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Grape pomace reduced reperfusion arrhythmias in rats with a high-fat-fructose diet

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Metabolic syndrome (MetS) is a risk factor for sudden cardiac death in humans, but animal models are needed for the study of this association. Grape pomace (GP), obtained from the winemaking process, contains phenolic compounds with potential cardioprotective effects. The aim of this study was to evaluate if a high-fat-fructose (HFF) diet facilitates the occurrence of arrhythmias during the reperfusion, and if a GP supplementation could counteract these effects. Wistar rats were fed with control (Ctrl), HFF diet and HFF plus GP (1 g kg^{-1} day^{-1}) for six weeks. The HFF diet induces characteristic features of MetS (higher systolic blood pressure, dyslipidemia and insulin resistance) which was attenuated by GP supplementation. In addition, HFF induced increased reperfusion arrhythmias that were reduced upon GP supplementation. GP also reduced the non-phosphorylated form of connexin-43 (Cx43) while enhancing heart p-AKT and p-eNOS protein levels and reducing Nox4 levels enhanced by the HFF diet, indicating that GP may increase NO bioavailability in the heart. We found a murine model of MetS with increased arrhythmogenesis and translational value. Furthermore, GP prevents diet-induced heart dysfunction and metabolic alterations. These results highlight the potential utilization of winemaking by-products containing significant amounts of bioactive compounds to prevent/attenuate MetS-associated cardiovascular pathologies.

Introduction

Metabolic syndrome (MetS), a cluster of symptoms characterized by dyslipidemia, hypertension and insulin resistance, is a risk factor for sudden death.[1] Changes in dietary habits such as high consumption of saturated fat and refined sugar predispose/contribute to the development of several metabolic alterations related to MetS. Thus, high fat diets may predispose individuals to a greater risk of cardiac arrhythmias, and it has been shown that cardiac arrhythmia in obesity is associated with an increased risk of sudden cardiac death.[2,3] One method to study the combination of chronic cardiovascular risk and the occurrence of lethal arrhythmias is to evaluate them in a model of ischemia/reperfusion in hearts from rats with dietary induction of MetS. Many strategies that were clearly protective at the experimental level failed to protect in the clinical setting, partly, due to the use of healthy and young animal models. Experiments during the last decade confirmed the therapeutic failures of promising agents when tested in diabetic,[4] obese, insulin resistant,[5,6] MetS[7] and aged[8] animals and highlighted the need for the development of animal models of disease that are representative of human clinical conditions.[9]

Epidemiological studies have established that moderate consumption of vegetables and fruit reduces the risk of cardiovascular disease (CVD).[10–15,16] Grape pomace (GP) is obtained from the winemaking process as the residue remaining after fermentation, mainly constituted of skins and seeds of berries. Interestingly, GP still contains high amounts of bioactive components such as dietary fiber (DF) and phenolic compounds[17,18] without the deleterious effect of alcohol, emphasizing the possible nutritive value of GP with a wide range of applications as food ingredients or dietary supplements.[19–21] The consumption of dietary fiber is associated with important health benefits,[22] including the reduction of cardiovascular disease risk factors.[23] On the other hand, the most abundant polyphenols identified in Malbec GP are flavanols, flavonols, anthocyanins, hydroxybenzoic and hydroxycinnamic acids, and stilbenes.[20,24] We recently show that GP and its extract attenuate high-fat-high-fructose (HFF) diet-induced adiposity and impaired insulin signaling in adipose, liver and muscle tissue in rats.[25]

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In the cardiovascular system as well as in other tissues most of the effects attributed to phenolic compounds are related to protecting/enhancing nitric oxide (NO) bioavailability. This is achieved by activating endothelial nitric oxide synthase (eNOS), an isoenzyme that generates NO and modulates various aspects of cardiac and vascular physiology[26] and/or indirectly protecting NO oxidation by inhibiting/reducing NADPH oxidase (Nox)-induced superoxide production and thus reactive oxygen species.[27,28] In the heart, Nox2 and Nox4 are the main sources of superoxide anion isoforms that could impair nitric oxide availability.[27,28] Nitric oxide behaves as an endogenous antiarrhythmic factor in the isolated rat heart during reperfusion.[29] One of the protective mechanisms of nitric oxide involves preserving intercellular communication through channels formed by protein connexin-43 (Cx43). Nitric oxide controls Cx43 expression through the activation of protein kinase A[30] and attenuated acute ischemia-induced ventricular arrhythmias by preventing Cx43 dephosphorylation.[31] On the other hand, Akt enhanced NO production through activation (phosphorylation) of the eNOS enzyme and has been shown to promote cardiac resistance to ischemia/reperfusion in rats fed the high fat-sucrose diet.[32]

In this study we propose: (i) to evaluate if a diet high in fat and fructose facilitates the appearance of arrhythmias during reperfusion; and (ii) to determine if a GP supplement could prevent the metabolic and electrophysiological effects of the HFF diet by increasing the bioavailability of nitric oxide.

Results

Anthocyanin and non-anthocyanin profiling of GP by HPLC-DAD

The non-anthocyanin and anthocyanin phenolic compositions of GP used for experiments are presented in Tables 1 and 2, respectively. As can be seen in Table 1, a total of fifteen non-anthocyanin compounds of different chemical classes were identified and quantified in the analysed GP. They corresponded to different non-flavonoids (hydroxybenzoic and hydroxycinnamic acids, stilbenes and phenylethanol analogs) and flavonoids (flavanols, including different tannins and flavonols). The maximum concentrations corresponded to the tannins (+)-catechin, (−)-epicatechin and (−)-epicatechin gallate. Syringic acid was also reported at high levels. The relative distribution of the concentration and profile of compounds is in agreement with our previous results achieved for grape pomace extracts.[25] Table 2 summarizes the profile and concentration of anthocyanins in the studied GP. Compounds are grouped based on the type of derivative (non-acylated, acylated and coumarylated). As expected, malvidin 3-*O*-glucoside was the main compound, followed by malvidin 3-*O*-*p*-coumaroylglucoside and petunidin 3-*O*-glucoside. The acetylated derivatives were the least abundant anthocyanins.

GP supplementation reduces SBP and attenuates metabolic parameters in HFF rats

Metabolic parameters are shown in Table 3. The daily food consumption and water intake were similar among groups.

Table 1 Levels of non-anthocyanin phenolic compounds in freeze-dried grape pomace of cv. Malbec

Compound	μg g^{-1} GP
Hydroxybenzoic acids	
Gallic acid	18.1 ± 2.3
Syringic acid	156.7 ± 6.6
Total	**174.8**
Hydroxycinnamic acids	
Caffeic acid	7.1 ± 0.6
p-Coumaric acid	55.1 ± 2.9
Ferulic acid	4.6 ± 0.3
Total	**66.8**
Stilbenes	
trans-Resveratrol	2.1 ± 0.3
Total	**2.1**
Flavanols	
Procyanidin B1	4.2 ± 0.6
(+)-Catechin	258.4 ± 28.8
Procyanidin B2	29.5 ± 6.4
(−)-Epicatechin	173.2 ± 23.3
(−)-Epicatechingallate	253.4 ± 41.3
(−)-Gallocatechin	7.6 ± 1.6
Total	**726.4**
Flavonols	
Quercetin	64.1 ± 10.6
Kaempferol-3-glucoside	12.6 ± 0.3
Total	**76.7**
Other compounds	
Tyrosol	7.8 ± 0.7
Total	**7.8**
Total non-anthocyanins	**1050.3**

Average contents (μg g^{-1} GP) with their standard deviations, n = 3 replicates.

Table 2 Levels of anthocyanins quantified in freeze-dried GP

Anthocyanins	μg g^{-1} GP
Delphinidin 3-*O*-glucoside	301 ± 10
Cyanidin 3-*O*-glucoside	28 ± 2
Petunidin 3-*O*-glucoside	497 ± 23
Peonidin 3-*O*-glucoside	81 ± 4
Malvidin 3-*O*-glucoside	2272 ± 157
Total glucosylated	**3179**
Delphinidin 3-*O*-acetylglucoside	26 ± 2
Peonidin 3-*O*-acetylglucoside	41 ± 3
Malvidin 3-*O*-acetylglucoside	13 ± 1
Total acetylated	**80**
Petunidin 3-*O*-*p*-coumaroylglucoside	92 ± 6
Peonidin 3-*O*-*p*-coumaroylglucoside	15 ± 1
Malvidin 3-*O*-*p*-coumaroylglucoside	1041 ± 77
Total coumaroylated	**1148**
Total anthocyanins	**4407**

Average contents (μg g^{-1} GP) with their standard deviations, n = 3 replicates.

The addition of the HFF diet clustered some features of the MetS, including higher blood pressure, insulin resistance, and elevated plasma triglycerides. Also, the HFF diet significantly increased the body weight compared with the control group. GP supplementation to HFF rats significantly reduced SBP, body weight and restored metabolic alterations such as insulin resistance (evaluated by the homeostatic model of insulin

Table 3 Effects of grape pomace (GP) supplementation on metabolic parameters in high fat fructose fed rats (HFF)

Parameter	Ctrl	HFF	HFF + GP
Food intake (g day^{-1})	23 ± 1	24 ± 1	22 ± 1
Liquid intake (mL day^{-1})	34 ± 3	33 ± 1	32 ± 1
Final body weight (g)	321 ± 5	376 ± 10*	334 ± 10
Final SBP (mmHg)	117 ± 4	136 ± 4*	120 ± 3
Glucose (mg dL^{-1})	96.0 ± 5	89.3 ± 2.3	91.7 ± 3.1
Insulin (ng ml^{-1})	1.10 ± 0.04	1.70 ± 0.04*	1.25 ± 0.10
HOMA:IR	3.5 ± 0.5	5.4 ± 0.6*	4.2 ± 0.8
Triglycerides (mg dL^{-1})	119 ± 3	145 ± 5*	127 ± 6
HDL (mg dL^{-1})	29.8 ± 2.0	23.6 ± 1.3	34.4 ± 2.0**
Total cholesterol (mg dL^{-1})	41.0 ± 3.8	37.5 ± 2.6	31.3 ± 2.8

SBP: systolic blood pressure; HOMA:IR: homeostatic model assessment of insulin resistance; HDL: high density lipoprotein. Values are shown as means ± SEM (n = 9) (one way ANOVA). *p < 0.05 *vs.* Ctrl and GP; **p < 0.05 *vs.* HFF.

resistance HOMA:IR), higher plasma triglycerides and lower HDL cholesterol. No differences were observed in total cholesterol levels among groups. Together, these data indicate that GP supplementation improves cardiovascular and metabolic parameters in rats receiving the HFF diet.

GP supplementation prevented severe reperfusion arrhythmias in HFF rats

The incidence and severity of reperfusion arrhythmias increased in isolated hearts from rats fed with the HFF diet and these changes were prevented by GP supplementation as shown in Fig. 1A and B. Hearts from control rats suffered transient arrhythmias like ventricular premature beats, bigeminy and salvos during the first minute of reperfusion and only ventricular premature beats during the following minutes. In the HFF diet group, hearts developed persistent ventricular fibrillation during reperfusion. These severe arrhythmias are suggested as the main cause of sudden cardiac death. Hearts from the GP supplemented group suffered brief episodes of ventricular tachycardia during the first minute of reperfusion followed by a recovery of sinus rhythm.

GP prevented non-phosphorylated state of Cx43 in rats' hearts

Next, we evaluated Cx43, a protein involved in the intercellular communication between myocytes. Hearts from HFF fed rats and GP supplemented rats showed lateralization of Cx43 in myocytes evaluated by immunohistochemistry (see black arrows in Fig. 2). However, we observed a reduction in the non-phosphorylated form of Cx43 only in hearts from GP supplemented rats.

Effect of GP on heart Akt, eNOS and Nox4 protein levels

Akt activation phosphorylates eNOS, an enzyme that produces NO. On the other hand, Nox activation may increase reactive oxygen species and reduce NO bioavailability. We evaluated the protein content of both enzymes as indicators of nitric oxide bioavailability in hearts (Fig. 3). Chronic fat and fructose consumption leads to a decreased p-eNOS and increased Nox4

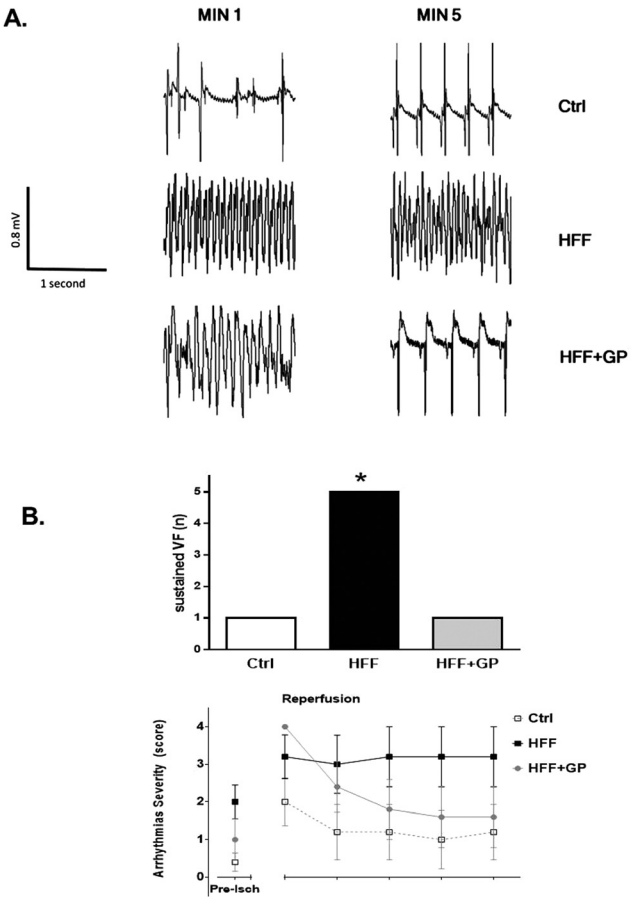

Fig. 1 Effect of dietary GP on arrhythmias during reperfusion. Section A shows pictures of typical electrocardiographic behavior during minutes 1 and 5 of reperfusion and B the incidence and duration of severe arrhythmias during reperfusion. Values are shown as means ± SEM (n = 9). * significantly different from Ctrl and HFF + GP (p < 0.05 by Fisher exact test).

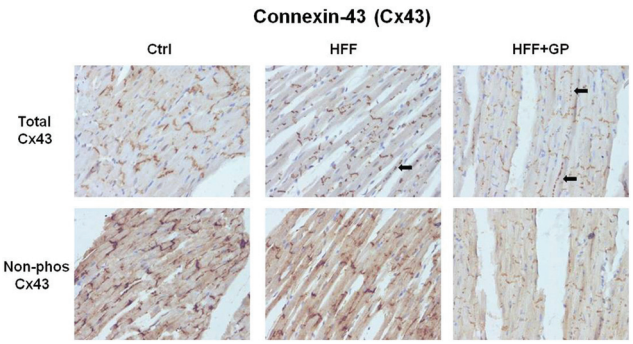

Fig. 2 Effect of GP supplementation on connexin-43 in the heart section. Representative images of connexin-43 (Cx43) and non-phosphorylated Cx43 (non-phos Cx43) evaluated by immunohistochemistry in heart myocytes of rats fed: Ctrl, HFF, and HFF + GP for 6 weeks. Magnification ×400.

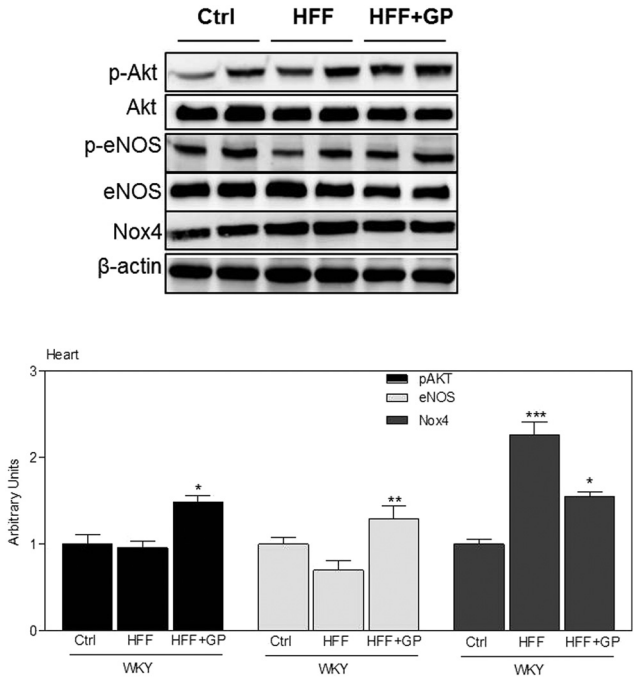

Fig. 3 GP supplementation increased NO bioavailability. Total and phosphorylated AKT and eNOS, and Nox4 were evaluated by western blot in heart homogenates in eight week old rats fed control (Ctrl) diets without or with high fat-fructose diet (HFF 20% each w/w), or HFF supplemented with GP 1 g per kg body weight per d (HFF + GP). Results were expressed as the ratio of phosphorylated/total protein level or protein/β-actin. Bands were quantified, and referred to control group values (Ctrl). Results are shown as the mean ± SEM of four animals/treatment. * significantly different from HFF and Ctrl group; ** significantly different with respect to HFF group and; *** significantly different with respect to Ctrl group ($p < 0.05$, one-way ANOVA).

protein levels, while no significant changes were observed in p-Akt protein levels. However, GP increased p-Akt, p-eNOS and reduced Nox4 protein levels restoring the balance disrupted by the HFF diet. These results indicate that GP enhanced heart eNOS and reduced Nox4 protecting NO bioavailability.

Discussion

This work presents evidence that the addition of fat and fructose to the diet for 6 weeks induced arrhythmias associated with MetS. Rats from the HFF group increased the incidence and duration of severe ventricular arrhythmias compared to the control group. Moreover, supplementation with GP to HFF rats protected the heart by reducing the duration of arrhythmias and restoring normal electrical activity. In addition, GP restores/counteracts HFF-induced body weight gain, insulin resistance, increased SBP, plasma TG levels, and lower HDL cholesterol levels.

Western diets, rich in saturated fat and sugars, are a risk factor for cardiovascular disease nonetheless it is difficult to induce murine models that replicate severe ventricular arrhythmias. For example, a high-fat diet increases the risk of ventricular tachycardia but not fibrillation in female rats.[2] Recently, it was shown that a high fat-fructose diet for six weeks causes increased ventricular fibrillation duration during reperfusion. In contrast to our results, the incidence of sustained ventricular fibrillation after 30 minutes of ischemia was observed only in five of the eleven fat-fructose fed rat hearts, not reaching statistical significance.[33] Other models like fructose fed rats and spontaneously hypertensive rats develop high incidence of sustained ventricular fibrillation during reperfusion after 15 min of regional ischemia, but also control hearts show high incidence.[34] Therefore, we highlight the value of the model described here as a reliable combination of MetS and severe arrhythmogenesis.

GP contains a mix of bioactive compounds such as flavanols, flavonols, anthocyanins, hydroxybenzoic and hydroxycinnamic acids, and stilbenes with important antioxidant[35] and anti-inflammatory[10] properties among others.[36] Experimental evidence suggests that some phenolic compounds exert beneficial effects on blood pressure by increasing endothelial-derived nitric oxide (NO) *via* modulation of eNOS activity and expression, changes in eNOS substrate availability, or the prevention of radical-induced NO conversion caused by enzymes such as NADPH oxidase.[12,37,38] Coronary reperfusion is generally associated with a reduction of endogenous NO production resulting from endothelial dysfunction and tissue damage. In this context, the flavonol quercetin reduces the contractile dysfunction, the infarct size and the pattern of protein expression changes (including iNOS and NOX) induced by cardiac ischemia.[39] In line with this, we observed that GP enhances p-Akt and eNOS and decreases Nox4, suggesting the cardioprotective capacity of bioactive compounds from GP. Accordingly, dealcoholized red wine as well as an important flavanol found in GP, (−)-epicatechin, was able to ameliorate fructose induced biochemical modifications in the heart through modulating the expression and/or activity of Nox and eNOS and thus favoring NO bioavailability.[28,40] Chronic consumption of grape phenolics improved oxidative stress markers by preventing in part NAD(P)H oxidase expression in the left cardiac ventricle in high-fat diet-induced obesity hamsters.[41] Rats supplemented with standard grape extract at a dose of 100 and 200 mg kg^{-1} day^{-1} for three weeks reduced myocardial ischemia reperfusion injury attributed to its *in vitro* antioxidant effect.[42] Moreover, supplementation with red wine polyphenols and red grape skin polyphenolic extract enriched with anthocyanins prevented hypertension, cardiac hypertrophy and production of reactive oxygen species in high fructose-fed rats.[43,44]

NO is an important antiarrhythmic factor in part attributed to its vasodilator effects and to some favorable ventricular repolarization effects. Also, it has been stated that NO could prevent in part arrhythmias *via* modulation of Cx43.[31] Cx43 is the main protein forming gap junctions in the heart. Changes in Cx43 expression have been associated with ischemia-reperfusion injury and ischemia-reperfusion arrhythmia.[30,45] Bian *et al.*,[46] showed that in a rat myocardial ischemia-reperfusion model, atorvastatin protected against myocardial ischemia-reperfusion injury and enhanced the expression of Cx43 by activating the

PI3K/Akt pathway and mitochondrial KATP channels. Thus, indicating that the activation of PI3K/AKT pathway signaling mediates Cx43 expression and phosphorylation. Our results indicate that the antiarrhythmic mechanisms associated with GP administration involve AKT, eNOS, and Cx43.

Overall, GP appears as a promising alternative for different biotechnological applications based on its high content of anthocyanins and non-anthocyanin compounds, all of them related to antioxidant and anti-inflammatory properties. These results highlight the potential utilization of winemaking by-products containing significant amounts of bioactive compounds (dietary fiber and phenolic compounds) to prevent/attenuate MetS-associated cardiovascular pathologies.

Conclusions

The above results suggest that a HFF diet facilitates the occurrence of arrhythmias during reperfusion and supplementation for 6 weeks with wine GP preventing this event, particularly with respect to the length of the arrhythmic episodes, probably secondary to a preventive effect against NO release and bioavailability, and as a consequence preserving Cx43 dephosphorylation and on the other hand, by protecting from altered metabolic parameters.

Methods

The Folin–Ciocalteu reagent was purchased from Merck (São Paulo, Brazil). Standards of gallic acid (99%), (−)-gallocatechin (≥98%), (−)-epicatechin gallate (≥98%), procyanidin B1 (≥90%), (+)-catechin (≥99%), procyanidin B2 (≥90%), (−)-epicatechin (≥95%), caffeic acid (99%), syringic acid (≥95%), *p*-coumaric acid (99%), ferulic acid (≥99%), *trans*-resveratrol (≥99%), quercetin hydrate (95%), kaempferol-3-glucoside (≥99%), and malvidin-3-*O*-glucoside chloride (≥95%) were purchased from Sigma-Aldrich. The standard of tyrosol (≥99.5%) was obtained from Fluka (Buchs, Switzerland). Stock solutions of compounds were prepared in methanol at the concentration levels of 1000 mg mL^{-1} and further dilutions were prepared monthly using methanol and stored in dark-glass bottles at −20 °C. Calibration standards were dissolved in the initial mobile phase of each method (LMW-PPs or anthocyanins). HPLC-grade acetonitrile (MeCN), methanol and formic acid (FA) were acquired from Mallinckrodt Baker (Inc. Phillipsburg, NJ, USA). Both primary–secondary amine (PSA) and octadecylsilane (C18) were obtained from Waters (Milford, MA, USA). Reagent grade NaCl, anhydrous Na_2CO_3, $MgSO_4$, and $CaCl_2$ were purchased from Biopack (Buenos Aires, Argentina).

The antibodies for Nox4 (sc-21860) and AKT (sc-8312) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for phospho-eNOS (#9571), eNOS (#9572), and phospho-AKT (#06248) were from Cell Signaling Technology (Danvers, MA, USA). Cx43 C 6219 was from Sigma-Aldrich and unphosphorylated Cx43 No. 13-8300 was from Zymed Technologies (San Francisco, CA, USA). Nitrocellulose membranes and the western blotting system were obtained from BIO-RAD (Hercules, CA, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Fructose was purchased from Saporiti Labs (Buenos Aires, Argentina). Bovine and porcine fat was from Recreo Refrigerating Industries S.A.I.C. (Santa Fe, Argentina). Commercial kits for the determination of HDL, and triglyceride concentrations were purchased from GTLab (Buenos Aires, Argentina). Unless otherwise noted, reagents were purchased from Sigma (St Louis, MO, USA).

Grape pomace sampling

Malbec (*Vitis vinifera* L.) GP from vineyards located in Gualtallary, Mendoza, Argentina was supplied by the Catena-Zapata local winery. The samples were collected after fermentation and carried to the laboratory in ice cooled boxes. Once in the laboratory, the fresh GP samples were stored at −20 °C prior to their use. GP was freeze-dried for 72 h at 0.12 bar and −45 °C. Freeze-dried GP was ground in a laboratory mixer. The obtained powder was placed in sealed tubes and kept at −20 °C under a dry atmosphere and in darkness prior to its use or analysis. 0.5 g of lyophilized GP and 5 mL of methanol were added with vigorous hand shaking, placed in an ultrasonic bath for 30 min (stirring every 5 min), and centrifuged for 2 min. TPC phenolic analysis was performed for the supernatant.

Malbec grape pomace contains a significant amount of dietary fiber (DF) (53.5%) mainly the insoluble one (94%) as we previously reported.[20,25] The anthocyanin and non-anthocyanin phenolic profiles of GP used for experiments are presented in Tables 1 and 2, respectively.

Total phenolic content

Absorbance measurements were made with a UV-vis spectrophotometer (Cary-50, Varian Inc.). Total phenolic content (TPC) was determined by the Folin–Ciocalteu assay according to Alonso *et al.*[47] TPC was expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE g^{-1}) by using a calibration curve with gallic acid as the standard (three replicates) in a range between 0–200 mg L^{-1} (R^2 = 0.999).

Low-molecular-weight phenolics and anthocyanin analysis

Target polyphenols and individual tannins were analyzed using a previously developed sample preparation approach with some modifications[48] and determined using a LC-MWD system (DionexSoftron GmbH, Thermo Fisher Scientific Inc., Germering, Germany). The Chromeleon 7.1 software was used to control all the acquisition parameters of the LC-MWD system and also to process the obtained data. LC separations were carried out in a reversed-phase Symmetry C18 column (4.6 mm × 250 mm, 5 μm particle sizes, Waters, Milford, MA, USA). Ultrapure water with 0.1% FA (A) and MeOH (B) was used as the mobile phase. Analytes were separated using the following gradient: 0 min, 20% B; 0–2 min, 30% B; 2–8 min, 32% B; 8–14 min, 80% B; 14–22 min, 20% B; 22–32 min, 20%

B. The mobile phase flow was 1 mL min^{-1} and the column temperature was 35 °C. The injection volume for standards and sample extracts was 10 μL. The quantification wavelengths for different families of analytes were 254 nm, 280 nm, 320 nm, and 370 nm.[49] The identification and quantification of the target polyphenols in GP was achieved by comparing of the retention times (R_t) and maximum absorbance values of detected peaks in samples of interest with those obtained with the injection of pure standards. Standard solutions were prepared at 6 concentration levels by diluting appropriate volumes of the working standard solution. For the calibration curve, linear ranges between 0.15 and 50 μg mL^{-1} were obtained, with coefficient of determination (R^2) higher than 0.9961 for all the studied polyphenols.

For anthocyanin determination, an aliquot of extract was diluted to 25 mL with an initial mobile phase of the anthocyanin method and analyzed by HPLC-MWD. Separations were carried out using a reversed-phase column, the same column as that for low-molecular-weight phenolics. The mobile phase consisted of ultrapure H_2O : FA : MeCN (87 : 10 : 3, v/v/v; eluent A) and ultrapure H_2O : FA : MeCN (40 : 10 : 50, v/v/v; eluent B) with the following gradient: 0 min, 10% B; 0–6 min, 25% B; 6–10 min, 31% B; 10–11 min, 40% B; 11–14 min, 50% B; 14–15 min, 100% B; 15–17 min, 10% B; 17–21 min, 10% B. The mobile phase flow was 0.8 mL min^{-1}, column temperature was 25 °C, and the injection volume was 5 μL. Quantifications were carried out *via* area measurements at 520 nm, and the anthocyanin content was expressed as malvidin-3-glucoside, using an external standard calibration curve (1–250 mg mL^{-1}, $R^2 \leq 0.9984$). The anthocyanin compounds detected *via* HPLC-MWD were confirmed by comparing with the elution profile and identified analytes achieved in our previous work.[24]

Animals and diets

Eight week male Wistar rats ($n = 27$) were used for this 6-week supplementation study. All animal studies were conducted in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the "Comité Institucional para el Cuidado y Uso de Animales de Laboratorio, (CICUAL)", protocol approval No. 36/2014. Rats weighing 300–350 g were housed under conditions of controlled temperature (21–25 °C) and humidity with a 12 hour light/dark cycle with access to standard rat chow (Gepsa-Feeds, Buenos Aires, Argentina) and water *ad libitum*. Rats were randomly assigned to the following 3 groups (9 rats per group): Control diet (Ctrl), High Fat-Fructose (HFF) diet (20% bovine fat and 20% fructose w/w added to the standard chow), and HFF supplemented with Grape Pomace (HFF + GP) in a dose of 1 g kg^{-1} day^{-1} added to the standard chow. The amount of GP used for dietary supplementation was based on the levels that are feasible to reach through dietary supplementation in humans, on our preliminary study using the same amount of GP,[25] and on a study performed in high fructose-feed rats.[50] The food was changed every two–three days. Food and water intakes were determined three times a week, and body weight was recorded weekly. The systolic blood pressure (SBP) was measured by the tail-cuff method and determined with a plethysmography Koda2® (Kent Scientific Corporation, USA) at the beginning, middle and last week of the protocol. After six weeks of treatment rats were sacrificed. Prior to an overnight fast, rats were weighed, anesthetized with ketamine (50 mg kg^{-1}) and acepromazine (1 mg kg^{-1}) and the blood was collected from the abdominal aorta into ice chilled EDTA tubes. Plasma was obtained after centrifugation at 3000 rpm for 15 min at 4 °C.

Biochemical determinations

Glucose was measured in the blood collected from the tail using a glucometer (Accu-Chek Performa, Roche, Argentina). Triglycerides (TG), high-density lipoprotein (HDL)-cholesterol, and total cholesterol concentrations were determined in plasma by enzymatic colorimetric methods using commercial kits (GTLab, Bs.As., Argentina) and measured with an automated spectrophotometer. Insulin was measured using the Ultra Sensitive Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA), following the manufacturer's guidelines. Insulin resistance was assessed using the homeostasis model assessment (HOMA-IR) parameter using the following formula: HOMA-IR (mg dL^{-1} × μU mL^{-1}) = fasting glucose (mg dL^{-1}) × fasting insulin (μU mL^{-1})/405.

Ischemia-reperfusion procedures

Hearts of 20 rats (7 from the Ctrl group, 6 from the HFF group, and 7 from HFF + GP) were immediately extracted and perfused according to the Langendorff technique with a modified Krebs–Henseleit solution, containing (mM): 121 NaCl, 25 $NaHCO_3$, 1.2 Na_2HPO_4, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 11 glucose, equilibrated with 5% CO_2 in O_2, as we previously described.[34] Hearts were stabilized for 15 min followed by three periods of surface electrogram recording: preischemia (5 min), regional ischemia (10 min), induced by ligation of the anterior descending coronary artery, and reperfusion (10 min). Surface electrograms equivalent of lead II were obtained using a Hewlett-Packard 1500A and digitized with an analog to digital converter NI PCI-6221 (National Instruments, Austin, TX, USA) and recorded using LabView Signal Express 2.5. Ventricular arrhythmias were classified according to the Lambeth convention. We evaluated the incidence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF). We also evaluated the arrhythmias severity each minute using the following score: 0 – sinus rhythm, 1 – premature ventricular beats or bigeminy, 2 – salvos, 3 – non-sustained VT (<30 s) 4 – sustained VT (>30 s) or VF.

Three hearts from each group were excised, washed, weighed, and immediately frozen in liquid nitrogen and then stored at −80 °C until further analysis, or stored in 4% paraformaldehyde for histological analysis.

Western blot analysis

Hearts were homogenized in (RIPA) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% w/v sodium dodecylsulfate, 1%

w/v Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate and protease inhibitors). Homogenates were centrifuged at 15 000*g* for 30 min, the supernatant collected, and protein concentration was measured using the Bradford method.[51] Aliquots of total cell lysates from rat's heart homogenates containing 30 μg of protein were denatured with Laemmli buffer, separated by reducing 8–10% (w/v) polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membranes as we previously described.[52] Membranes were blotted for 2 h in 5% (w/v) nonfat milk, and subsequently incubated in the presence of the corresponding primary antibodies p-AKT, AKT, eNOS, and Nox4 (1 : 1000 dilution for all the antibodies) overnight at 4 °C. After incubation for 90 min at room temperature in the presence of the secondary antibody (either HRP or biotinylated antibody (one hour with Extravidin afterward when it's biotinylated)) the conjugates were visualized and quantified by chemiluminescence detection in a Luminescent Analyzer Image Reader (LAS-4000) Fujifilm. The densitometric analysis was performed using the Image J Program.

Immunohistochemistry analysis

Heart tissue sections for histology were prepared from paraffin-embedded samples and cut transversely on a microtome (Microm HM325, Walldorf, Germany) with a thickness of 5 μm each, and dewaxed in xylol, rehydrated, and incubated with 1.5% hydrogen peroxide (H_2O_2) for 30 min to quench endogenous peroxidase activity. After washing in tris-buffered saline 0.05% Triton X-100 (0.05 M Tris, 0.15 M NaCl; pH 7.6), and nonspecific blocking with 1% BSA, nonfat milk (3.5%) for 30 min at room temperature, the tissue was incubated overnight with primary antibody anti-connexin 43 rabbit monoclonal (diluted 1 : 2000) to recognize the phosphorylated (P-Cx43) and unphosphorylated (P0-Cx43) forms of Cx43; and with unphosphorylated Cx43 mouse monoclonal (diluted 1 : 200) to recognize the unphosphorylated form of Cx43 at room temperature. After additional washings, sections were incubated with the corresponding biotinylated-secondary antibody (dilution 1/100) for 2 hours at room temperature and then incubated with Extravidin-HRP (dilution 1/100) for 1 hour. Finally, the color of the immune stained sections was enhanced using diaminobenzidine, glucose oxidase, and nickel ammonium sulfate. Slices were mounted, dehydrated, and cover slipped with synthetic Canada balsam (Laboratorio Cicarelli, Buenos Aires, Argentina). The images were examined under an optical microscope (Nikon Optiphot-2, Kanagawa, Japan) and digitalized with a digital camera (Panasonic GP-KR222 color CCD, Panasonic, Osaka, Japan).

Statistical analysis

Variables measured from GP analysis were expressed as mean ± SD, and data from the *in vivo* study were expressed as mean ± SEM. The statistical significance was assessed by one-way ANOVA followed by Bonferroni's multiple comparison post-test or Fisher's exact test, as appropriate. GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. Differences were considered significant at $p < 0.05$.

Conflicts of interest

There are no conflicts to declare.

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