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Dietary supplementation of a sulforaphane-enriched broccoli extract protects the heart from acute cardiac stress

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ABSTRACT

Cardiac arrythmias play a critical role in several pathological conditions. Importantly, increased arrhythmic risk is associated with systemic oxidative stress and activation of the autonomic nervous system. Thus, we hypothesized that dietary antioxidant supplementation may help in reducing cardiac stress-induced arrhythmias. Sulforaphane (SFN), an isothiocyanate present in *Brassicaceaes,* is recognized as a powerful health-promoting compound with known antioxidant properties. Then, we aimed to generate a broccoli extract (BE) enriched in SFN and determine whether oral BE supplementation induced cardio-protection during acute cardiac stress in rats. BE decreases cardiac sympathetic drive and increases parasympathetic cardiac modulation as evidenced by heart rate variability (HRV) shifts. In addition, isoproterenol-induced cardiac stress (a sympathomimetic agent) induced a ~ 4-fold increase in arrhythmia incidence and this effect was almost completely abolished by BE treatment. In conclusion, dietary supplementation with a BE regulates cardiac autonomic drive and protects the heart from acute cardiac stress.

1. Introduction

Reactive oxygen species (ROS) are physiologically relevant signal molecules. However, ROS accumulation due to production/elimination rate mismatch lead to the loss of redox homeostasis precipitating the development of pathophysiological conditions (Sies & Jones, 2020). Importantly, ROS accumulation has been proposed to represent a corner stone in the development of cardiovascular diseases such as hypertension and heart failure (Florea & Cohn, 2014; Haspula and Clark, 2018). More importantly, cardiac arrhythmogenesis is closely linked to ROS formation (Neuman et al., 2007; Jeong et al., 2012). It has been shown that acute administration of sympatho-mimetic agents, like the β-adrenergic receptor agonist Isoproterenol (Iso), results in cardiac local increase in oxidative stress which triggers the development of cardiac arrhythmias (Zhang et al., 2005). The latter is of clinical relevance since adrenergic stimulation during cardiac stress may lead to life-threatening events. Then, molecules that can protect the heart from the adverse consequences of acute cardiac stress may have beneficial effects.

Abbreviations: BP, Blood pressure; CTRL, Control; DBP, Diastolic BP; ECG, Electrocardiogram; FRAP, Ferric ion reducing ability; FRSA, Free radical scavenging ability; GFN, Glucoraphanin; HR, Heart rate; HRV, HR variability; Iso, Isoproterenol; Keap-1, kelch-like ECH-associated protein 1; LV, Left ventricle; LVCO, LV cardiac output; LVEDP, LV end diastolic pressure; LVEDV, LV end diastolic volume; LVEF, LV ejection fraction; LVESV, LV end systolic volume; LVSV, LV stroke volume; MABP, Mean arterial BP; Nrf-2, Nuclear factor erythroid 2–related factor 2; PP, Pulse pressure; ROS, Reactive oxygen species; SBP, Systolic BP; SFN, Sulforaphane; TPC, Total phenolic compounds.

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Natural antioxidants products from fruits and vegetables has been extensively proposed as non-pharmaceutical approaches to reduce oxidative stress levels (Kaur & Kapoor, 2001). One potential molecule that fulfills these criteria is sulforaphane (SFN). SFN is normally present in *Brassicaceaes*, where broccoli represents a main source for this compound (Zhang & Tang, 2007; Podsędek, 2007). Interestingly, it has been suggested that extracts made from broccoli sprouts or seeds may have salutary effects since SFN can regulate upstream molecular targets including but not limited to the activation of the nuclear factor erythroid 2–related factor 2 (Nrf-2), a master regulator of antioxidant enzymes gene expression (Zhang & Tang, 2007). Despite broccoli extracts has been considered as a promising molecular antioxidant supplement, limited data showing its role in physiologically relevant scenarios are available. Accordingly, in the present study we first generate an SFN-enriched broccoli extract (BE) and tested whether BE dietary supplementation protect the heart during acute cardiac stress.

2. MATERIAL AND methods

2.1. Preparation of the broccoli extract

Broccoli heads (*Brassica oleracea* var. *italica*) cv. Imperial were kindly provided by *Agrocesar Ltda.* (Curacaví, Chile). Broccoli heads were washed, cut into 3-cm width pieces, and 400 g of broccoli pieces were blanched at 57 °C for 13 min in a thermostatic water bath (Stuart, United Kingdom). After that, broccoli pieces were air-dried at 60 °C, until attaining 40% moisture content. Dehydrated broccoli was homogenized, pulverized and mixed with methylene dichloride and sodium sulfate anhydrous (4 L methylene dichloride and 40 g sodium sulfate anhydrous per 100 g rehydrated broccoli). The extract was then recovered by filtration (0.22 μm PVDF filter) and concentrated in a rotatory evaporator.

2.2. Analytical determinations and characterization of broccoli extract

2.2.1. Sulforaphane and glucoraphanin content

SFN and glucoraphanin (GFN) were quantified by reverse phase HPLC using the method proposed by Kenneth (1990) and Liang, Yuan, Dong, and Liu (2006) and Francisco et al. (2009), respectively. All chemicals were HPLC grade and purchased from Merck (Darmstadt, Germany).

2.2.2. Total phenolic compounds

The Folin-Ciocalteau method was used to estimate total phenolic compounds (TPC) (Faller & Fialho, 2009). Results were expressed as mg of gallic acid equivalents per gram of dry matter (mg GAE/g DM).

2.2.3. Free radical scavenging ability

The free radical scavenging ability (FRSA) was measured using 2,2-diphenyl-1-picryhydrazyl (DPPH·) (Brand-Williams, Cuvelier, & Berset, 1995). Results were expressed in Trolox equivalents.

2.2.4. Ferric reducing ability

The ferric ion reducing ability (FRAP) was measured according to Zhou et al. (2016).

2.3. Animal studies and ethical considerations

All animals were housed at 25–27 °C and received *ad libitum* access to food and water. All experiments were made in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guía para el Cuidado y Uso de los Animales de Laboratorio from ANID. Experimental protocols were approved by the Ethics Committee for Animal Experiments of the Pontificia Universidad Católica de Chile (Authorization No. 170914006).

2.4. Physiological experiments

Adult male Sprague-Dawley rats (n = 10) of 250–300 g were randomly assigned to broccoli extract (BE) and control (CTRL) treatment. BE group received two weeks of BE by oral gavage at 3 mg/kg on a daily basis. CTRL group (n = 5) received saline (0.9% NaCl) by gavage. Blood pressure (BP), electrocardiogram (ECG) and left ventricular hemodynamic parameters were measured in anaesthetized animals (α-chloralose 800 mg/Kg; urethane 40 mg/Kg) before ($CTRL_{Iso}$- and BE_{Iso}-) and after ($CTRL_{Iso}$ + and BE_{Iso} +) the induction of cardiac stress with intravenous injections of isoproterenol (1 mg/kg). At the end of the experiments, all animals were euthanized via anesthetic overdose (sodium pentobarbital 100 mg/kg i.p.).

2.4.1. Cardiac function assessment

Left ventricular (LV) cardiac parameters were measured as previously described (Andrade et al., 2019). Briefly, a conductance catheter (Millar, USA) was placed inside the right carotid artery to record baseline systolic (SBP), diastolic (DBP), mean arterial (MABP), and pulse pressure (PP). Then, the catheter was advanced into the LV to obtain end-diastolic volume (LVEDV), end-systolic volume (LVESV), LVED pressure (LVEDP), stroke volume (SV), cardiac output (CO), ejection fraction (EF) and maximum and minimum value of first derivative of LVP (dP/dt_{max} and dP/dt_{min}, respectively).

2.4.2. Arrhythmia incidence

Arrhythmias were defined as a premature or delayed beats that produce a change in heart rate >2.5 standard deviation (SD) from the mean (Andrade et al., 2019). Arrhythmia index was calculated over 30 min before and after Iso administration.

2.4.3. Heart rate variability (HRV)

Autonomic balance was indirectly evaluated through HRV analysis. R-R time series obtained from 10 min ECG baseline recordings were submit to autoregressive frequency domain analysis using the following cut-off frequencies: low frequency (LF; 0.04–0.6 Hz) and high frequency (HF; 0.6 –2.4 Hz). LF/HF ratio was used as an indicator of cardiac sympathovagal balance. LF and HF were expressed as normalized units (n.u.). HRV analysis was performed in Kubios Premium version 3.0.1 (Kubios, Finland).

2.4.4. Electrocardiography analysis

ECG waveforms were analyzed from 15 to 20 cardiac cycles using the ECG plug-in from LabChart 7.0 software (ADinstruments, New Zeland).

2.4.5. Analysis of cardiac oxidative stress

Superoxide levels in the LV were measured using the superoxide-sensitive fluorescent dye dihydroethidium (DHE). Snap-frozen LV biopsies were sectioned on a cryostat at 50 μm thickness at −20 °C and placed onto electrostatic-charged microscope slides (Superfrost, USA). LV sections were incubated with 1 μM DHE (Life technology, USA) in PBS for 30 min at 37 °C, and washed three times for 5 min in PBS and mounted with fluorescent mounting media (Vector Laboratories, USA). Images were acquired with a laser confocal microscope (LSM710, Zeiss, Germany). Slides were scanned (10 fields per heart section, 20 × magnification) and images were processed using ImageJ software (NIH image) to estimate fluorescence intensity.

2.5. Data analysis

Data is presented as mean ± SD. Unpaired and parametric T-test analysis or Two-Way ANOVA followed by Holm-Sidak post hoc was performed according to data structure. A p value < 0.05 was set as the level of statistical significance.

Table 1
Effect of broccoli extract (BE) on cardiac hemodynamic parameters.

	CTRL (n = 5)	BE (n = 5)
LVESV (μl)	35.8 ± 6.1	50 ± 17.6
LVEDV (μl)	108.6 ± 30.0	142.7 ± 32.3
SV (μl)	72.8 ± 28.1	97.7 ± 18.3
HR (bpm)	337.2 ± 11.1	348.1 ± 24.2
CO (ml min^{-1})	24.4 ± 8.9	32.1 ± 5.8

Data is presented as mean ± S.D. CTRL: Control; BE: broccoli extract; LVESV: left ventricle end systolic volume; LVEDV: left ventricle end diastolic volume; SV: Stroke Volume; HR: heart rate; CO: cardiac output. Unpaired T-test analysis.

3. Results

3.1. Results

3.1.1. Characterization of lyophilized broccoli extract

Extracts from natural sources usually contain several compounds, depending on the solvent and the solubility of the molecules. Broccoli extract was analyzed in terms of SFN and GFN content as bioactive compounds of interest, which exhibit indirect antioxidant activity that cannot be detected by traditional *in vitro* assays. Since the extract most likely contained other molecules that may contribute to its antioxidant activity, we conducted *in vitro* assays in order to quantify antioxidant properties unattributable to SFN and GFN present in the extract. Dehydrated broccoli (before extraction) contained 0.130 ± 0.002 mg SFN per gram while the lyophilized extract contained 149.1 ± 1.9 mg SFN per gram, representing a 1,146-fold increase in SFN concentration. Then, delivering SFN as an extract allows reaching therapeutic doses unlike administering dehydrated broccoli, since a 3 mg/kg gavage equals 0.020 g of lyophilized extract and 23 g of dehydrated broccoli.

Glucoraphanin content in the lyophilized extract was 0.16 ± 0.01 mg per gram, being negligible compared to SFN content. This result suggest two facts: (1) GFN conversion to SFN during preprocessing of broccoli before extraction was close to 100% and therefore there were only traces of GFN in the vegetal material before extraction; and (2) the first step of extraction used an organic solvent, and SFN solubility in this solvent is much higher than GFN solubility; therefore only traces of GFN were recovered in back extraction. Probably our results obey to a combination of both effects.

Total polyphenols content (TPC) in the lyophilized extract was 8.8 ± 0.5 mg GAE per gram. This value is only slightly higher than that observed for dehydrated broccoli (5.9 ± 0.09 mg GAE per gram), suggesting that the recovery of phenolic compounds in the extract was significantly lower than SFN recovery. The reason is the low solubility of polyphenols in organic solvents. FRAP of the lyophilized extract was 5.3 ± 0.1 mg TE per gram, being significantly higher than FRAP found in dehydrated broccoli (1.8 ± 0.2 mg TE per gram). FRSA of the extract was 2.9 ± 0.04 mg TE per gram, agreeing with that of dehydrated broccoli (2.9 ± 0.2 mg TE per gram). Antioxidant activity of the extract is significantly lower than that found in widely recognized antioxidant foods, such as *Berberis microphylla* (34.8 ± 1.5 mg TE per gram dry weight).

The chemical characterization of the lyophilized broccoli extract indicates that the main compound is SFN, with low TPC and antioxidant activity, and therefore the physiological effects of administering the lyophilized extract can be attributed to sulforaphane.

3.1.2. Effects of BE supplementation on baseline cardiovascular parameters

Broccoli extract dietary supplementation have no negative/adverse effects on vascular and cardiac physiology on healthy animals (Table 1). Compared to untreated animals, BE-treated group showed similar SBP, DBP, MABP, PP and HR values (**Table S1**). In addition, BE-treated rats showed no changes in cardiac chamber dimensions compared to CTRL rats (Table 1). Also, active and passive properties of the heart were similar between groups (**Table S1**). In contrast, cardiac autonomic

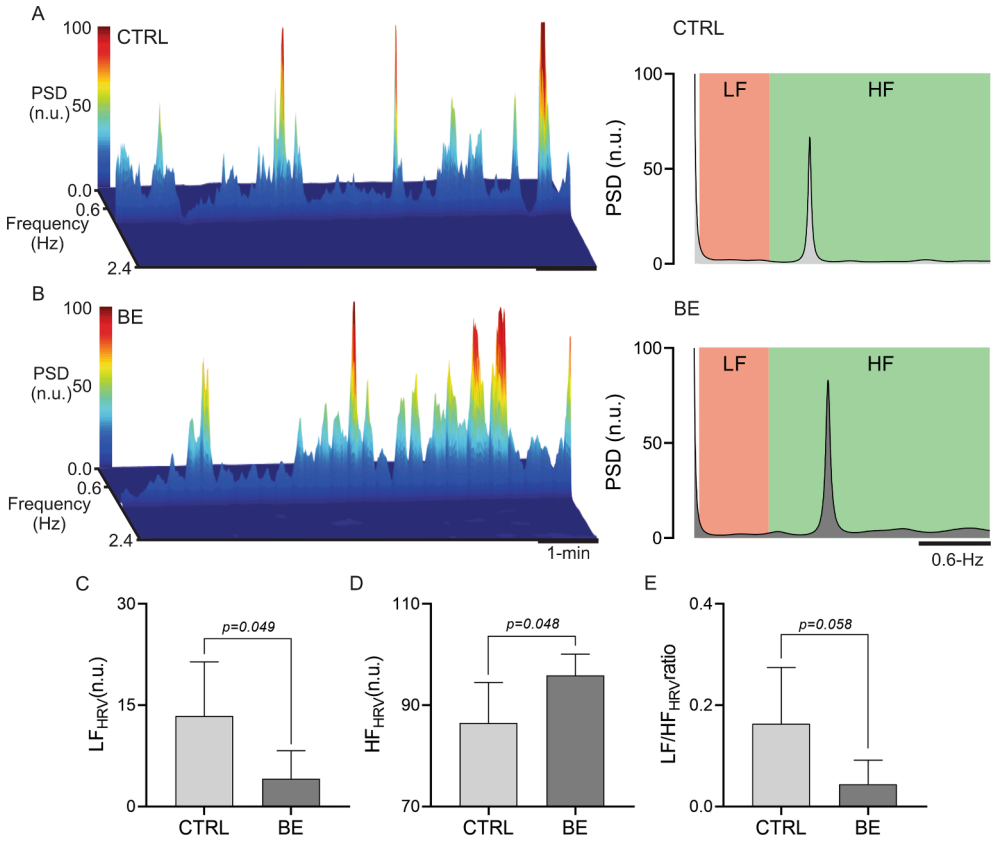

Fig. 1. *BE dietary supplementation shifts autonomic balance toward cardiac parasympathetic modulation.* (A) Colormap of representative time-varying domain analysis of heart rate variability (HRV) (left) and representative HRV spectra (right) during baseline recording in one CTRL rat (LF, low frequency of HRV; HF, high frequency component of HRV). (B) Colormap of representative time-varying domain analysis of HRV (left) and representative HRV spectra (right) during baseline recording in one BE-treated rat. (C) Summary data showing LF and HF component, and LF/HF ration of HRV. Unpaired T-test analysis. CTRL, n = 5; BE, n = 5.

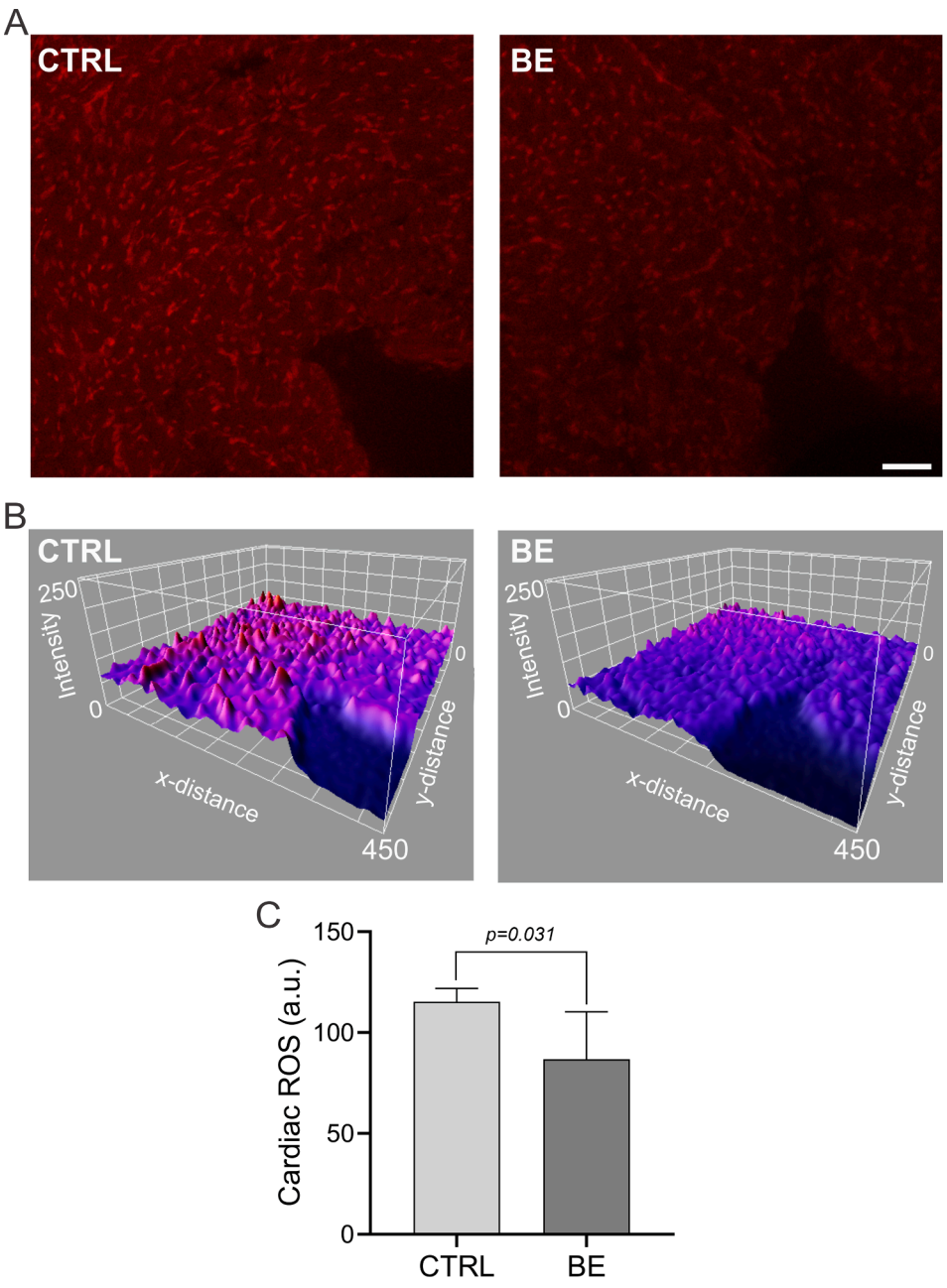

Fig. 2. *Dietary broccoli extract (BE) supplementation reduce cardiac oxidative stress.* (A) Representative images of ROS formation determined by dihydroethidium (DHE) staining (20x magnification). Note that BE treated rats showed a decrease DHE staining compared to CTRL group. Scale bar, 50 μm. (B) Representative DHE fluorescence intensity colormap from one CTRL rat and one BE rat. (C) Summary data showing the effects of BE supplementation on cardiac tissue ROS formation in arbitrary units (a.u.). Unpaired T-test analysis. CTRL, n = 5; BE, n = 5.

balance in BE-treated animals showed a significant shift towards vagal modulation compared to untreated rats (Fig. 1**A**). Indeed, BE rats showed a decreased LF component of HRV compared to CTRL group (4.1 ± 4.2 vs. 13.4 ± 8.0 n.u., BE vs. CTRL group, respectively) (Fig. 1**B**) and a significant increase in the HF component of HRV (86.5 ± 8.0 vs. 95.9 ± 4.2 n.u., CTRL vs. BE, respectively) (Fig. 1**C**). Accordingly, the LF/HF ratio decreases in BE groups compared to CTRL group (Fig. 1**D**). Total power of HRV showed a trend to decrease in BE animals compared to CTRL animals but this was not statistically significant (**Table S2**). Cardiac autonomic effects in BE group were not associated with changes in cardiac electrophysiology since no alterations in resting ECG were found in BE compared to CTRL animals (**Table S2**).

3.1.3. BE dietary supplementation reduce cardiac oxidative stress

Cardiac oxidative stress was evaluated through quantification of DHE staining in heart biopsies (Fig. 2**A and B**). Compared to untreated rats, heart from rats that underwent dietary BE supplementation showed a significant reduction in oxidative stress levels (Fig. 2**C**). Indeed, hearts from CTRL animals display ~ 25% more DHE staining compared to the hearts obtained from BE-treated animals (115.4 ± 2.9 vs. 86.7 ± 10.5 a.u.; CTRL vs. BE, respectively).

3.1.4. Protective effects of BE during acute cardiac stress

Both groups showed similar arrhythmia incidence during baseline recordings (12.0 ± 9.9 vs. 9.0 ± 9.6 events/hour, CTRL vs. BE group, respectively, $p > 0.05$) (Fig. 3**A and B**). However, dietary broccoli extract supplementation for two weeks significantly protects the heart from acute stress-induced cardiac arrhythmogenesis. Indeed, CTRL animals showed a ~ 3-fold increase in arrhythmia index following acute Iso stimulation (38.4 ± 19.6 vs. 12.0 ± 9.9 events/hour, $CTRL_{Iso+}$ vs. $CTRL_{Iso-}$, respectively; $p < 0.05$) and this response was no longer present in rats that underwent BE treatment before Iso administration (4.0 ± 3.2 vs. 8.9 ± 9.9 events/hour, BE_{Iso+} vs. BE_{Iso-}, respectively; $p > 0.05$). Importantly, broccoli extract did not modify cardiac chronotropic nor

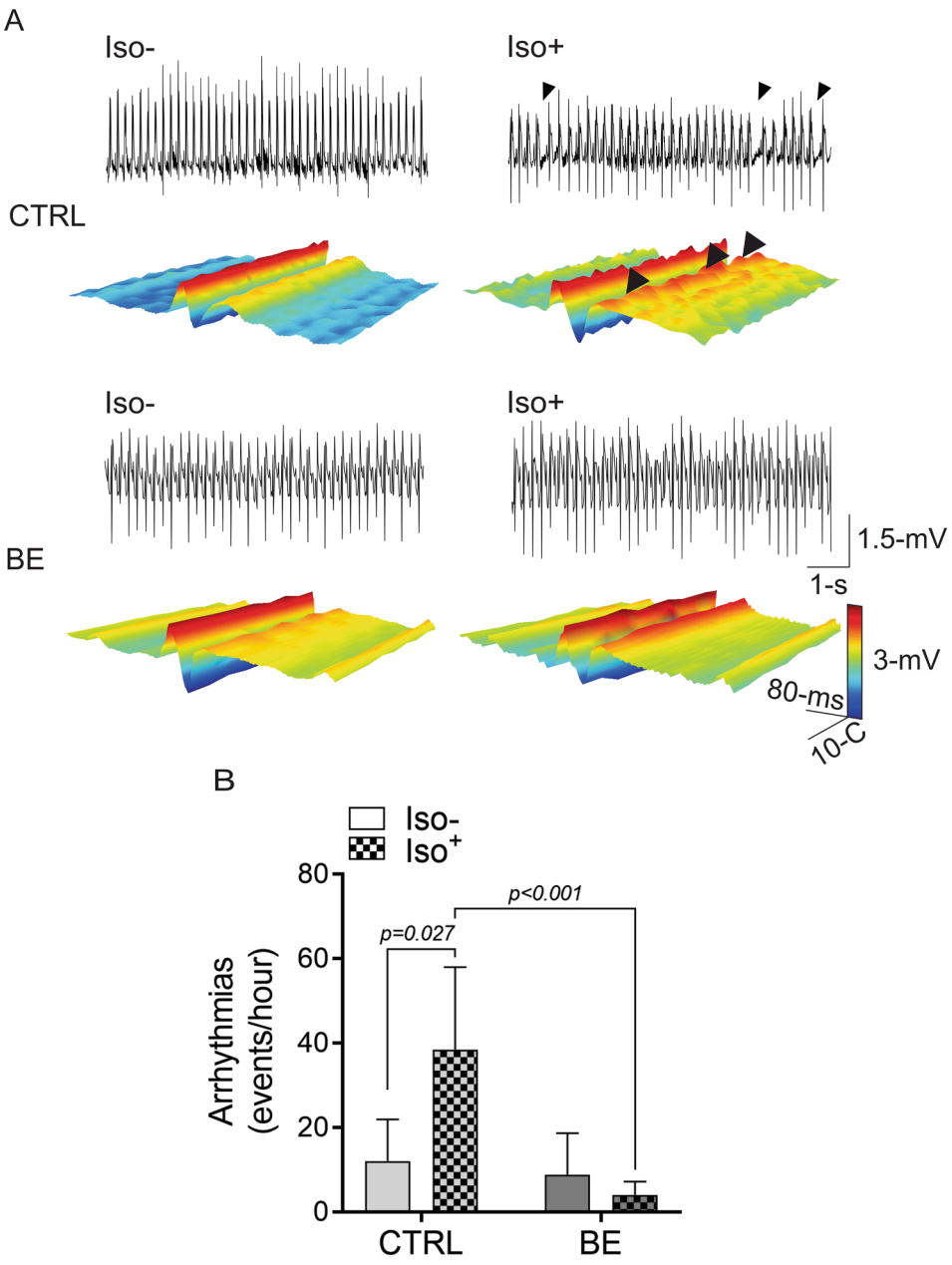

Fig. 3. *Oral treatment with a broccoli extract protects against the heart from cardiac stress-induced cardiac arrhythmias.* (A) Representative recording of electrocardiogram (ECG) and waterfall plot of continuous ECG waves for one CTRL rat (up) and BE-treated rat (bottom) before and after isoproterenol (Iso) -induced cardiac stress. Arrowheads showed the presence of arrhythmic events; (B) Summary data showing arrhythmia incidence during Iso. Two-Way ANOVA followed by Holm Sidak *post hoc*. CTRL, n = 5; BE, n = 5.

ionotropic response to β-adrenergic stimulation then no compromising cardiac function (Fig. 3 **and Table S2**).

4. Discussion

The main findings of the present study are: (i) we produced a BE enriched in SFN, (ii) dietary supplementation with BE prevent cardiac arrhythmogenesis during acute cardiac stress, (iii) BE treatment shifts HRV towards parasympathetic modulation at baseline conditions and reduced cardiac oxidative stress, and (iv) BE supplementation in the diet is safe and have no detrimental effects on hemodynamics and/or cardiac function. Together, our findings support a salutary effect of BE as a dietary supplement to prevent the adverse consequences of acute cardiac stress.

Broccoli extracts with biologically relevant functions (i.e. antioxidants) has been previously described (Farag & Motaal, 2010). However, only limited data is available regarding *in-vivo* functional studies using animals or humans since low SFN concentration has been previously achieved. In the present study we developed an efficient extraction process that gives higher levels of SFN per gram of broccoli tissue. Indeed, extraction/purification method was optimized to obtain up to ~ 150 mg of SFN per gram of broccoli tissue. Oral administration of BE in supplementation with normal dietary regimen in rats for 2-weeks did not modified any gross baseline cardiovascular parameter (i.e. blood pressure, heart rate). However, we cannot rule out the possibility that longer periods of oral BE supplementation may result in different outcomes. In our hands, oral BE administration for 2 weeks was safe but long-term interventions warrant further investigations.

Antioxidants intake has been associated with several health benefits in cardiovascular disease since oxidative stress is considered a pivotal mechanism underlying disease development and progression (Maxwell and Lip, 1997). Indeed, free radical accumulation can initiate lipid peroxidation and DNA damage of the heart leading to cardiac hypertrophy (Franssen, Chen, Hamdani, & Paulus, 2016). Furthermore, oxidative stress-related functional changes have also been described since ROS accumulation alters ionic currents in cardiomyocytes

contributing to arrhythmias (Sovari, 2016). Importantly, cardiac arrhythmias are considered as risk factors for developing life-threating events (Huikuri, Castellanos, & Myerburg, 2001). Here we reported that 2-weeks of BE treatment markedly reduced the incidence of cardiac arrhythmias following acute isoproterenol stimulation *in-vivo* and reduced cardiac oxidative stress as evidenced by reduction in baseline DHE levels. Several mechanisms may partially explain the observed beneficial effects of BE on cardiac physiology during acute stress. SFN, the main bioactive compound found in broccoli and present in higher amounts in BE, may induce Nrf-2 pathway (Lawrence, 2009). Then, BE may have increased the expression of one or more molecular components of the endogenous antioxidant defense system through the regulation of the Nrf-2 signaling pathway in the heart. In line with this, it has been shown that up-regulation of Nrf-2 has cardioprotective effects (Chen & Maltagliati, 2018). Then, it is plausible to hypothesized that the protective effects of BE on cardiac stress-related arrhythmia generation may be associated with improved redox balance within the cardiac tissue. Whether this effect may be mediated by SFN or any other antioxidant/protective bioactive compound present in the BE remain to be determine. Future studies should focus on the cellular/molecular effects of this BE in the heart redox balance and antioxidant capacity.

Additionally, we found that BE supplementation significantly shifted HRV towards a more parasympathetic modulation of the heart. The autonomic nervous system orchestrates cardiac adjustments to cope metabolic demand in a beat-to-beat basis. Normally, activation of the sympathetic nervous systems drives increases in cardiac output while activation of the parasympathetic limb of the autonomic nervous system decreases cardiac output (Paton, Boscan, Pickering, & Nalivaiko, 2005). However, autonomic imbalance often leads to cardiac function impairment and the development of cardiovascular diseases. Interestingly, pathological autonomic imbalance is characterized by withdrawal of cardiac parasympathetic modulation and increases in sympathetic drive (Paton et al., 2005). Here we reported that BE increases parasympathetic modulation of the heart in healthy animals. How BE may change the activity of autonomic nervous system neurons is completely unknown. However, it has been shown that SFN can cross the blood brain barrier (Jazwa et al., 2011). Therefore, BE may exert its effects at the central nervous system in areas associated with autonomic regulation through the regulation of redox balance by a SFN-mediated mechanism. Future research should assess the effects of BE supplementation on central nervous system structures related to autonomic control.

5. Conclusions

Oral dietary supplementation with a SFN-enriched BE protects the heart during acute cardiac stress by limiting the incidence of cardiac arrhythmias. Importantly, BE protective effects take place without compromising cardiac function. Finally, BE also shifts HRV towards parasympathetic modulation, suggesting that BE supplementation may help improving cardiovascular health by sympathetic nervous system unloading.

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Ethical considerations

All experiments involving the use of laboratory animals were made in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guía para el Cuidado y Uso de los Animales de Laboratorio from ANID. Experimental protocols were approved by the Ethics Committee for Animal Experiments of the Pontificia Universidad Católica de Chile (Authorization No. 170914006).

CRediT authorship contribution statement

Katherin V. Pereyra: Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. **David C. Andrade:** Methodology, Formal analysis, Data curation, Writing - original draft. **Camilo Toledo:** Methodology, Writing - original draft. **Karla G. Schwarz:** Writing - review & editing. **Atenea Uribe-Ojeda:** Writing - review & editing. **Angélica P. Ríos-Gallardo:** Writing - review & editing. **Rodrigo A. Quintanilla:** . **Samuel Contreras:** Methodology. **Andrea Mahn:** Methodology, Formal analysis, Writing - review & editing. **Rodrigo Del Rio:** Conceptualization, Investigation, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104267.

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