



Profile of pterostilbene-induced redox homeostasis modulation in cardiac myoblasts and heart tissue

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This study was designed to investigate the effect of pterostilbene (PTS) on cardiac oxidative stress *in vitro*, as this is a simple and promising methodology to study cardiac disease. Cardiac myoblasts (H9c2 cells) and homogenised cardiac tissue were incubated with the PTS and cyclodextrin (PTS + HPβCD) complex for 1 and 24 h, respectively, at concentrations of 50 μM for the cells and 25 and 50 μM for cardiac tissue. The PTS + HPβCD complex was used to increase the solubility of PTS in water. After the pretreatment period, cardiomyoblasts were challenged with hydrogen peroxide (6.67 μM) for 10 min, while cardiac tissue was submitted to a hydroxyl radical generator system (30 min). Cellular viability, oxidative stress biomarkers (e.g. total reactive oxygen species (ROS), carbonyl assay and lipoperoxidation) and the antioxidant response (e.g. sulfhydryl and the antioxidant enzyme activities of superoxide dismutase, catalase and glutathione peroxidase) were evaluated. In cardiomyoblasts, the PTS + HPβCD complex (50 μM) increased cellular viability. Moreover, the PTS + HPβCD complex also significantly increased sulfhydryl levels in the cells submitted to an oxidative challenge. In cardiac tissue, lipid peroxidation, carbonyls and ROS levels were significantly increased in the groups submitted to oxidative damage, while the PTS + HPβCD complex significantly reduced ROS levels in these groups. In addition, the PTS + HPβCD complex also provoked increased catalase activity in both experimental protocols. These data suggest that the PTS + HPβCD complex may play a cardioprotective role through a reduction of ROS levels associated with an improved antioxidant response.

Keywords. Antioxidant enzymes; cardiac myoblasts; cardiac tissue; cyclodextrin; H9c2 cells; oxidative damage; oxidative stress; pterostilbene

1. Introduction

Cardiovascular diseases are among the major causes of death and disability worldwide (WHO 2017). Many experimental models can be used to explore the mechanisms of cardiac disease and to suggest new therapeutic alternatives. Cell culture using cardiomyoblasts is a simple and promising methodology to amplify our understanding of cardiac cellular physiology (Freshney 2006). In addition, with intentions of studying cardiac alteration in a more complex environment, an *in vitro* model using homogenate tissue may be used as a preliminary approach before starting more structured studies. Moreover, preclinical *in vitro* evaluation seems to be essential

in order to develop a comprehensive objective regarding the mechanism of action and toxicity of a new drug (von Moltke *et al.* 1998). In this sense, natural antioxidative molecules (i.e. molecules derived from food sources such as black fruits) have been implicated as playing important functions.

Phytochemicals, including pterostilbene (PTS) (trans-3,5-dimethoxy-4'-hydroxy stilbene), are a limb of the class of stilbenes. PTS is commonly found in black fruits (e.g. blueberries, grapes, plums and blackberries) and has been considered as a promising molecule halting the progression of several diseases due to its antiinflammatory and anticancer activities which improve cellular functions (Estrela *et al.* 2013; McCormack and McFadden 2013; Lameiro 2012). Moreover, PTS seems to

increase antioxidant defences and reduce reactive species-induced oxidative damage (McCormack and McFadden 2013; Wu 2010). Although the therapeutic effects of PTS appear promising, they may be limited due to the reduced hydrosolubility of the compound (Bethune *et al.* 2011). Nevertheless, this physical–chemical characteristic of PTS can be changed through combination with hydroxypropyl- β -cyclodextrin (HP β CD), which reduces the lipophilicity of this molecule and improves its bioavailability (Loftsson and Brewster 1996; Lopez-Nicolas *et al.* 2009; Pinho *et al.* 2014). In addition, the formed complex can identify novel therapeutic effects and may be used *in vitro* (cell culture) or *in vivo* (oral and parenteral administration) (Loftsson *et al.* 2016). There is a paucity of information regarding the action of the PTS + HP β CD complex on the cardiovascular system. Wang *et al.* (2015) demonstrated that PTS had an antiinflammatory role on ischemia- and reperfusion-induced cardiac damage via inhibition of the TLR4/NF- κ B pathway (Wang *et al.* 2015); however, the antioxidant effects of PTS and its modulation of redox homeostasis in cardiac tissue remains unclear.

Oxidative stress represents disturbed redox homeostasis that is provoked by an increased production of reactive oxygen species (ROS) or a reduced antioxidant response (Halliwell 2012). The enzymatic antioxidant system, which includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), is a relevant mechanism of defence against oxidative damage (Barbosa *et al.* 2010). In addition, there is also a nonenzymatic antioxidant system that acts as a scavenger and quencher of free radicals (Halliwell and Gutteridge 1986), such as glutathione (GSH), tocopherol and ascorbic acid (Patterson 1988). The redox imbalance seems to be involved in many cardiac alterations and leads to adverse remodelling and heart failure (Khullar *et al.* 2010). In this context, natural product-derived molecules (i.e. PTS) can be used as novel therapeutic approaches to mitigate the disruption of redox homeostasis.

Therefore, this study aimed to explore the effects of the PTS + HP β CD compound on cellular viability (*in vitro*), the ROS production response (in a cardiomyoblast cell culture model) as well as the antioxidant response and protection against oxidative stress in a more complex biological environment (homogenate cardiac tissue protocol).

2. Materials and methods

The experimental design was divided into experiment I (cardiac myoblasts analyses) and experiment II (cardiac tissue analyses).

2.1 Preparation of PTS + HP β CD

PTS (98% purity) was acquired from Changha Organic Herb (Changha, China), while HP β CD was supplied by Roquette

Frères (Lestrem, France). All reagents were of analytical or HPLC grade. The complex was prepared using 0.3 M HP β CD dissolved in water with an excess of PTS (Dos Santos Lacerda *et al.* 2017; Yeo *et al.* 2013). The complex was solubilised in Dulbecco's modified Eagle's medium (DMEM) and tested at concentrations of 50, 100 and 150 μ M to establish the optimal concentration for the pretreatment of cardiac myoblasts (experiment I). The PTS + HP β CD complex was solubilised in distilled water and used at doses of 25 and 50 μ M for the pretreatment of cardiac tissue (experiment II) (Dewi *et al.* 2015).

2.2 Experiment I

2.2.1 Cell culture: The cardiac myoblast lineage H9c2 (rat embryonic ventricular cells purchased from ATCC) was cultured in DMEM supplemented with 10% foetal bovine serum and 1% antibiotic–antifungal reagent (penicillin 10,000 U/mL, streptomycin 10,000 μ g/mL and 25 μ g/mL Fungizone[®]). Cells were incubated at 37 °C in 5% CO₂ and 100% humidity. In the experimental protocol, cells were seeded at 10⁴ cells/well for the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (96-well plates) and 8 \times 10⁵ cells/10 cm dish (55 cm²) for biochemical assays. After seeding, cells were maintained under culture conditions for 24 h to allow them to reach the desired adherence and confluence for the experimental protocols (Fernandes *et al.* 2015; Mallik *et al.* 2011; Shewchuk *et al.* 2010).

2.2.2 Cell viability assay: Cardiomyoblast viability was determined via the MTT assay. After 24 h of incubation with different concentrations of the PTS + HP β CD complex, the culture medium was withdrawn and hydrogen peroxide (H₂O₂; 6.67 μ M prepared in DMEM) was added for 10 min to promote oxidative stress. This was the incubation time that reduced approximately 50% of cellular viability under the experimental conditions. After removal of the H₂O₂ solution, fresh DMEM was added to the wells to proceed with the MTT assay. Incubation with MTT (5 mg/ml in PBS to produce a final 10% (v/v) concentration of MTT) occurred at 37 °C for 4 h. The supernatant was carefully removed before 50 μ L of DMSO was added to each well. After a 10 min incubation, the cytotoxicity of each treatment was quantified by determining the absorbance in each well at 570 nm (650 nm correction wavelength) using a microplate reader (Zenyth 200rt, Biochrom Inc, UK).

2.2.3 Experimental groups: Three experimental groups were defined following the analysis of cell viability: group 1 (DMEM) contained H9c2 cells in DMEM culture, group 2 (H₂O₂) contained H9c2 cells with H₂O₂-induced oxidative stress (6.67 μ M/L for 10 min) and group 3 (P50 + H₂O₂)

contained H9c2 cells pretreated with the PTS + HP β CD complex (50 μ M for 24 h) and stressed with H₂O₂. For experiment I, three replicates were performed for all biochemical analyses (i.e. ROS levels; SOD, CAT and GPx activities and sulphydryl content).

2.3 Experiment II

2.3.1 Animals and ethical approval: Male Wistar rats (200 \pm 50 g) were obtained from the Center for Reproduction and Experimentation of Laboratory Animals, Federal University of Rio Grande do Sul, Brazil. The rats were housed in polypropylene cages and received pelleted food and water ad libitum. They were kept under standard conditions including 12-h light–dark cycles and a controlled temperature of 22°C. The project was submitted to and approved by the Ethics Committee on Use of Animals, Federal University of Rio Grande do Sul (CEUA-UFRGS; registered under protocol number 29835).

2.3.2 Preparation of heart tissue for in vitro assays: The right and left ventricles of adult Wistar rats (n = 5) were homogenised for 40 s (OMNI Tissue Homogenizer, OMNI International, USA) in the presence of RIPA buffer (5 mL/g of tissue) containing phenylmethylsulfonyl fluoride [100 mM; 1% (v/v)]. The homogenate was centrifuged at 1000g for 10 min at 4°C (Sorvall RC 5 B, Rotor SM 24), and the supernatant collected (Llesuy *et al.* 1985).

2.3.3 Homogenised cardiac tissue plus PTS + HP β CD and the hydroxyl radical generation system: Initially, 500 μ L of homogenised heart supernatant was incubated with 500 μ L of the PTS + HP β CD complex, at final concentrations of 25 and 50 μ M, for 1 h at 37°C. The cardiac supernatant group, which did not receive the PTS + HP β CD complex, was incubated in the same volume of distilled water. Samples were subsequently incubated with FeCl₂, H₂O₂ and ascorbic acid to induce oxidative stress (hydroxyl radical generation system) for 30 min at 37°C (Halliwell 1990).

2.3.4 Experimental groups: Control group contained the heart tissue homogenate. P25 and P50 groups contained heart tissue incubated with PTS + HP β CD complex that corresponded to 25 or 50 μ M PTS, respectively. Stress group contained heart tissue submitted to the hydroxyl radical generation system. P25 + stress group contained heart tissue incubated with 25 μ M of the PTS + HP β CD complex and submitted to the hydroxyl radical generation system. P50 + stress group contained heart tissue incubated with 50 μ M of the PTS + HP β CD complex and submitted to the hydroxyl radical generation system. The biochemical analyses performed for experiment II determined ROS levels,

antioxidant enzyme activity and carbonyl, TBARS, nitrite and sulphydryl contents.

2.4 Biochemical analyses

2.4.1 Total ROS determination: The concentrations of ROS in heart tissue and H9c2 cells were detected using 2',7'-dichloro-dihydrofluorescein diacetate (DCFH-DA) as previously described (Lebel 1992). DCFH-DA is membrane permeable and rapidly oxidised to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS. Samples were excited at 488 nm, and emissions were collected at 525 nm. Results were expressed in nmol of DCF/mg of protein.

2.4.2 Thiobarbituric acid reactive substances (TBARS): Thiobarbituric acid (TBA) reacts with lipid peroxidation products including malondialdehyde and other aldehydes. In this assay, 50 μ L of 8.1% sodium dodecyl sulphate, 375 μ L of 20% acetic acid (pH 3.5) and 375 μ L of 0.8% TBA were added to 200 μ L of homogenate and incubated in a boiling water bath for 60 min. The supernatant was removed, and the absorbance measured at 535 nm using a spectrophotometer (Anthos Zenyth 200 rt, Biochrom, UK). The results were expressed as μ mol of TBARS/mg of protein (Ohkawa *et al.* 1979; Esterbauer *et al.* 1991).

2.4.3 Carbonyl assay: This technique is based on the reaction of oxidised proteins with 2,4-dinitrophenylhydrazine (DNPH). Briefly, samples were added to a 10mM DNPH in 2.5M HCl solution for 1 h in the dark at room temperature with shaking every 15 min. A 20% trichloroacetic acid (w/v) solution was subsequently added to the samples, which were centrifuged at 1000g for 5 min to collect protein precipitates. The pellets were dissolved in ethanol:ethyl acetate (1:1 v/v) and incubated for 10 min at 37°C with 6 M guanidine hydrochloride solution. Absorbance was measured at 360 nm using a spectrophotometer (Anthos Zenyth 200 rt), and results were expressed as nmol of DNPH derivatives/mg of protein (Reznick 1994).

2.4.4 Nitrite analysis: Nitrite levels were measured in cardiac tissue using the Griess reaction (1% sulfanilamide, 0.1% N-(1-Naphthyl)ethylene diamine and 2.3 mL 85% phosphoric acid) according to the procedure described by Granger *et al.* (1999). Total tissue nitrite was estimated at 540 nm from a standard curve of sodium nitrite (10⁸ to 10⁻³ M). Results were expressed in mM/mg of protein.

2.4.5 Measurement of antioxidant enzyme activities: The SOD assay is based on pyrogallol autoxidation inhibition by SOD in the sample. It uses a buffered solution (50 mM Tris and 1 mM EDTA; pH 8.2), 24 mM pyrogallol (in 10 mM

HCl) and 30 mM CAT (CAT from bovine liver, 10,000–40,000 U/mg of protein, Sigma Aldrich). The results were expressed as U SOD/mg of protein (Marklund 1985). CAT activity is directly proportional to the rate of H₂O₂ decomposition, therefore its activity can be measured through the evaluation of H₂O₂ consumption at 240 nm. The results were expressed in nm of H₂O₂ decomposed/min/mg of protein (Aebi 1984). The GPx enzyme catalyses the reaction of hydroperoxides with reduced GSH. GPx activity was determined by measuring the consumption of NADPH at 340 nm in the presence of glutathione reductase and GSH. The results were expressed in nmol NADPH/min/mg of protein (Flohé 1984).

2.4.6 Determination of sulfhydryl: The sulfhydryl content assay was described by Aksenov and Markesberry (2001). Briefly, 0.1 mM of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) was added to heart samples (14 µL), and samples were incubated for 30 min at room temperature in the dark. Absorbance was measured at 412 nm (Anthos Zenyth 200 rt), and the results were expressed as nmol TNB/mg of protein.

2.4.7 Dosage of proteins: Proteins were quantified using the method described by Lowry et al (1951) with bovine albumin as a standard. Absorbance was measured at 625 nm using a spectrophotometer (Anthos Zenyth 200 rt), and results were expressed in mg/mL.

2.5 Statistical analysis

Data were analysed using a Shapiro–Wilk normality test. Once the data were found to be parametric, one-way ANOVA complemented with Tukey's post hoc test was performed to compare the different groups. Nonparametric data were analysed using Kruskal–Wallis followed by Dunn's post hoc test. The differences were considered significant when $P < 0.05$ (two-sided probability). Results were expressed as mean \pm SEM for parametric data and as the median and interquartile range for nonparametric data. GraphPad Prism 6.0 software was used for data analysis.

3. Results

3.1 Determination of cell viability

Cell viability was increased in the P50 and P100 groups compared to the control. Cyclodextrin alone did not have a negative effect on cell viability. These data were used to select the lowest concentration that improved viability (50 µM) in order to evaluate other oxidative stress parameters (figure 1).

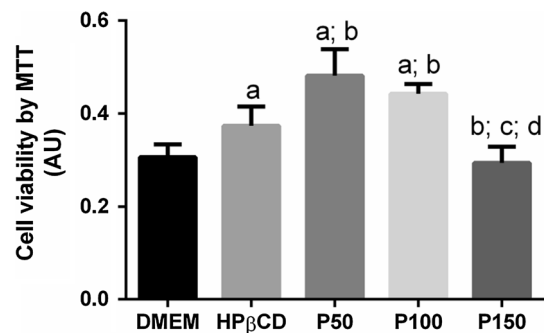


Figure 1. Cell viability analyses. Colorimetric MTT assay in H9c2 cardiac cells pretreated with pterostilbene complexed with cyclodextrin (50µM – P50, 100 µM – P100, 150 µM – P150) before incubation with hydrogen peroxide (H₂O₂). AU: Arbitrary Units. Values are expressed as mean \pm SEM; 8 independent experiments were performed. Statistical analysis was performed using One-way ANOVA. Comparisons between groups were made using the Tukey's post-test. ^a vs. Control, ^b vs. HPβCD, ^c vs. P50 group, ^d vs. P100 group, $p < 0.05$.

3.2 Oxidative stress analyses

3.2.1 Determination of total ROS: In experiment I, there were no significant differences between the DMEM, H₂O₂ and P50 + H₂O₂ groups (figure 2A); however, in experiment II, ROS levels were increased in the stress and P50 + stress groups compared to the P25 and P50 groups. In addition, the P25 + stress group showed decreased ROS levels compared to the stress and P50 + stress groups ($P < 0.05$; figure 2B).

3.2.2 TBARS concentration: According to the data from experiment II, the stress, P25 + stress and P50 + stress groups showed increased TBARS levels when compared to the control and P50 groups. In addition, the P50 group also showed a decreased TBARS content compared to the P25 group ($P < 0.05$; figure 3A).

3.2.3 Carbonyl content: There was a three-fold increase in the carbonyl content in the stress group compared to the control group ($P < 0.05$; experiment II); however, the P25 + stress and P50 + stress groups did not demonstrate significant differences when compared to their respective control groups (P25 and P50) (figure 3B).

3.2.4 Nitrite analysis: There were increased nitrite concentrations in the stress and P50 + stress groups when compared to the P25 and P50 groups in experiment II. It is important to note that the 25 µM dose showed protective effects under stress conditions, as nitrite levels did not increase significantly in the P25 + stress group compared with the P25 group ($P < 0.05$; figure 3C).

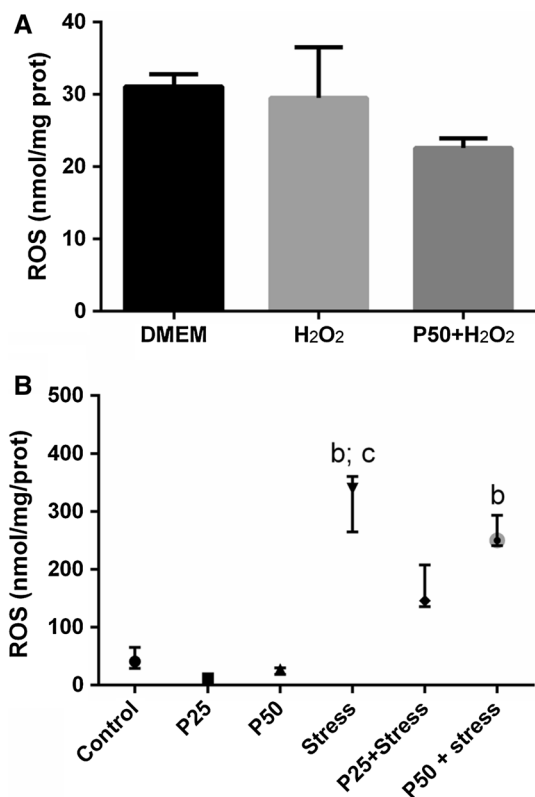


Figure 2. Oxidative stress determined by total ROS levels. (A) In H9c2 cardiac cells pretreated with pterostilbene complexed with cyclodextrin (50 μ M – P50) before incubation with hydrogen peroxide (H₂O₂). Values are expressed as mean \pm SEM; 3 independent experiments were performed. (B) Cardiac tissue *in vitro* pretreated with pterostilbene complexed with cyclodextrin (25 and 50 μ M) for a period of 1 h before oxidative damage induction using a hydroxyl radical generator system. Values are expressed as mean \pm SEM; 5 independent experiments were performed. Statistical analysis was performed using One-way ANOVA. Comparisons between groups were made using the Tukey's post-test. ^b vs. P25 group, ^c vs. P50 group, ($p < 0.05$).

3.3 Antioxidant defence analyses

3.3.1 Measurement of antioxidant enzyme activities: SOD, CAT and GPx: In experiment I, SOD activity was not significantly different between the groups; however, in experiment II, the P25, P50 and stress groups showed elevated SOD activity compared to the control group ($P < 0.05$; figure 4A and 4B). In cardiac myoblasts (experiment I), increased CAT activity was observed in the H₂O₂ and P50 + H₂O₂ groups compared to the control group (figure 4C). In experiment II, CAT activity was decreased four-fold when comparing the stress group to the control group. CAT levels increased in the P25 + stress group when compared to the stress group. It is important to note that

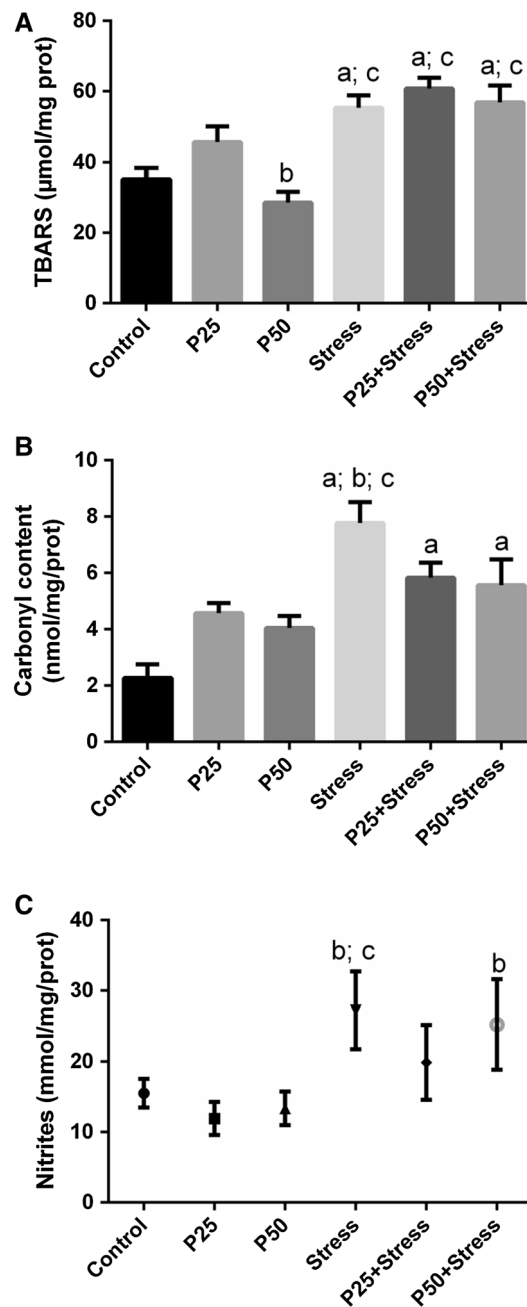


Figure 3. Markers of oxidative stress measured in homogenates of cardiac tissue pretreated with pterostilbene complexed with cyclodextrin (25 μ M – P25 e 50 μ M – P50) for a period of 1 h, before oxidative damage induction using a hydroxyl radical generator system. (A) Lipid peroxidation measured by determination of TBA-RS. (B) Protein oxidative damage determined by carbonyl content. (C) Analysis of nitrites. Values are expressed as mean \pm SEM; 5 independent experiments were performed. Statistical analysis was performed using One-way ANOVA. Comparisons between groups were made using the Tukey's post-test. ^a vs. Control, ^b vs. P25 group, ^c vs. P50 group, ($p < 0.05$).

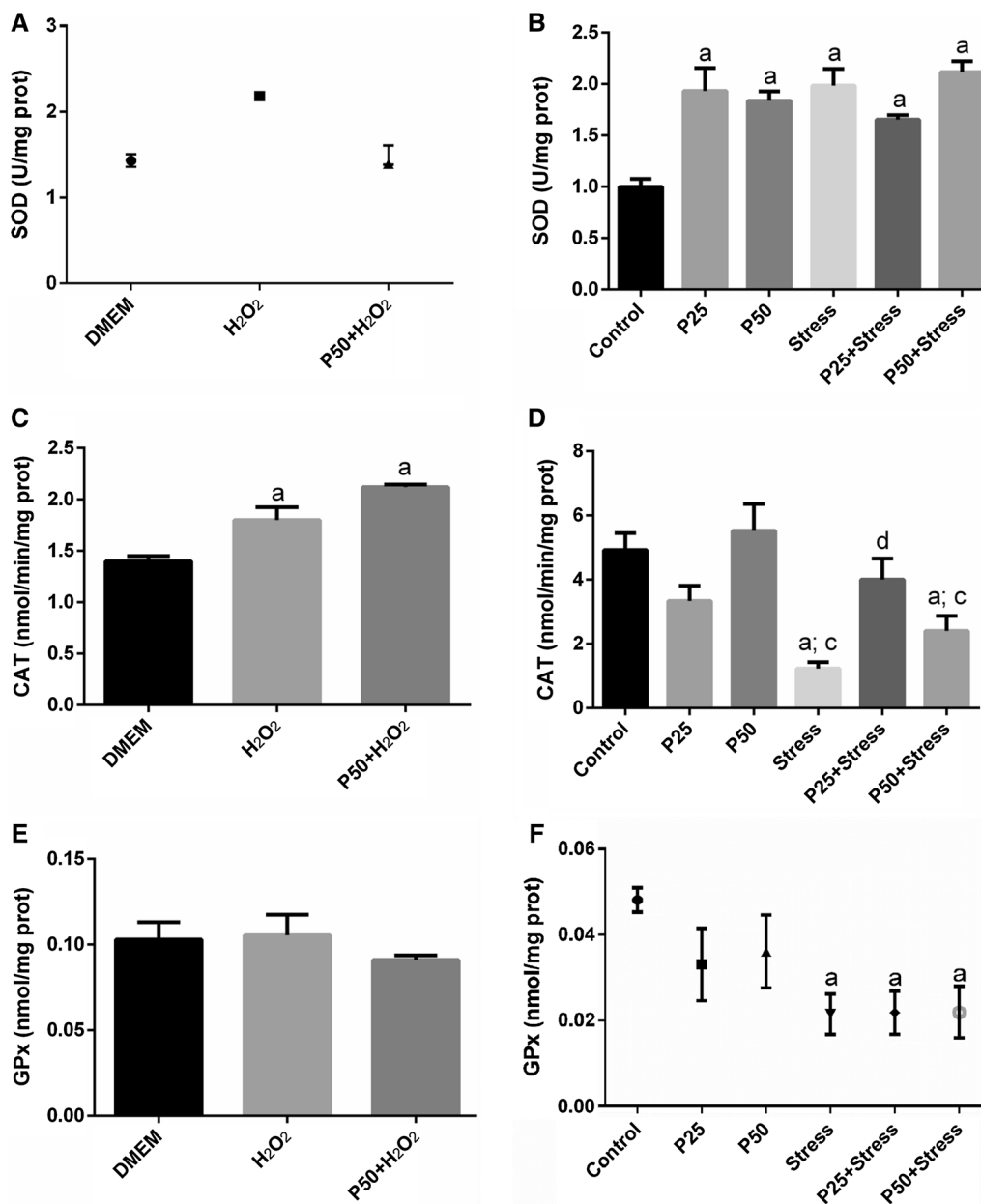


Figure 4. Analysis of enzymatic antioxidant defenses. (A) SOD, (C) CAT, (E) GPx in H9c2 cardiac cells pretreated with pterostilbene complexed with cyclodextrin (50 μ M – P50) during a period of 24 hours, before incubation with hydrogen peroxide. Values are expressed as mean \pm SEM; 3 independent experiments were performed. Statistical analysis was performed using One-way ANOVA. Comparisons between groups were made using the Tukey's post-test. ^a vs. Control group, ($p < 0.05$). (B) SOD, (D) CAT, (F) GPx in homogenates of cardiac tissue pretreated with pterostilbene complexed with cyclodextrin (25 μ M – P25 e 50 μ M – P50) for a period of 1 h, before oxidative damage induction using a hydroxyl radical generator system. Values are expressed as mean \pm SEM; 5 independent experiments were performed. Statistical analysis was performed using One-way ANOVA. Comparisons between groups were made using the Tukey's post-test. * ^a vs. Control group, ^c vs. P50 group, ^d vs. Stress group, ($p < 0.05$).

there was no significant difference between the P25 and P25 + stress groups, thus demonstrating a protective effect of PTS (25 μ M) against oxidative stress ($P < 0.05$; figure 4D). Treatment with the PTS + HP β CD complex did not change GPx activity in experiment I (figure 4E);

however, in experiment II, GPx activity was reduced in the stress, P25 + stress and P50 + stress groups when compared to the control ($P < 0.05$; figure 4F).

In experiment I, the sulfhydryl content was increased two-fold in the P50 + H₂O₂ group compared to the DMEM

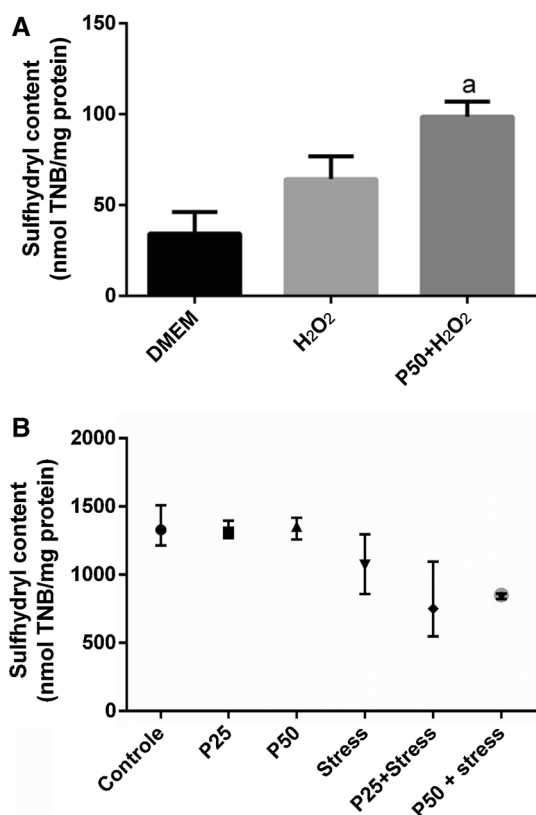


Figure 5. Analysis of antioxidant non-enzymatic defenses by measuring levels of sulfhydryl content. (A) In H9c2 cardiac cells pretreated with pterostilbene complexed with cyclodextrin (50 μ M) during a period of 24 hours, before incubation with hydrogen peroxide. Values are expressed as mean \pm SEM. Statistical analysis was performed using One-way ANOVA. Comparisons between groups were made using the Tukey's post-test. ^a vs. Control group, ($p < 0.05$). (B) In homogenates of cardiac tissue pretreated with pterostilbene complexed with cyclodextrin (25 μ M – P25 e 50 μ M – P50) for 1 h, before oxidative damage induction using a hydroxyl radical generator system. Values are expressed as mean \pm SD. Statistical analysis was performed using One-way ANOVA. Comparisons between groups were performed using the Tukey's post-test.

group (figure 5A). In experiment II, using cardiac tissue, there were no changes between the groups (figure 5B).

4. Discussion

The present study is avant-garde. It explored the effects of pretreatment with the PTS + HP β CD complex (a pharmaceutical technology to improve the physical–chemical properties of PTS) on cardiomyoblasts and cardiac tissue homogenates exposed to oxidative stress. The PTS + HP β CD complex was able to increase the cellular viability

and the sulfhydryl content in cardiac myoblasts in culture. Moreover, the PTS + HP β CD complex also decreased ROS levels and increased CAT activity in cells and cardiac tissue.

PTS has been well studied in diseases such as cancer, diabetes and fungal infections and has demonstrated significant and satisfactory effects, especially as an antioxidant and antiinflammatory agent (Estrela *et al.* 2013; Kosuru 2016; Manickam 1997). The low aqueous solubility of PTS meant that it was developed into a new formulation through combination with HP β CD. The complexation with cyclodextrin protects the biologically-active phenol from degradation, thus preserving its pharmacological effects (Wei *et al.* 2010). Moreover, the cyclodextrins contribute to improved cellular absorption as they inhibit molecular efflux across the cellular membrane (Borbas *et al.* 2015). However, the role of the PTS + HP β CD complex in cardiac redox homeostasis remains unclear. Two *in vitro* protocols were developed to explore the antioxidant mechanisms of the PTS + HP β CD complex: a cell culture experiment (a simple system using H9c2 cells) and a cardiac tissue experiment (a more complex model using a ROS production system). Both systems were able to trigger oxidative stress, thus resulting in an increase of ROS, lipid peroxidation and protein oxidation.

The PTS + HP β CD complex (50 μ M) was able to induce cellular viability; however, the same profile was not observed when H₂O₂ was added to the cell culture. It is possible that H₂O₂-induced stress provoked intense and irreversible cellular damage in cardiomyoblasts, as the ROS levels remained increased. Unlike in cardiomyocytes, the PTS + HP β CD complex was effective at reducing ROS levels in the cardiac tissue exposed to oxidative stress. Such an effect is important as elevated ROS concentrations cause damage to the structure and function of macromolecules, alter cellular signalling pathways and impair the function of cardiac cells (Taverne *et al.* 2013).

In this context, PTS administration seems to induce an antioxidant response as it stimulates increased counterregulation of thiol groups in cell culture. Similarly, Mikstacha *et al.* (2010) reported that both PTS and resveratrol increased nonenzymatic defences in erythrocytes. Phenolic compounds also increase the availability of nonenzymatic antioxidants through the regulation of glutathione synthesis (Moskaug *et al.* 2005). PTS can act as an antioxidant through radical scavenging as its molecular structure (based on the aromatic ring and phenolic hydroxyl) stabilises free radicals by donating hydrogen (Fraga *et al.* 2010). Moreover, previous results suggest that the PTS + HP β CD complex is as effective as noncomplexed PTS in terms of its antioxidant activity, thus indicating that the combination with HP β CD does not change this property (Dos Santos Lacerda *et al.* 2017). Therefore, the decision was made to explore the classical enzymatic antioxidant system in both *in vitro* protocols. The results showed significantly increased

SOD activity in groups treated with the PTS + HP β CD complex in experiment II. SOD defends against superoxide anions and reduces the possibility of superoxide via the nitric oxide reaction and peroxynitrite synthesis. Therefore, the PTS + HP β CD complex may play a key role in the induction of enzymatic antioxidation and provoke cardioprotective effects. In right heart failure, oral administration of the PTS + HP β CD complex reduced NADPH oxidase activity (diminishing the production of the superoxide anion) associated with the inductor effect of the SOD response. This resulted in the prevention of maladaptive remodelling and the protection of systolic function (Dos Santos Lacerda et al. 2017). Moreover, some data described increased SOD activity in colon cancer cells (Chiou 2011) as well as in hepatic tissue treated with PTS (Wu 2010), thus demonstrating that this molecule has a modulatory role in the antioxidant response. CAT and GPx are other relevant antioxidant enzymes that may be involved in redox control in cardiac tissue, and the PTS + HP β CD complex seems to stimulate CAT activity. The CAT-induced reduction of H₂O₂ levels represents a relevant protection mechanism against oxidative events produced by H₂O₂, which could react with transition metals (i.e. Fe or Cu) and generate hydroxyl radicals, as ROS are known to be intensely reactive. The hydroxyl radical-dependent rupture of redox homeostasis may lead to cellular damage, as the oxidation of lipids and proteins may be increased. In addition, recent evidence suggests that PTS mediates the activation of nuclear factor (erythroid-derived 2)-like 2, a redox-sensitive transcription factor that is a key regulator of the antioxidant response (Bhakkialakshmi et al. 2016; Xue et al. 2017). Thus the possibility that this pharmacological mechanism also contributes to the activation of antioxidant systems in the heart by PTS cannot be ruled out; however, this signalling was not the focus of this study. The PTS-induced improved antioxidant response, which was verified by *in vitro* protocols, is associated with increased cardiac function, which was observed during *in vivo* experiments. The echocardiographic parameters of the right ventricle were enhanced after PTS treatment in rats subjected to monocrotaline-induced heart failure (Dos Santos Lacerda et al. 2017).

With regards to nitric oxide metabolism, PTS reduced nitrite levels in cardiac tissue. This effect is important as increased nitrite synthesis is positively correlated with the glycosylation of proteins, which leads to the formation of free radicals (i.e. peroxynitrite) and oxidative stress (Searles 2002; Taverne et al. 2013). Therefore low doses of the PTS + HP β CD complex could be beneficial to heart tissue and protect against nitrosative stress (Halliwell 2012).

Therefore, these results show that pretreatment with the PTS + HP β CD complex is promising, as a positive redox response was observed in cardiomyoblasts and cardiac tissue. This phytochemical plays a critical role in modulating ROS production in cells and cardiac tissue. Moreover, CAT

seems to be a key enzyme in the antioxidant response, being relevant at both the cellular and cardiac tissue level. Nevertheless, other studies are necessary (i.e. *in vivo* studies) to understand the impact of the PTS + HP β CD complex on functional aspects of the cardiovascular system.

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