamber, and occluding cuffs and pneumatic pulse transducers were placed on their tails. A sphygmomanometer was inflated and deflated automatically, and tail-cuff signals from the transducer were automatically recorded using an IITC apparatus (IITC, Inc., Woodland Hills, CA) connected to a computer. For each blood pressure measurement session, the mean of three arterial blood pressure readings was recorded for each rat.

Plant Material

The plant material of choice (*Punica granatum* L.) popularly known as pomegranate, belonging to the family Lythraceae, was collected in the city of Vitoria, state of Espirito Santo, Brazil. Plant samples were authenticated by Dra. Valquíria Ferreira Dutra at the Department of Biological Sciences, Federal University of Espirito Santo, where a sample (voucher specimen number 37631) was deposited in the herbarium of the VIES/UFES in botany sector.

Preparation of Pomegranate Peel Extract

The peel of *Punica granatum* L. was removed and dried in shade for 10 d before grinding. Extract was prepared according to Lapornik et al. (2005)

with modification. Briefly, pomegranate was collected; peel was removed, dried for 5 d, and then ground. The ground material (85.71 g) was mixed in 1000 ml ethanol (95°GL) in an amber bottle until the complete extraction of peel compounds. Subsequently, the sample was vacuum filtered, supernatant was collected, and alcohol was evaporated in a rotary evaporator at 60°C. The resultant crude extract (hydroalcoholic extract) (68%, w/w) was kept at 4°C in an amber bottle. Because the hydroalcoholic extract undergoes a certain degree of hydration, a dry weight determination was made. The hydroalcoholic extract was diluted in filtered water and administered for 30 d orally by gavage at a concentration of 25 mg/100 g rat.

Determining Estrous Cycle Phase

Daily vaginal smears were taken from each female rat as previously described (Marcondes et al., 2002) to confirm that estrous cycles were proceeding normally. The vaginal epithelial cells were examined by microscopy for at least 7 consecutive days before the experiment. The swabs were performed between 8:00 and 10:00 a.m. to maintain consistency. The females exhibiting normal estrous cycles were killed at proestrus between 9:00 a.m. and 1:00 p.m.

Isolation of Coronary Arteries

At the end of treatment, animals were anesthetized with sodium thiopental (50 mg/kg, ip) and euthanized via decapitation. The thorax cavity was opened, and the heart was removed and placed in a buffer solution of Tris-HCl, pH 7, with 50 m*M* NaCl (Carmona et al., 2006). The left anterior descending branch and septal branch coronary were isolated using a dissection microscope (D.F. Vasconcelos M900, São Paulo, Brazil). Subsequently, samples were stored at –80°C until protein quantification (Furieri et al., 2011).

Measurement of Angiotensin-Converting Enzyme (ACE) Coronary Activity

Angiotensin-converting enzyme (ACE) coronary activity was determined using the fluorescence resonance energy transfer (FRET) peptide Abz-FRK(Dnp)P-OH (Aminotech Pesquisa and Desenvolvimento, SP, Brazil) as a substrate (Alves et al., 2005). Coronary samples were homogenized in 0.1 *M* Tris-HCl buffer, pH 7, containing 50 m*M* NaCl and centrifuged at 1000 × g for 10 min. The hydrolysis rate of the Abz-FRK(Dnp)P-OH substrate (10 μ*M*) after incubation for 30 min at 37°C in coronary homogenate aliquots was assessed to obtain ACE enzymatic activity. The assay methodology was adapted for a 96-well plate reader. Fluorescence was measured at 320 nm excitation and 420 nm emission wavelengths (Synergy2 BiotekR USA). Assays were performed in triplicate, and results were averaged. Coronary ACE activity was expressed in arbitrary fluorescence units (AFU/μg protein). The protein content was determined by the Lowry et al. method (1951) method.

Histological Analysis of Anterior Septal Coronary

At the end of treatments, each rat was anesthetized with sodium thiopental (50 mg/kg, intraperitoneal injection) and the heart was removed. For each animal, the medial portion from the septal coronary artery was used. Subsequently, 10 sections every 100 μm were obtained. The morphometric analyses of total vascular and wall areas corresponded to average values obtained from 10 cross sections. Subsequently, tissues were embedded in optical cutting temperature (OCT) compound and cross-sectioned on a cryostat (Jung CM1850; Leica, Wetzlar, Germany) at a thickness of 8 μm. For each animal, the coronary cross sections were mounted on gelatin-coated slides and stained with hematoxylin–eosin for morphometric analysis as shown previously (Borgo et al., 2016).

Morphometry

Images of the coronary arteries were captured using a 20× objective with a color video camera (VKC150, Hitachi, Tokyo, Japan) connected to a microscope (Olympus AX70, Olympus, Center Valley, PA) and analyzed employing a National Institutes of Health (NIH) imaging program. An examiner blind to the experimental groups performed the image analysis to prevent any bias in the interpretation of the results.

Detection of Superoxide Production

Dihydroethidium (DHE) staining was used to determine ROS generation. Unfixed frozen coronary sections (8 μm) after dehydration with a 30% sucrose solution were incubated with 2 μmol DHE (Molecular Probes, Sigma, D7008) in modified Krebs solution containing 20 mmol HEPES for 30 min in a light-protected chamber at 37°C. Subsequently, the samples were subjected to three washes with phosphate-buffered saline (PBS), air dried (light-protected), and mounted with neutral glycerine. The levels of ROS were determined using microscopy, and coronary fluorescence was quantified with microscope software (NIS-Elements BR 7.0, Nikon Instruments, Inc., Champigny-sur-Marne, France). An enhanced red fluorescence suggested elevated levels of superoxide anion. The fluorescence intensity of the coronaries was quantified in 10 arbitrarily selected coronaries, and the mean value for each islet was calculated.

Statistical Analysis

All data are expressed as the mean ± SEM. To identify possible outlier data, a two-sided Grubbs test was used to identify whether at least one outlier was present in each dataset. When Grubbs' test identified one outlier, an adapted ROUT method was used to detect any outliers from that column data and remove them according to the Q setting at 1% (alpha = .01). For each data set, the D'Agostino–Pearson omnibus normality test was also performed. If data passed the normality test, then one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons was applied. The significance was set at $p < .05$.

Results

Figure 1 shows the systolic blood pressure (SBP) on d 30 of treatment with water or pomegranate peel extract in the 8-wk-old animals (A) and 32-wk-old animals (B). A significant decrease was noted in SBP in the SHR-PG group compared with the SHR alone after 30 d in both 8-wk-old and 32-wk-old animals. Data in Figure 2 demonstrate the effect of treatment with pomegranate peel extract on coronary ACE activity. The activity of ACE was significantly reduced in SHR-PG compared with SHR in both age groups.

Figure 3 summarizes the data obtained from coronary morphology analyses of 8-wk-old (left panels) and 32-wk-old animals (right panels). An increase in total vascular (1.86 ± 0.19 vs. 1.13 ± 0.1 μm^2, Figure 3D) and wall areas (1.06 ± 0.11 vs. 0.56 ± 0.04 μm^2, Figure 3E) was noted in SHR with 8-wk-old animals compared to age-matched controls. Similarly, an elevation in total vascular (3.75 ± 0.16 vs. 2.27 ± 0.16 μm^2, Figure 3J) and wall areas (1.82 ± 0.13 vs. 0.78 ± 0.06 μm^2, Figure 3K) was found in SHR at 32 wk compared to age-matched controls. However, treatment of SHR with pomegranate peel extract resulted in a return to control values in total vascular area in SHR at both 8 wk (1.86 ± 0.19 to 0.89 ± 0.07, Figure 3D) and 32 wk (3.75 ± 0.16 to 2.25 ± 0.29 μm^2, Figure 3J) respectively. Further, treatment of SHR with pomegranate peel extract also produced a return to control levels in wall areas in SHR at 8

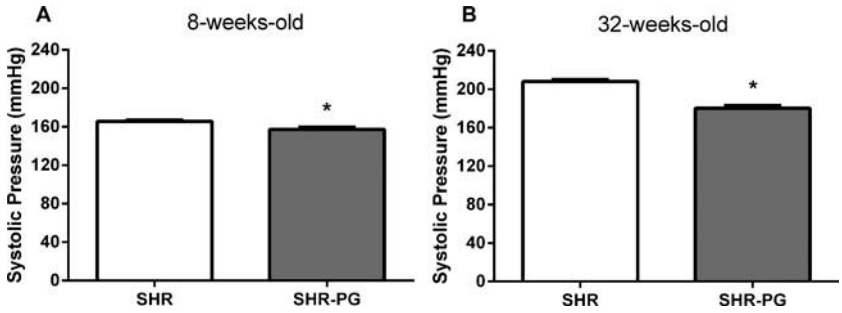

Figure 1. Effect of pomegranate extract on systolic blood pressure (SBP) on d 0 and on d 30 in 8-wk-old (A, $n = 18$) and 32-wk-old animals (B, $n = 16–18$). Values are expressed as means ± SEM. Asterisk indicates significant at $p < .05$ compared with SHR alone (unpaired Student's *t*-test).

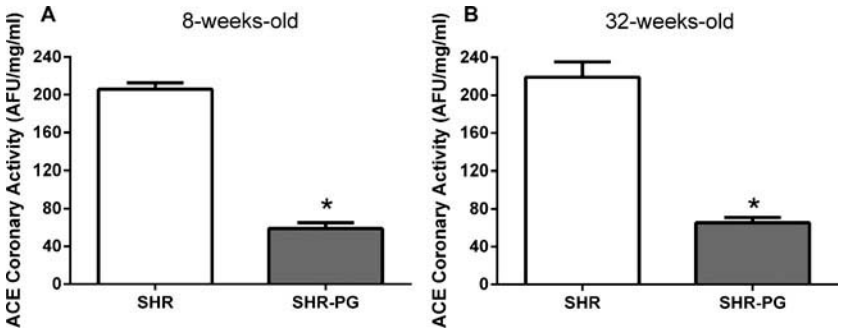

Figure 2. Influence of pomegranate extract on coronary ACE activity on d 30. Eight-week-old (A) SHR rats and SHR-PG, and 32-wk-old (B) SHR rats and SHR-PG. Values are expressed as means ± SEM; $n = 5$ animals in each group. Asterisk indicates significant at $p < .05$ compared with SHR alone.

wk (1.06 ± 0.11 to 0.64 ± 0.03 μm^2, Figure 3E) and 32 wk (1.82 ± 0.13 to 1.13 ± 0.09 μm^2, Figure 3K). An increase in wall/lumen ratios was found in SHR at 8 wk (1.38 ± 0.10 vs. 0.89 ± 0.07 μm^2, Figure 3F) and 32 wk (1.28 ± 0.05 vs. 0.57 ± 0.05 μm^2, Figure 3L) compared to age-matched controls. The treatment of SHR with pomegranate peel extract were also able to lower these values in SHR at 8 wk (1.38 ± 0.1 to 0.97 ± 0.09 μm^2, Figure 3F) and 32 wk (1.28 ± 0.05 to 0.8 ± 0.13 μm^2, Figure 3L).

The antioxidant potential effect of the pomegranate peel extract treatment was observed in the coronary arteries using DHE staining, as illustrated in the microphotographs in Figure 4. A DHE oxidative assay revealed intense fluorescence in SHR animals but none in SHR-PG. Pomegranate peel extract administration reduced vascular oxidative stress in coronary arteries in the SHR at ages 8 wk (11.3 ± 0.9 AU vs. 3.66 ± 0.9 AU) and 32 wk (55.7 ± 4.9 AU vs. 25.96 ± 5.0 AU) compared with respective controls. On average, coronary arteries from SHR-PG animals exhibited approximately 50% less ethidium fluorescence than SHR.

Discussion

Our main finding was that treatment with hydroalcoholic extracts of *Punica granatum* peel was able to reduce oxidative stress and coronary ACE activity in SHR rats. Further, the treatments lowered SBP and prevented vascular remodeling in coronary arteries in this hypertension model. In the present study, 30 d of treatment with pomegranate peel extract significantly decreased SBP in 8- and 32-wk-old animals. Among the possible causes of hypertension, an overproduction of ROS is implicated. Oxidative stress is defined as an imbalance between ROS levels and antioxidant defenses, which worsens with aging and hypertension (Wind et al., 2010; Ghio et al., 2012). Further, oxidative stress was shown to be intimately related with endothelial dysfunction (Hamilton et al., 2001). Wind et al. (2010) found that ROS production increased in the aortas of aged SHR compared with aged Wistar-matched control aortas. This augmented ROS production may lead to elevated SBP directly by vasoconstrictor effects or indirectly by reduced activities of vasodilators (Reckelhoff and Romero, 2003). Although the animal model used in this study was characterized by enhanced generation of ROS, oxidative stress appeared to increase in conditions of hypertension and aging. Thus, greater oxidative stress was expected in 32-wk-old versus 8-wk-old animals. Treatment with pomegranate peel extract in both 8-wk and 32-wk SHR may have led to lower ROS production, which may have been sufficient to lower SBP. Further, as ROS contribute to endothelium-dependent contraction and increase in vascular resistance, antioxidant substances found in pomegranate extracts may be associated with the observed decrease in SBP (Kitiyakara and Wilcox, 1998). This finding is in agreement with other studies of advanced aging, which demonstrated a 5% decline in SBP with a daily consumption of pomegranate juice for 2 wk (Aviram and Dornfeld, 2001).A decrease in coronary ACE activity was noted in the SHR-PG group compared with SHR alone at 8 and 32 wk.

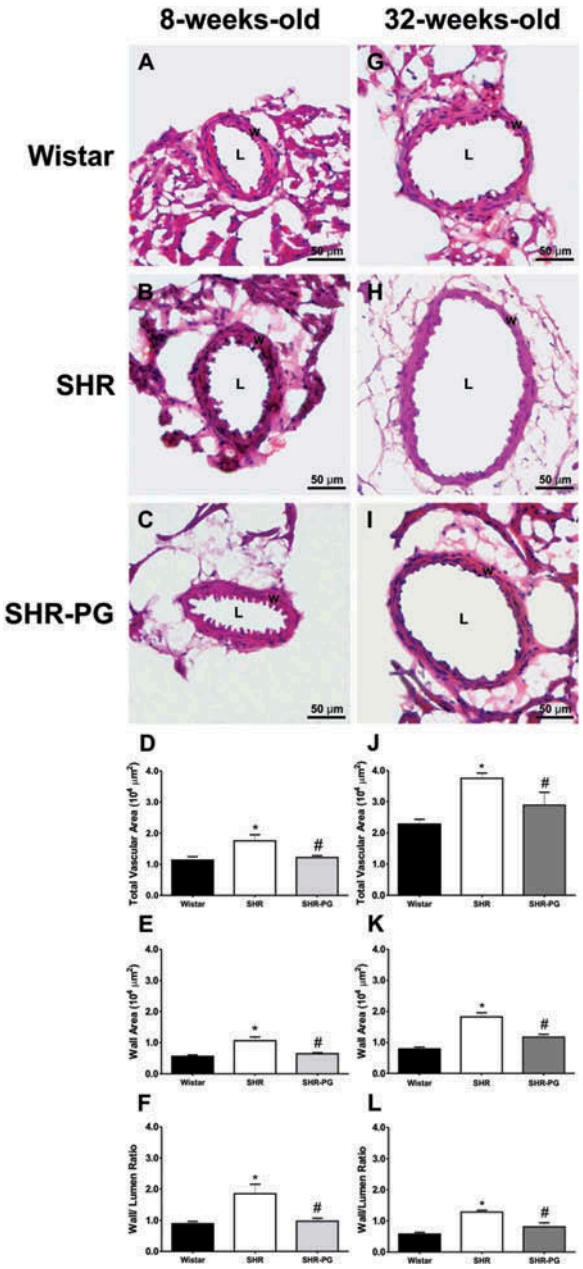

Figure 3. Effect of pomegranate extract on morphometric parameters of coronary arteries. Top panel, microphotographs are typical cross sections of coronary arteries in SHR and SHR-PG rats at 8 (left panel) and 32 (right panel) wk, respectively. Bar = 100 µm. Bottom bar graphs show influence of pomegranate peel extract on total vascular and wall area at 8 (left panel) and 32 (right panel) wk old, respectively. Values are means ± SEM, n = 4–7 per group. Asterisk indicates significant at $p < .05$, and double asterisk at $p < .01$ compared with control, and $^{\#}p < .05$ compared with SHR alone.

Angiotensin-converting enzyme, a zinc (Zn) metallopeptidase, possesses two large homologous domains, the N and C domains. These domains are often the targets of ACE inhibitors that act by binding to the Zn (Comini et al., 2007). Polyphenols have chemical structures that favor chelation of redox-active metals (Fraga, 2007) which may have favored ACE inhibition observed in this study. In addition, Mohan et al. (2010) demonstrated that consumption of pomegranate juice for 4 wk in diabetic hypertensive rats decreased serum ACE levels. In hypertension, angiotensin II is known to play a role in vascular remodeling. This effect seems to be mediated by a rise in production of free radicals (Ushio-Fukai et al., 1996; Zalba et al., 2000). Thus, coronary ACE inhibition produced by pomegranate peel extract may have influenced the morphology of the cells of the coronary arteries as noted in this investigation. Althunibat et al. (2010) reported that administration of *Punica granatum* peel methanol extract improved antioxidant enzyme activities in diabetic rats. Aviram et al. (2000) demonstrated marked antioxidant capacity of pomegranate juice to scavenge free radicals. Based on the antioxidant properties of *Punica granatum* described in the literature (Gil et al., 2000) and its scavenging capacity, it was postulated that treatment with pomegranate peel extract might decrease oxidative stress in this experimental model. Data demonstrated that coronary arteries of SHR-PG exhibited a marked fall in generation of $O_2^{\cdot-}$. These results support the notion that one of the mechanisms involved in attenuation of damage derived from hypertension may be related to reduced oxidative stress. Cardiovascular morpho-physiological abnormalities have been associated with increased oxidative stress (Lima et al., 2012). Consequently, interventions that are able to potentiate tissue antioxidant capacity, including pharmacological therapies, have been used to provide vascular and cellular benefits (Bazargani-gilani et al., 2014; Danesi et al., 2014). Evidence indicates that pomegranate juice extract may also exert beneficial actions in hypertensive individuals.

Our morphometric analyses showed that pomegranate extract treatment prevented alterations induced by hypertension and/or aging on total vascular and coronary wall area. The renin–angiotensin–aldosterone system has trophic actions on the components of the arterial wall, and angiotensin II might initiate hypertrophic processes on vascular smooth muscles (Touyz et al., 2003). Not surprisingly, ACE inhibition was found to affect hypertrophied arterial walls in SHR animal models (Dedkov et al., 2006). A novel finding

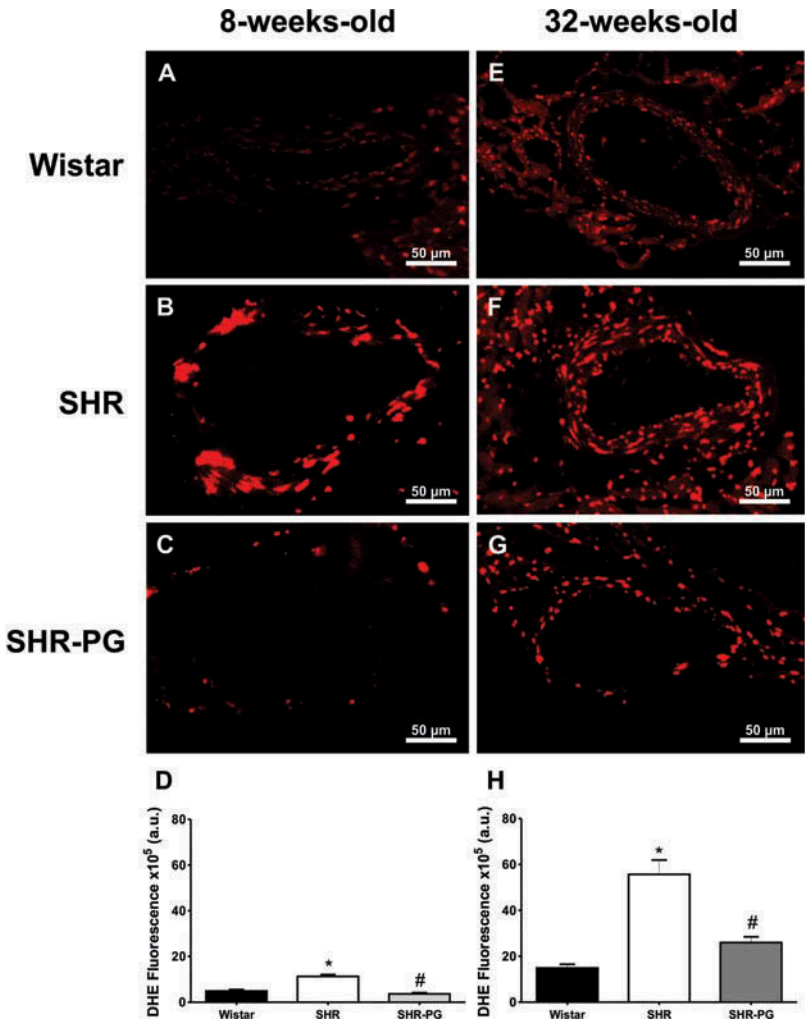

Figure 4. Influence of pomegranate peel extract treatment on superoxide anion production in coronary arteries. The top panels show higher fluorescence intensity (red) in SHR rats when compared to SHR-PG rats using dihydroethidium (DHE) staining. The bar graph shows the average DHE fluorescence (AU: arbitrary units) comparing all groups ($n = 6$). Left panel, 8-wk-old animals and right panel, 32-wk-old animals. Values are means ± SEM. Asterisk indicates significant at $p < .01$ compared with controls, and $^{\#\#}p < .01$ compared with SHR-PG group. Scale bar: 100 µm.

of this study revealed that in SHR that received pomegranate extract, the morphological alterations induced by hypertension and/or aging were diminished. This finding may be attributed to a reduction in oxidative stresses mediated by coronary ACE inhibition and the antioxidant actions of polyphenol compounds. These effects may be correlated with the potent antioxidant activity of pomegranate associated with high polyphenol content and to the specific type of polyphenols present in pomegranate, specifically, hydrolyzable tannins, which display a high scavenging capacity for free radicals (Aviram and Rosenblat, 2012).

Our study corroborated the observations of Touyz et al. (2003), which indicated that ROS might induce morphological alterations. Indeed, ROS promoted these alterations by (i) modifying the activity of tyrosine kinases, metalloproteinases, and mitogen-activated protein kinases (Baas and Berk, 1995; Intengan and Schiffrin, 2001); (ii) acting on gene and protein expression mechanisms by activating transcription factors, such as nuclear factor (NF)-κB and AP-1 (Touyz and Schiffrin, 2000); and (iii) stimulating ion channels, such as plasma membrane Ca^{2+} and K^{+} channels, leading to changes in cation concentrations (Lounsbury et al., 2000).

Data demonstrated for the first time the influence of pomegranate peel extract administration on the vascular remodeling process of coronary arteries for young and elderly hypertensive female

rats. The coronary vascular remodeling was characterized by increased wall and cross-sectional vascular areas. Thus, the coronary artery remodeling in SHR might reflect an adaptive response to elevated arterial pressures to normalize increased wall tension (Dedkov et al., 2006; Irwin et al., 2014).

In conclusion, this study showed that treatment with pomegranate peel extracts was able to prevent morphological alterations in coronary arteries induced by hypertension and/or aging, which likely occurred through antihypertensive actions such as antioxidant effects and decreasing coronary ACE activity. Therefore, the beneficial effects of pomegranate peel extracts in coronary arteries may be considered in the development of better therapies for hypertension.

Acknowledgments

The authors thank Nazare S. Bissoli for critical review and Polyana L. M. Dalpiaz for performing the measurement of ACE activity.

Conflicts of Interest

All the authors disclose no financial and personal relationships with other people or organizations that could inappropriately influence their work.

Funding

This work is supported by grants from the FAPES (54687578/2011) and CNPq (55262312011-3).

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