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Protective Effects of Grape Juice on Vascular Damage Induced by Chlorine Free Radical in Rats

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ABSTRACT: Grapes and their derivatives have antioxidant and cardioprotective properties. Therefore, we hypothesized that grape juice (GJ) could improve vascular oxidative damage caused by chlorine radicals (OCl^-), which are excessively produced in vascular tissue during cardiovascular diseases (mainly diabetes and hypertension). The antioxidant capacity of GJ was analyzed by an electrochemical method, followed by administration in rats (100 or 300 mg/kg/d, via the oral) for seven days. Then, rats were sacrificed, and their aortas were isolated and subjected to isometric recordings or immunohistochemical analyses with or without exposure to OCl^- (5, 20, or 100 μM, 60 min). Concentration-effect curves for acetylcholine (ACh) and sodium nitroprusside (SNP) were derived to analyze endothelium-dependent or independent vasorelaxation. The GJ presented with high antioxidant capacity, and treatment with GJ did not alter vascular relaxation induced by ACh or SNP. After exposure to OCl^-, endothelium-denuded arteries showed preserved relaxation with SNP, whereas endothelium-intact arteries showed reduced relaxation with ACh. OCl^- at various concentrations induced significantly decreased relaxation of arteries (80.6±4.2%, 55.4±4.7%, and 28.1±5.9%, respectively) vs. control arteries (96.8±2.4%). However, treatment with GJ prevented loss in relaxation caused by 5 and 20 μM OCl^- and improved relaxation after exposure to 100 μM OCl^-. Exposure to OCl^- induced increased nitrotyrosine immunostaining of endothelial cell layers, which was improved by GJ treatment. Altogether, vascular damage caused by OCl^- was prevented by treatment with GJ, and GJ prevented nitrosative stress in these vessels.

Keywords: endothelial dysfunction, grape juice, hypochlorite, vascular relaxation

INTRODUCTION

Oxidative stress plays a central role in endothelial dysfunction leading to impaired cardiovascular function in pathophysiological processes such as hypertension, diabetes, atherosclerosis, and aging (Yung et al., 2006; Radovits et al., 2013). Reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and reactive chlorine species, such as hypochlorous acid (HOCl) and hypochlorite (OCl^-), are major oxidizing agents that contribute to injuries observed in the cardiovascular system (Hamilton et al., 2001; Lassègue and Griendling, 2004; Radovits et al., 2013). Vascular peroxidase 1 and myeloperoxidase transform up to 70% of produced ROS to reactive chlorine species (OCl^-) in the vasculature, leading to increased toxicity (Li et al., 2012; Davies and Hawkins, 2020).

Reactive chlorinate compounds are highly toxic to the vascular system and lead to endothelial dysfunction, chronic inflammation, and impaired endothelium-dependent vascular relaxation (Stocker et al., 2004; Kawai et al., 2006; Radovits et al., 2013; Davies and Hawkins, 2020). Chlorinated reactive species (OCl^-) can reach high concentrations in many pathological processes of the cardiovascular system that generate an active inflammatory process (e.g., hypertension, diabetes, atherosclerosis, and infarction, etc.), reaching micromolar levels in local circulation and affected tissues (Zhang et al., 2001).

Grapes are rich in compounds with high antioxidant potential (mainly polyphenols and flavonoids) that reduce the risk of cardiovascular diseases and improve endothelial dysfunction in animal models and in patients with severe cardiovascular disease (Kris-Etherton et al., 2002; Porteri et al., 2010; Blumberg et al., 2015). Moreover,

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grape juice (GJ) consumption can increase important endogenous antioxidant enzymes, such as glutathione, catalase, and superoxide dismutase (O'Byrne et al., 2002; Toaldo et al., 2016). Consumption of both red wine and GJ show comparable antioxidants benefits and vascular relaxant effects (Anselm et al., 2007; Mudnic et al., 2012). Therefore, GJ may benefit patients discouraged from drinking alcohol, such as psychiatric patients, the elderly, children, and pregnant women (Vinson et al., 2001). In this study, we aimed to examine the protective effects of GJ consumption on vascular relaxation and endothelial dysfunction caused by OCl^- in the arteries of rats.

MATERIALS AND METHODS

Grape juice analysis

GJ sample was selected from GJ produced and marketed in different regions of Brazil. The GJ's eligibility criteria included: i) formed from whole red grapes; ii) no added preservatives, stabilizers, or antioxidants; iii) no added water; iv) no added sugar; and v) the ingredients described on the label were "100% grape juice". The GJ selected for this study had the best antioxidant activity measured by several different methods (Britto, 2019). The chosen GJ is produced in the Rio de Janeiro State and is commercially available throughout Brazil.

The total phenolic content was estimated using Folin-Ciocalteu reactions as previously described (Lino et al., 2014). Briefly, 2.5 mL of 10% Folin-Ciocalteu reagent was added to a small volume of GJ (25～100 μL) and then treated with sodium carbonate solution. The absorbance was measured at 760 nm, and the total phenolic content was calculated in relation to gallic acid equivalents (GAEs) based on a standard curve of gallic acid. All experiments were performed at least three times. Results were expressed in μg GAE per mL of GJ.

The antioxidant capacity of samples was assessed by electroanalytical assays (differential pulse voltammetry, DPV) according to previously standardized methods (Lino et al., 2014; de Souza et al., 2017). Voltametric experiments were performed using a potentiostat/galvanostat μAutolab III® and GPES 4.9® software (Eco Chemie, Utrecht, The Netherlands). Measurements were performed using 50 μL of GJ with 0.1 M phosphate buffer solution (pH 6.0) in a one-compartment electrochemical cell (5 mL), with a three-electrode system consisting of a carbon paste electrode, a piston-driven holder [containing graphite powder (70%) and purified mineral oil (30%), diameter=2 mm], a platinum wire, and the Ag/AgCl/KCl_{sat} (all purchased from Analyser, São Paulo, Brazil), representing the electrode of work, the counter electrode, and the reference electrode, respectively. The experimental conditions were as follows: pulse amplitude, 50 mV; pulse width, 0.4 s; and scan rate, 5 mV/s. Vitamin C (10 μM) was used as positive control. All experiments were conducted at room temperature (21±1°C) in triplicate and analysed with software Origin 8® (OriginLab Corporation, Northampton, MA, USA).

Animals and treatment

Male Wistar rats (200～230 g) were obtained from the Central Bioterium at the Federal University of Goias. The animals were housed at a temperature of 22±2°C in a light-controlled room (12-h dark/light cycle) with free access to filtered water and rat chow. Rats were acclimatized for at least 7 days before starting the experiment. Rats were handled in agreement with globally established standard guidelines for the use of laboratory animals. All procedures were accepted by the Animal Research Ethics Committee at the Federal University of Goiás, Goiânia, Brazil (protocol: 044/17).

Prior to starting the experiments, the dry weight (w/w) of the GJ was determined according to methods standardized by the Brazilian pharmacopoeia (Agência Nacional de Vigilância Sanitária, Fundação Oswaldo Cruz, 2010). After determining the content of the solids (18% of dry weight), GJ was administered orally (100 or 300 mg/kg/d by gavage, diluted in distilled water; total volume 1 mL) over one week, always at the same hour of the day. The control group received only vehicle (distilled water) in the same volume (n=6 rats per experimental group).

Isolated artery preparation

After seven days of treatment, rats were anaesthetized by inhaling isoflurane and euthanized. The aortas (thoracic branches) were removed, cleaned, and cut into rings approximately 4 mm in length. Samples were separated for immunoreactivity analysis (in 10% buffered formalin), or placed in an isolated organ bath between two stainless-steel stirrups and coupled to a computerized system and a WinDaq Resource data achievement unit (DATAQ Instruments, Akron, OH, USA) to measure isometric vascular tone. Aortic rings were placed in a 10-mL organ chamber containing a physiological salt solution of the following composition: 4.7 mM KCl, 130 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 14.9 mM $NaHCO_3$, 5.5 mM glucose, and 1.6 mM $CaCl_2$ at 36±1°C with 95% O_2 and 5% CO_2 (pH 7.4). The vascular rings were stretched to a basal tension of 1.5 g before allowing them to equilibrate in the bathing solution. Each rat supplied only one arterial ring for the different protocols by following earlier standardized methods (de Souza et al., 2017; Jordão et al., 2017).

Some preparations had the endothelial cells layer mechanically removed by rubbing the inner artery surface with a fine metallic wire (200 μm in diameter). The effectiveness of the removal was confirmed by the absence of

dilation in response to acetylcholine (ACh, 1 μM) precontracted with phenylephrine (Phe, 0.1 μM, 50% effective concentration determined earlier in our laboratory) (Lobo de Andrade et al., 2015).

After stabilization, artery rings were subjected to oxidative stress, characterized by the addition of OCl^- in the bath solution at concentrations of 5, 20, and 100 μM for 60 min (Radovits et al., 2013). After successive rinsing, vascular rings with or without endothelial cells were precontracted (Phe, 0.1 μM), and cumulative concentration-response curves were derived for dilation induced by ACh (0.1 nM to 10 μM) or nitric oxide donor sodium nitroprusside (SNP, 0.01 nM to 1 μM).

Immunohistochemical staining

Immunohistochemical staining for nitrotyrosine (NT, a general marker of nitrosative stress) was performed in 20 μM OCl^--exposed arteries from rats pretreated with 0 (control), 100, or 300 mg/kg/d of GJ. Arterial sections (3 μm) partitioned by microtome (Leica 2165 model, RM Microsystems Inc., Bannockburn, IL, USA) were mounted on polarized slides (Surgipath® X-tra® Clipped Corner Leica Biosystems Richmond Inc., Richmond, IL, USA) and subjected to immunohistochemistry. Samples were deparaffinized and rehydrated, and then antigens were retrieved using Trilogy (Cell Marque, Rocklin, CA, USA) at a concentration of 1:100 in distilled water and at a temperature of 97°C for 30 min in a digital water bath (DeLeo, Porto Alegre, Brazil). Slides were incubated with the primary antibody anti-NT (clone 39B6, Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:50). The anti-mouse IgG light chain binding protein-horseradish peroxidase secondary antibody (Santa Cruz Biotechnology) was used to amply the antibody signal. Reactions were chemically detected with 3,3'-diaminobenzidine (DAB, Dako, Carpinteria, CA, USA) in a darkroom for 2 min. Sections were then counterstained with Harris's hematoxylin and mounted on coverslips. Control artery sections were exposed to 1% bovine serum albumin in buffer solution instead of primary antibodies.

Immunostaining of endothelial layers was performed as previously described (Radovits et al., 2013), whereby a scoring system was calculated by multiplying staining intensity (SI) by the amount of positive endothelial cells (PCs). The SI was scored as follows: 0, no positive staining; 1 to 3, increasing degrees of intermediate staining; and 4, extensive staining. The PC was scored as follows: 1, up to 10% of PCs; 2, 11% to 50% of PCs; 3, 51% to 80% of PCs; and 4, >80% of PCs.

Statistical analysis

The data are presented as mean±standard error of the mean. The statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Comparisons among groups were conducted using ANOVA (plus Newman-Keuls post hoc test), and values of $P<0.05$ were considered to be significantly different.

RESULTS

Grape juice analysis

The polyphenol content (expressed as μg GAE/mL sample) of the GJ was 2.28±0.06 μg/mL. The high antioxidant activity observed using the DPV method makes it possible to detect the presence of potent electroactive compounds in the GJ, which presented two oxidation peaks (1a and 2a) at E_{p1a} ~0.13 V and E_{p2a} ~0.59 V (vs. Ag/AgCl/KCl) (Fig. 1). It is well documented that peaks below 0.5 V (pH 5.0) are related to substances with elevated antioxidant power (reducing power).

Vascular reactivity

In the absence of exposure to OCl^-, GJ did not change endothelium-dependent or independent vascular relaxation to ACh or SNP, respectively, in isolated arteries (Fig. 2).

Endothelial dysfunction caused by OCl^- exposure was demonstrated by reduced maximal vasorelaxation (final time point of the curve) of isolated arteries in response to ACh vs. the control group (96.8±2.4%, n=6). Impairment caused by OCl^- was concentration-dependent (80.6±4.2%, n=6 and 55.4±4.7%, n=6 in 5 and 20 μM OCl^-, respectively), and relaxation was almost completely inhibited by the higher tested OCl– concentration (100 μM OCl^-, 28.1±5.9%, n=6) (Fig. 3A). Endothelium-independent vasodilation induced by the nitric oxide

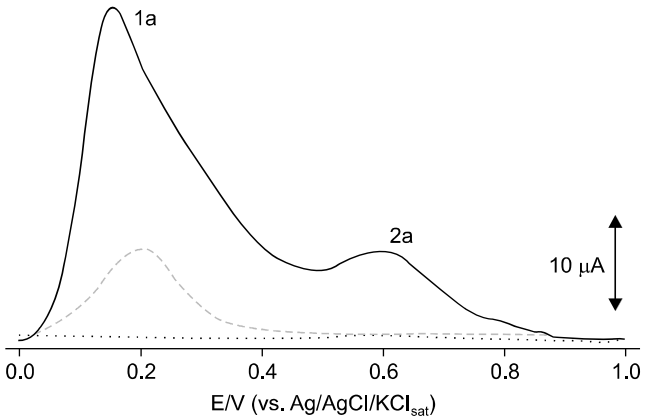

Fig. 1. Electrochemical index. Average differential pulse voltammograms obtained for grape juice (continuous line), standard vitamin C (positive control; dashed line), and blank (dotted line) samples. Samples were diluted in 5 mL of 0.1 M phosphate buffer (21±1°C; pH 6.0) solution and characterized using carbon paste electrodes (diameter = 2 mm). Other parameters included a pulse width of 5 mV, a pulse amplitude of 50 mV, and a scan rate of 5 mV/s. E/V, electric potential/voltage.

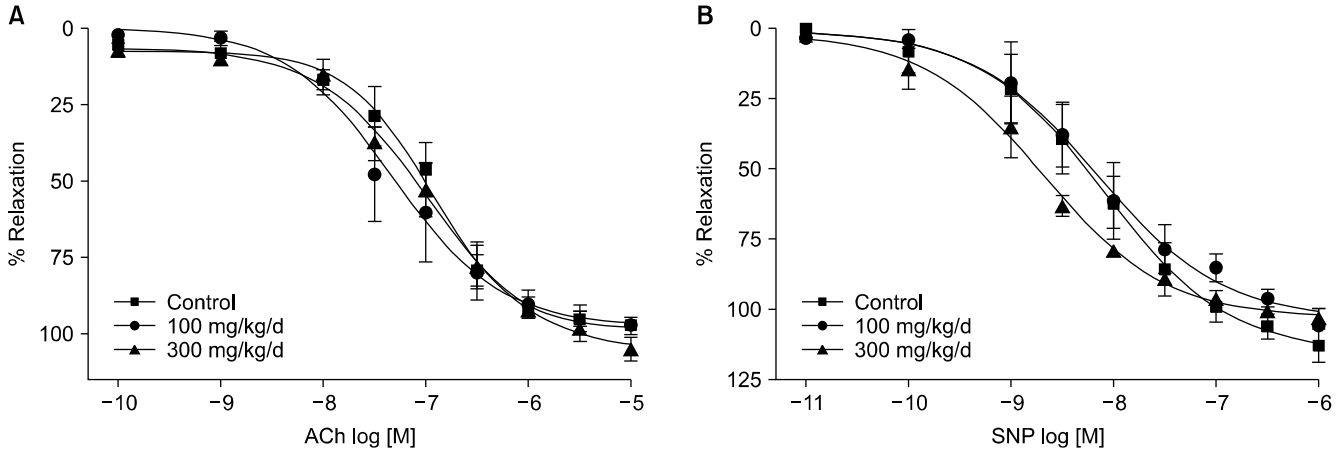

Fig. 2. Vascular reactivity in arteries of rats treated with grape juice (GJ). Vascular relaxation studies of isolated artery rings in rats treated with GJ at 100 and 300 mg/kg/d and controls (n=6 per group). (A) Endothelium-dependent relaxation in response to acetylcholine (Ach). (B) Endothelium-independent relaxation in response to sodium nitroprusside (SNP). Data are presented as mean±SEM, analysed using one-way ANOVA followed by Newman-Keuls post-hoc test.

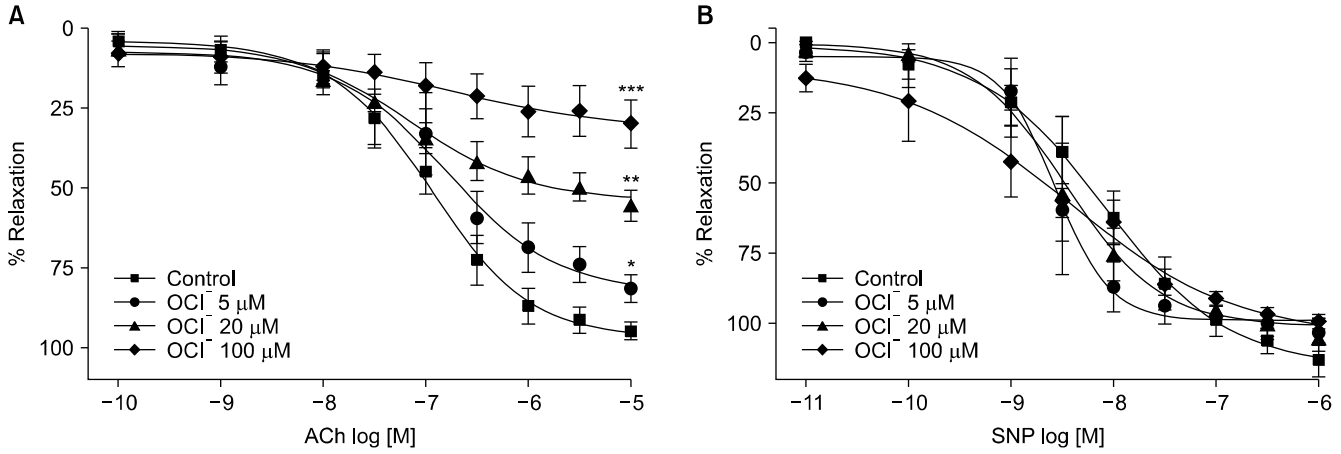

Fig. 3. Effects of pretreatment with OCl^- on the vascular function. Vascular relaxation studies of isolated artery rings from non-treated rats exposed to 5, 20, and 100 μM OCl^- for 60 min, or controls (n=6 per group). (A) Endothelium-dependent dilation in response to acetylcholine (Ach). (B) Endothelium-independent dilation in response to sodium nitroprusside (SNP). Data are presented as mean±SEM, analysed using one-way ANOVA followed by Newman-Keuls post-hoc test. *P<0.05, **P<0.01, and ***P<0.001 vs. controls.

donor SNP was not altered after exposure to oxidative stress, regardless of the OCl^- concentration used (Fig. 3B).

Endothelium-dependent vasodilation induced by ACh is presented in Fig. 4. The reduction in maximal relaxation (final time point of the curve) induced by OCl^- was followed by a recovery in the capacity of ACh to induce relaxation. Upon exposure to 5 and 20 μM OCl^-, treatment with GJ prevented impairment in vascular relaxation, with results comparable with the control group (Fig. 4A and 4B). Furthermore, impaired relaxation caused by exposure to 100 μM OCl^- (28.1±5.9%, n=6) was attenuated in the arteries of rats treated with GJ (to 53.4±5.2% and 50.1±6.1% in 100 and 300 mg/kg, respectively). However, GJ treatment was not able to reverse maximum relaxation to levels observed in the control group (96.8±2.4%, n=6) (Fig. 4C).

Immunohistochemical analysis

Immunohistochemistry analysis of NT in the endothelial cells layer showed significantly (P<0.05) increased immunoreactivity (brown staining) in arteries exposed to OCl^- compared with controls. A similar level of immunoreactivity was observed in the medial smooth muscle cell layers in all groups. However, treatment with both 100 and 300 mg/kg GJ decreased NT-immunoreactivity in endothelial cells layers (Fig. 5).

DISCUSSION

Epidemiological studies have demonstrated that red grapes and their products (such as wines and GJ) have high antioxidant potential, and their consumption shows protective effects against the onset of cardiovascular dis-

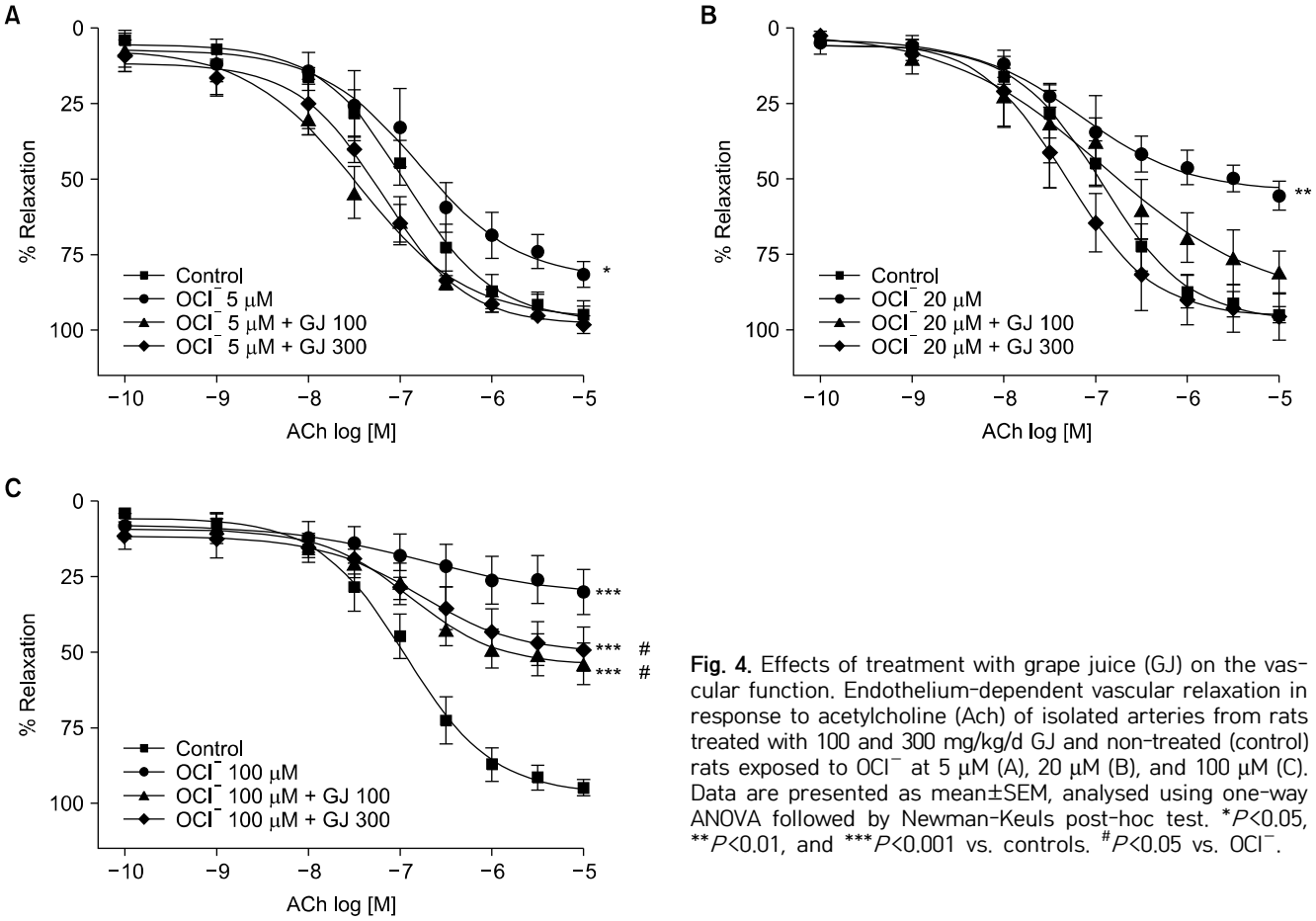

Fig. 4. Effects of treatment with grape juice (GJ) on the vascular function. Endothelium-dependent vascular relaxation in response to acetylcholine (Ach) of isolated arteries from rats treated with 100 and 300 mg/kg/d GJ and non-treated (control) rats exposed to OCl^- at 5 μM (A), 20 μM (B), and 100 μM (C). Data are presented as mean±SEM, analysed using one-way ANOVA followed by Newman-Keuls post-hoc test. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs. controls. $^{\#}P<0.05$ vs. OCl^-.

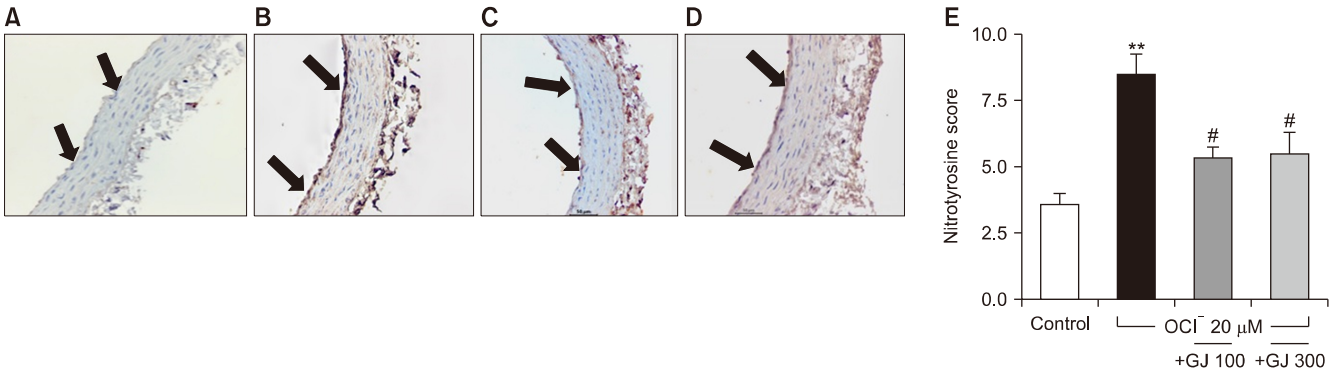

Fig. 5. Vascular immunohistochemistry for nitrotyrosine. Representative photomicrographs of nitrotyrosine (brown staining, ×400; black arrows) immunoreactivity of arteries from control rats (A), non-treated rats exposed to 20 μM OCl^- (B), rats treated with 100 mg/kg/d grape juice (GJ) (C), and rats treated with 300 mg/kg/d GJ (D). (E) Immunohistochemical scores for nitrotyrosine stained in brown on the endothelial cells layer of arterial segments from rats in the different treatment groups (n=6 per group). $^{**}P<0.01$ vs. controls. $^{\#}P<0.05$ vs. OCl^-.

eases (Porteri et al., 2010; Toaldo et al., 2016; Tsai et al., 2017). The present study showed that dietary GJ prevents oxidative lesions induced by OCl^- in the vasculature, thus improves vascular relaxation induced by endothelial factors.

Since antioxidant compounds are electroactive elements, electrochemical analysis can be considered a major tool in determining radical scavenging capacity. Indeed, good electron-donating agents (i.e., antioxidants) can reversibly oxidize at lower peak potentials ($E_{pla}<0.5$ mV, pH=7). Therefore, the concept of an electrochemical index was previously proposed to classify compounds as having antioxidant capacity (Lino et al., 2014). Using the DPV, it was possible to observe the presence of electroactive compounds in GJ, which presented with two oxidation peaks. It is well established that peaks below 0.5 V (pH 5.0) are related to compounds with high reducing power (Lino et al., 2014; de Souza et al., 2017). Antiradical compounds are normally reduced in living organisms at 0.8 V. Therefore, electroactive substances that

exhibit peak potentials lower than 0.7 V (pH 7.0) can scavenge free radicals. Furthermore, since ascorbic acid and tocopherol have reduced potential of below 0.5 V (pH 7.0), treatment with GJ could restore these compounds as endogenous antioxidants, making cells less susceptible to oxidative stress (Madrakian et al., 2014). This antioxidant effect probably results from the combined action of compounds present in the GJ, which (in addition to containing a high concentration of polyphenols) contains significant amounts of antioxidant compounds, such as anthocyanins (total monomeric anthocyanins, 12.45 mg/100 mL), total flavonoids (14.8 mg/100 mL), ascorbic acid (0.98 mg/100 mL), resveratrol (4,3',5'-trihydroxystilbene, 0.164 mg/100 mL), and malic acid (284 mg/100 mL) (Britto, 2019; Monteiro, 2020).

Immediate systemic arterial pressure control is measured by a refined physiological system involving neural and hormonal regulations that powerfully affect vascular tone. Substances that interfere with the function of blood vessels in terms of contraction/relaxation rapidly alter the blood pressure (Thrasher, 2004; Lobo de Andrade et al., 2015). Our results showed that treatment with GJ did not alter vascular responsivity to endothelium-derived nitric oxide (stimulated by ACh) or nitric oxide-donor (SNP) in healthy vessels. Therefore, GJ was unable to modify vascular function.

Robust evidence shows that ROS play a crucial role in the pathogenesis of vascular/endothelial dysfunction observed in progression of diseases such as hypertension, diabetes, ischemia, atherosclerosis, and aging (Yung et al., 2006; de Souza et al., 2017). In addition to ROS (mainly O_2^- and H_2O_2), the reactive species of chlorine, such as HOCl and OCl^-, are major oxidizing compounds that contribute to cardiovascular damage (Hamilton et al., 2001; Lassègue and Griendling, 2004; Radovits et al., 2013). Exposure to reactive species of chlorine leads to endothelial dysfunction and impaired endothelium-dependent vasorelaxation (Summers et al., 2012; Radovits et al., 2013).

Dietary consumption of grapes and their by-products is related to prevention of oxidative diseases, such as cardiovascular and degenerative diseases (Evans et al., 2014), diabetes (Rasines-Perea and Teissedre, 2017), arteriosclerosis (Kris-Etherton et al., 2002), senescence, DNA damage (Balu et al., 2006), and certain cancers (Kris-Etherton et al., 2002). Accordingly, in earlier works we showed protective effects of natural compounds with high antioxidant power in the cardiovascular systems of animals with increased oxidative stress induced by hypertension (de Souza et al., 2017; Jordão et al., 2017). Most of the ROS generated are transformed into OCl^- through enzymatic reactions in the vasculature, leading to increased toxicity (Li et al., 2012). In this study, we showed that treatment with GJ can prevent vascular damage induced by OCl^-. Treatment with GJ at 100 and 300 mg/kg prevented impairments in vascular relaxation caused by OCl^- at the same concentration as found in vascular tissue in oxidative diseases (Zhang et al., 2001; Li et al., 2012).

Several studies have shown that *in vivo* and *in vitro* exposure of blood vessels to OCl^- results in impaired endothelial cell function (Zhang et al., 2001; Li et al., 2012; Radovits et al., 2013). In agreement with these findings, we showed that OCl^- decreases endothelium-dependent vascular relaxation of isolated arteries. However, endothelium-independent vasodilation stimulated by the nitric oxide donor SNP was not affected by OCl^- at 5, 20, or 100 μM, indicative of normal vasodilatory capacity of vascular smooth muscle cells in response to exogenous nitric oxide. However, GJ treatment prevented endothelium dysfunction induced by OCl^-. Indeed, GJ preserved the impaired relaxation induced by 5 and 20 μM OCl^-, and improved endothelium dysfunction induced by 100 μM OCl^-. This effect may be attributed to the high antioxidant potential of GJ and by its capacity to increase important endogenous antioxidant enzymes (such as catalase, superoxide dismutase, glutathione, and glutathione peroxidase) in human and animals (O'Byrne et al., 2002; Toaldo et al., 2016; Bedê et al., 2021), which may prevent oxidative damage of endothelial cells induced by chorine species.

Increased oxidative stress (along with nitrosative stress) plays a central role in the toxic actions of chorine species on the cardiovascular system (Zhang et al., 2001; Summers et al., 2012). Increased oxidation could interact with endothelial nitric oxide production, further reducing its bioavailability and producing the potent toxic oxidant $ONOO^-$, an inducer of protein tyrosine residue nitration (Eiserich et al., 1998). Immunohistochemical assessment of OCl^--exposed arteries showed strong immunoreactivity with the NT antibody, demonstrating upregulated nitrosative stress (Cigremis et al., 2009; Radovits et al., 2013). However, treatment with GJ reduced nitrosative stress and prevented endothelial dysfunction, thus improving endothelium-dependent vascular relaxation.

In conclusion, we showed that OCl^--exposed arteries present with impaired endothelium-dependent vasodilation and increased NT immunoreactivity. Furthermore, GJ can prevent nitrosative stress in vascular tissues, thus helping to avoid endothelial dysfunction induced by chlorine species. These results will contribute to the body of knowledge about GJ and its use as a supplement for prevention of cardiovascular and oxidative diseases.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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