UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

FARMACOLOGIA E TERAPÊUTICA

Tese de Doutorado

Potencial farmacológico do pterostilbeno sobre diferentes alvos no remodelamento ventricular cardíaco: efeitos sobre o ventrículo direito e esquerdo

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Tese submetida ao Programa de Pós-Graduação em Ciências Biológicas: Farmacologia e Terapêutica, da Universidade Federal do Rio Grande do Sul-UFRGS, como requisito para obtenção do título de Doutor.

Orientador: Dr. Alex Sander da Rosa Araujo
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Porto Alegre, 2018.

“Em algum lugar, alguma coisa incrível está esperando para ser descoberta.”

Carl Sagan

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Resumo

O estilbenoide pterostilbeno (PTS), um constituinte fenólico altamente lipofílico, vem se destacando devido ao seu potencial farmacológico evidenciado por seus efeitos antitumoral, antidiabético e anti-inflamatorio, dentre outros, descritos tanto em estudos pré-clínicos quanto clínicos. O objetivo deste estudo foi avaliar a toxicidade, determinar dose terapêutica, potencial antioxidante do pterostilbeno complexado à ciclodextrina (complexo PTS:HPβCD), bem como seu efeito sobre parâmetros funcionais e modulação de vias redox sensíveis no ventrículo direito e esquerdo de ratos com insuficiência cardíaca. O complexo PTS:HPβCD foi preparado por método em solução e caracterizado por calorimetria diferencial de exploratória (DSC), espectroscopia por infravermelho e por ressonância magnética nuclear. O teor de PTS no complexo foi quantificado por cromatografia líquida de alta eficiência (CLAE). Posteriormente, os efeitos farmacológicas do complexo, nas doses correspondentes a 25, 50 e 100 mg/kg de PTS, foram testadas sobre o estresse oxidativo e vias de sinalização redox sensíveis no tecido hepático (Experimento I), no ventrículo direito (VD) (Experimento II) e esquerdo (VE) (Experimento III). No experimento I, a fim de determinar toxidade e identificar a dose terapêutica, foram avaliados no tecido hepático de ratos alguns parâmetros oxidativos de dano tecidual, nível de antioxidante não enzimático e a expressão de proteínas redox sensíveis como proteína cinase B (AKT) e glicogênio sintase cinase-3β (GSK-3β) após 14 dias da administração oral (gavage) do complexo PTS:HPβCD (25, 50 e 100 mg/kg/dia). A complexação demonstrou ser capaz de aumentar a solubilidade aparente da PTS tornando possível a administração da curva de dose, sem induzir dano hepático ou apoptose pelas doses administradas. Além disso, o complexo mostrou efeitos antioxidantes dose-dependentes no fígado dos ratos, evidenciados por uma redução na peroxidação lipídica e de espécies reativas de oxigênio, bem como por aumento no conteúdo de antioxidante não enzimático. O complexo PTS: HPBCD também aumentou a expressão de proteínas redox sensíveis, como AKT e GSK-3β, relacionadas à via de sinalização da insulina no fígado. No experimento II, a mesma curva de dose (25, 50 e 100 mg/kg/dia) administrada 1x ao dia durante 14 dias, foi testada sobre as alterações inerentes ao remodelamento do VD induzido por hipertensão arterial pulmonar (*Cor pulmonale*), após a administração do alcaloide monocrotalina (MCT). Ao final do protocolo experimental, para avaliar a função cardíaca os animais foram submetidos ao cateterismo do VD e ecocardiografia. Os achados sugerem que complexo PTS:HPβCD,

de maneira dose-dependente, protege a função sistólica, previne a hipertrofia do VD, reduz a concentração de ânion superóxido dependente de NADPH oxidase, restaurou a atividade da superóxido dismutase (SOD) e glutationa peroxidase (GPx), além de reduzir a atividade da catalase (CAT). Adicionalmente, o complexo PTS: HPβCD aumentou o conteúdo de glutationa reduzida (GSH) e a razão glutationa reduzida/glutationa oxidada, e ainda restaurou a atividade da glutationa-S-transferase (GST) e glutaredoxina (GRx) no VD de ratos com *Cor pulmonale.* Além disso, em doses mais elevadas, também evitou a redução do volume sistólico e do débito cardíaco, reduziu a lipoperoxidação e aumentou a expressão da proteína SERCA no VD de ratos tratados com MCT. Por outro lado, no experimento III, foi testado o efeito do complexo PTS:HPBCD sobre o remodelamento do VE pós infarto agudo do miocárdio (IAM) em ratos. Neste protocolo experimental, o IAM foi produzido por meio da ligadura cirúrgica dos ramos anteriores descendentes da artéria coronária esquerda. Sete dias após o procedimento cirúrgico, o complexo PTS:HPβCD, na dose de 100 mg/kg/dia, foi diariamente administrado por via oral durante 8 dias. Os resultados mostraram que o complexo PBS:HPβCD diminuiu a peroxidação lipídica, preveniu a redução na atividade da tioredoxina redutase (TRxR) e aumentou a atividade da GST e da GRx. Além disso, a expressão do fator nuclear eritróide 2 (Nrf2) e p-GSK-3β foi aumentada, enquanto a relação GSK-3β fosforilada/total foi reduzida no VE dos animais infartados. Portanto, o conjunto de resultados da presente Tese destaca o papel antioxidante do complexo PTS:HPβCD em ratos, especialmente na dose de 100 mg/kg, e sua modulação sobre a expressão proteínas de sinalização redox sensíveis tanto no fígado quanto em ambos ventrículos sob condições de remodelamento patológico. As ações cardioprotetoras do complexo PTS:HPβCD parecem prevenir o remodelamento ventricular e melhoram a função sistólica direita e esquerda, efeitos que provavelmente foram favorecidos pelo aumento da biodisponibilidade oral promovido pela presença da HPβCD no complexo. Diante disso, o complexo PTS:HPβCD poderia ser usado como um tratamento complementar a terapia farmacológica disponível a fim de minimizar os efeitos deletérios do desbalanço oxidativo sobre a homeostase e função cardíaca.

Abstract

The stilbenoid pterostilbene (PTS), a highly lipophilic phenolic constituent, has been highlighted due to its pharmacological potential evidenced by its antitumor, antidiabetic and anti-inflammatory effects, among others described in both preclinical and clinical studies. The aim of this study was to evaluate toxicity, determine therapeutic dose and antioxidant potential of pterostilbene complexed to cyclodextrin (PTS:HPβCD), as well as its effect on functional parameters and modulation of sensitive redox pathways in the right and left ventricles of rats with heart failure. The PTS:HPβCD complex was prepared by solution method and characterized by differential scanning calorimetry (DSC), infrared spectroscopy and nuclear magnetic resonance. The PTS content in the complex was quantified by high performance liquid chromatography (HPLC). Subsequently, the pharmacological properties of the complex at doses corresponding to 25, 50 and 100 mg/kg of PTS were tested on oxidative stress and redox signaling pathways in the hepatic tissue (Experiment I) in the right ventricle (RV) (Experiment II) and left ventricle (LV) (Experiment III). Regarding Experiment I, in order to determine toxicity and to identify the therapeutic dose, oxidative parameters of tissue damage, non-enzymatic antioxidant levels and expression of Protein kinase B (AKT) and Glycogen synthase kinase-3β (GSK-3β) sensitive redox proteins were evaluated in rat hepatic tissue after 14 days of the PTS:HPβCD complex (25, 50 and 100 mg/kg/day) oral administration (via gavage). Complexation has been shown to increase the apparent solubility of PTS without inducing hepatic damage or apoptosis at the doses administered. In addition, PTS:HPβCD complex showed dose-dependent antioxidant effects on rats liver, evidenced by a reduction in lipid peroxidation and reactive oxygen species, as well as by an increase in non-enzymatic antioxidant content. The PTS:HPBCD complex also increased the expression of sensitive redox proteins, such as AKT and GSK-3β, related to insulin signaling pathway in the liver. In Experiment II, same doses (25, 50 and 100 mg/kg/day) of PTS:HPBCD were administered daily for 14 days and its effects were tested on the inherent RV remodeling triggered by pulmonary arterial hypertension (*Cor pulmonale*) after administration of the monocrotaline (MCT) alkaloid. At the end of the experimental protocol, animals were submitted to RV catheterization and echocardiography to evaluate cardiac function. The results suggest that the PTS:HPβCD complex, in a dose-dependent manner, protects systolic function, prevents RV hypertrophy, reduces NADPH oxidase-dependent superoxide anion concentration, and restores superoxide dismutase (SOD) and

glutathione peroxidase (GPx) activity, in addition to reducing catalase activity (CAT). Moreover, PTS:HPβCD complex increased reduced glutathione (GSH) content and reduced glutathione/oxidized glutathione ratio, recovering glutathione-S-transferase (GST) and glutaredoxin (GRx) activities in the RV of rats with *Cor pulmonale*. At higher doses, PTS:HPβCD also avoided reduction of systolic volume and cardiac output, reduced lipoperoxidation and increased expression of SERCA in the RV of MCT treated rats. On the other hand, in Experiment III, the effect of the PTS:HPBCD complex was tested on the LV remodeling after acute myocardial infarction (AMI) in rats. In this experimental protocol, AMI was triggered by surgical ligation of the descending anterior branches of left coronary artery. Seven days after the surgical procedure, 100 mg/kg/day of PTS:HPβCD complex were administered orally for 8 days. Results showed that the PTS:HPβCD decreased lipid peroxidation, prevented the reduction of thioredoxin reductase (TRxR) activity and increased GST and GRx activities. Furthermore, expression of erythroid nuclear factor 2 (Nrf2) and p-GSK-3β was increased, while the phosphorylated/total GSK-3β ratio was reduced in the LV of infarcted animals treated with the complex. Therefore, the set of results of the present thesis highlights the antioxidant role of the PTS:HPβCD complex in rats, especially at the dose of 100 mg/kg, and its modulation on the expression of sensitive redox signaling proteins in the liver and both ventricles under conditions of pathological remodeling. The cardioprotective actions of the PTS:HPβCD complex seem to prevent detrimental ventricular remodeling and improve right and left systolic function, effects that were probably favored by the increased oral bioavailability promoted by the presence of HPβCD in the complex. In view of the presented evidences, the PTS:HPβCD complex could be used as a complementary treatment to the available pharmacological therapy in order to minimize the deleterious effects of oxidative imbalance on homeostasis and cardiac function.

Lista de figuras

Lista de abreviaturas

AKT: serina/treonina quinase

Ca^{2+}: íon cálcio

CAT: catalase

CLAE: cromatografia líquida de alta eficiência

CoA: malonil coenzima A

COX2: ciclo-oxigenase-2

CTR: Controle

Cys-SOH: ânion tiolato, gerando a forma sulfênica

ERK1/2: quinase regulada por sinal extracelular 1 e 2

ERNs: espécies reativas de nitrogênio

EROs: espécies reativas de oxigênio

FAC: variação da área fracional

GSK-3β: glicogênio sintase quinase 3β

GPx: glutationa peroxidase

Grx: glutaredoxina

GSH: reduzida da glutationa

GSSG: glutationa oxidada

GST: glutationa-S-transferase

H_2O_2: peróxido de hidrogênio

HO-1: heme oxigenasse-1

HPβCD: hidroxipropil-β-ciclodextrina

IAM: infarto agudo do miocárdio

IC: insuficiência cardíaca

ICD: insuficiência cardíaca direita (ICD)

iNOS: óxido nítrico sintase induzível

JNK: quinase c-Jun N-terminal

Keap1: kelch-like ECH-associated protein-1

MAPK: proteínas quinases ativadas por mitógenos

MCT: monocrotalina

MMPs: metaloproteinases da matriz mitocondrial

NADPH: nicotinamida adenina dinucleotídeo fosfato

NF-κB: fator nuclear kappa

NO: óxido nítrico

Nrf2: nuclear factorerythroid 2-related factor 2

$O^{2\bullet-}$: ânion superóxido

$^{\bullet}OH$: radical hidroxil

$ONOO^-$: peroxinitrito

p-38: p-38 MAP-quinase

PKA: proteína cinase A

PTS: pterostilbeno

RyR: receptor de rianodina

SOD: superóxido dismutase

TAPSE: excursão sistólica do plano do anel da tricúspide

TRx: tiorredoxina

TRxR: tiorredoxina redutase

VD: ventrículo direito

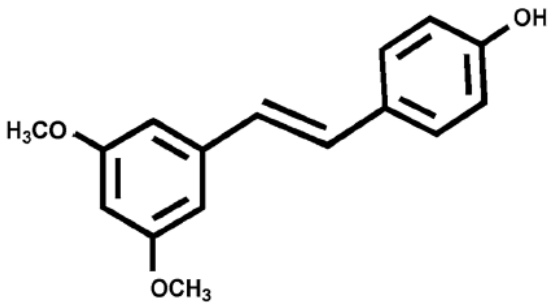
VE: ventrículo esquerdo

1. Revisão da literatura

1.1 Pterostilbeno: aspectos farmacológicos

O pterostilbeno (*trans*-3,5-dimethoxy-4'-hydroxystilbene,) é um constituinte fenólico encontrado numa ampla variedade de frutas, tais como mirtilo, uva, ameixa dentre outras (RIVIERE et al., 2012; RIMANDO et al., 2004). Biologicamente, o pteroslibeno (PTS) pode ser classificado como uma fitoalexina, por participar do sistema de defesa da planta. Este constituinte é sintetizado a partir de moléculas precursoras como malonil coenzima A (CoA) e p-cumaroil Coa catalisada pela enzima estilbeno sintetase, em resposta a infecções por patógenos (vírus, bactérias e fungos) ou por excessiva exposição à radiação ultravioleta (BAVARESCO et al., 1999).

Quimicamente, o PTS pertence ao grupo dos estilbenos, devido à presença de dois anéis benzenos ligados por uma porção de isopropileno (KASIOTIS et al., 2013), sendo estruturalmente análago ao resveratrol. Contudo, diferencia-se deste por apresentar dois grupamentos metóxi e um grupo hidroxila (Figura 1), enquanto que o resveratrol possui três hidroxilas (LIN et al., 2009). A presença dos grupos metoxi na estrutura do pterostilbeno promove sua maior estabilidade metabólica, bem como aumenta sua lipofilia, resultando em melhores perfis farmacocinéticos, promovendo sua absorção intestinal, aumento da biodisponibilidade e potencial de penetração através das membranas teciduais (MCCOMARK; MCFADDEN 2012). Neste contexto, o PTS tem demonstrado farmacocinética mais favorável e superior potência farmacológica do que o resveratrol, mesmo quando administrado em doses menores (CHOO et al., 2014).



Fórmula Molecular: C16H16O3

Peso molecular: 256.99 g/mol

Figura 1: Estrutura química do pterostilbeno. Extraído e adaptado de Pterostilbene Monograph 2010.

O PTS vem se destacando devido aos seus efeitos biológicos benéficos, recentemente descritos tanto em abordagens pré-clínicas quanto clínicas (WU et al., 2017; RICHE et al., 2014).O potencial farmacológico deste composto inclui os seguintes efeitos: antitumoral, uma vez que este constituinte fenólico estimula a sinalização de apoptose; anti-inflamatória, dada a sua inibição nas atividades COX2 e iNOS; antidiabética, devido à ação inibitória sobre gliconeogênese, e insulinotrópica; assim como antioxidante (ACHARYA; GHASKADBI, 2013; LV et al., 2014; MCCORMACK; MCFADDEN, 2013); PARI; SATHEESH, 2006; PAUL et al., 2009). Os efeitos antioxidantes são atribuídos, em parte, a sua capacidade de neutralização e inibição da produção de espécies reativas, assim como ativação de sistemas antioxidantes endógenos (MCCOMARK; MCFADDEN, 2012). Adicionalmente, outro importante efeito citoprotetor do PTS está relacionado a ativação do nuclear factor erythroid 2-related factor 2 **(**Nrf2), um fator de transcrição, regulador chave da resposta antioxidante. Esse mecanismo está relacionado à inibição da interação da proteína Kelch-like ECH-associated protein-1 (Keap1) com o Nrf2 (Keap1-Nrf2), com consequente a ativação de Nrf2, resultando em sua translocação para o núcleo e ativação transcricional de genes de antioxidantes de fase II e de agentes redutores como a glutationa (ELANGO et al., 2016). Esses efeitos despertam, cada vez mais, o interesse científico para a identificação de novos mecanismos de ação do PTS e o torna um candidato para utilização como alimento funcional ou composto farmacêutico terapêutico (KOSURU et al., 2016).

Nesse contexto, torna-se importante conhecer as características físico-químicas e farmacocinéticas do PTS, a fim de determinar a dose terapêutica, biodisponibilidade, perfil de distribuição, efeitos no tecido alvo e grau de toxicidade, fatores que desempenham um importante papel no desenvolvimento de novas formas farmacêuticas e produtos terapêuticos (LIN et al., 1997; YEO et al., 2013). Evidências obtidas a partir de estudos farmacocinéticos pré-clínicos revelam que a absorção oral do PTS pode ser consideravelmente maximizada quando coadministrado durante ou logo após uma refeição, enquanto que no jejum sua biodisponibilidade é reduzida, sendo inferior a 5 % (YEO et al., 2013). Em relação a mecanismos de excreção, Remsberg e cols. sugerem que o PTS seja excretado predominantemente por meio de vias de eliminação não renal, já que a fração intacta excretada na urina foi inferior a 1% (REMSBERG et al., 2008). Recentes estudos evidenciaram que o PTS sofre metabolismo hepático de fase II, predominantemente metilação, glicuronidação e sulfatação, considerada sua principal via de eliminação (KAPETANOVIC et al., 2011; REMSBERG et al., 2008). As

concentrações de metabólitos plasmáticos de PTS (ambos conjugados com sulfato e glicuronídeos) foram substancialmente mais elevadas do que as concentrações plasmáticas do composto original (KAPETANOVIC et al., 2011). O PTS exibe uma farmacococinética não linear, ou seja, a velocidade de eliminação é constante independente da quantidade de composto presente (YEO et al., 2013). Assim, pequenas mudanças na dose resultam em mudanças desproporcionais nas concentrações do composto no sangue, sejam por concentrações obtidas após dose única ou múltiplas doses (SHARGEL; ANDREW, 1999). Neste contexto, a cinética de eliminação de PTS pode ser saturada, levando a uma maior exposição aos tecidos (CHOO et al., 2014; YEO et al., 2013).

Estudos que avaliem a segurança da administração terapêutica do PTS são escassos. Em roedores a administração intraperitoneal (30 mg/kg/dia) durante três semanas, não demonstrou toxicidade sistêmica ou tecidual (RUIZ et al., 2009). Em seres humanos, Riche de cols. (2013) não encontraram efeitos tóxicos ou reações adversas do PTS após a administração de doses de 250 mg/dia.

1.2 Pterostilbeno: o impacto da hidrossolubilidade na biodisponibilidade

Particulamente para compostos administrados pela via oral, a efetividade terapêutica depende de fatores como solubilidade no fluido gastrointestinal, extensão de absorção pelo epitélio intestinal, biodisponibilidade, estabilidade metabólica, dentre outros fatores (CHAN; STEWART, 1996; HORTER; DRESSMAN, 2001; JAMBHEKAR; BREEN, 2013). A maioria dos compostos fenólicos apresentam baixa biodisponibilidade oral (cerca de 10%) em decorrência de suas características químicas (HU, 2007). Estas particularidades poderiam explicar, em parte, a inobservância de efeito farmacológico frequentemente observada em estudos de intervenção *in vivo*, apesar de inúmeros estudos destacarem os efeitos farmacológicos destes compostos *in vitro*, nos quais estratégias de solubilização são empregadas para a realização dos testes, como a dissolução em dimetilsulfóxido. A aparente discrepância entre estes estudos pode ser parcialmente justificada pela baixa absorção e fraca biodisponibilidade oral, bem como pelo extenso metabolismo hepático destes compostos (WILLIAMSON; MANACH, 2005).

Similarmente, o uso farmacológico do PTS, especialmente quando administrado pela via oral pode ser limitado, devido ao seu caráter lipofílico e consequente baixa

hidrossolubilidade (aproximadamente 21 ug/mL) (BETHUNE et al., 2011). Segundo o sistema de classificação biofarmacêutica, esta característica química categoriza substâncias lipofílicas, como o PTS, em compostos de classe II, contemplando substâncias que apresentam alta permeabilidade e baixa solubilidade e estão sujeitos a problemas relacionadas com a biodisponibilidade (FDA, 2017). Portanto, uma melhor compreensão das propriedades físico-químicas de bioativos é relevante para o desenvolvimento de um sistema de liberação otimizado para administração oral de compostos fenólicos (BLUME et al., 2000; PENG et al., 2018).

Neste contexto, a baixa hidrossolubilidade do PTS no trato gastrointestinal pode ser considerada uma barreira para biodisponibilidade oral, reduzindo o seu efeito terapêutico (YEO et al., 2013). Diante disso, mudanças nas propriedades físicas e químicas do PTS promovem melhora no perfil farmacocinético e aumentam sua biodisponibilidade oral, intensificando potenciais efeitos farmacológicos (PINHO et al., 2014). Assim, estratégias farmacotécnicas, como a formação de complexos de inclusão com ciclodextrinas, que possibilitem o aumento da hidrossolubilidade aparente do PTS, sem reduzir suas propriedades biológicas podem ser consideradas uma alternativa para minimizar a baixa hidrossolubilidade (CHOO et al., 2014; LOFTSSON; BREWSTER, 2012).

As ciclodextrinas são oligossacarídeos cíclicos de origem natural, que apresentam uma cavidade interna hidrofóbica e uma superfície externa hidrofílica, possibilitando o encapsulamento molecular de fármacos lipofílicos (LOFTSSON; BREWSTER, 2012; PINHO et al., 2014). A complexação de moléculas com ciclodextrinas promove modificações nas propriedades físico/químicas das moléculas hospedes, aumentando sua hidrossolubilidade e estabilidade (SZETJLI, 1998). Derivados de ciclodextrinas naturais, como a hidroxipropil-β-ciclodextrina (HPβCD) apresenta características que permitem sua utilização, tanto para estudos *in vitro* quanto *in vivo,* devido a sua baixa toxicidade e alta hidrossolubilidade (LOFTSSON; BREWSTER, 2011; LÓPEZ-NICOLÁZ et al., 2009). Neste sentido, a utilização de ciclodextrinas como agentes promotores de hidrossolubilidade do PTS revela-se oportuna podendo facilitar a entrega e permeação dessa molécula nas camadas de difusão existentes na superfície do epitélio gastrintestinal, aumentando sua concentração plasmática (LOFTSSON; BREWSTER, 2011; YEO et al., 2013). Os achados de Yeo e cols. (2013) evidenciam que o PTS complexado com HPβCD, administrado pela via oral,apresenta-se cerca de 4 vezes mais biodisponível do que o PTS em suspensão (não-complexado), confirmando que a baixa hidrossolubilidade constitui-

se numa barreira para a sua biodisponibilidade. Ainda, o perfil de biodistribuição de PTS foi investigado utilizando uma solução formulada com HPβCD. Verificou-se que esta formulação facilita o alcance do PTS ao fígado, coração e pulmões de modo abundante (CHOO et al., 2014). Outra vantagem adicional do uso de ciclodextrinas em formulações farmacêuticas, se dá porque a complexação protege moléculas bioativas contra a degradação decorrentes de fatores tais como temperatura, pH e exposição à luz, podendo manter a estabilidade química de alguns compostos bioativos (PINHO et al 2014; SZETJLI 1998).

Embora vantajoso e promissor, poucos estudos se dedicam a explorar os efeitos terapêuticos do PTS complexado a ciclodextrina (Figura 2) em modelos biológicos especialmente relacionados a disfunções cardiovasculares.

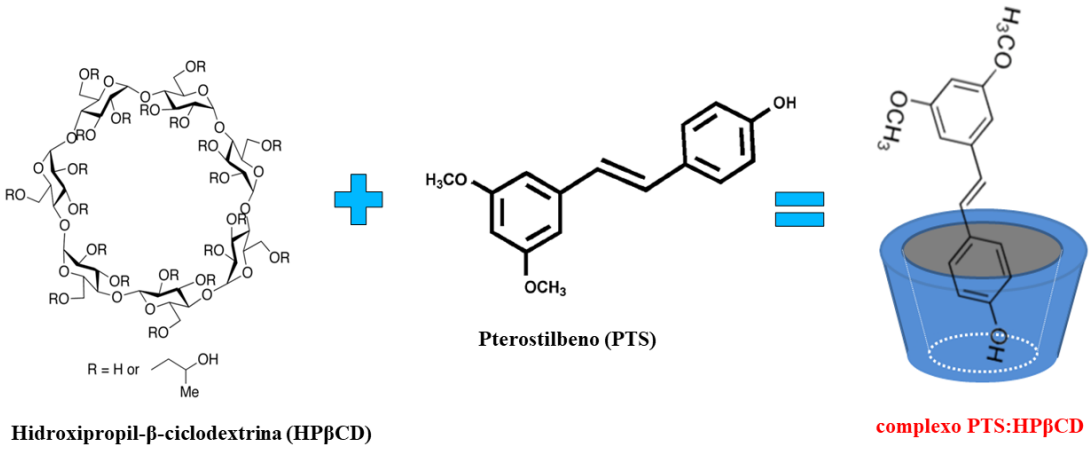


Figura 2: Desenho esquemático do complexo de inclusão PTS:HPβCD

1.3 Remodelamento ventricular e insuficiência cardíaca

A insuficiência cardíaca (IC) é uma doença crônica caracterizada por incapacidade do coração de manter o fluxo sanguíneo necessário para satisfazer as demandas metabólicas do corpo, representando uma das principais causas de morte no mundo (GOLDBERG, 2010). A IC é considerada uma pandemia global em rápido crescimento, com uma prevalência estimada de 37,7 milhões de indivíduos em todo o mundo. O comprometimento funcional cardíaco pode ser secundário a muitas etiologias, acarretando sintomas que afetam a qualidade de vida dos indivíduos doentes, incluindo dispneia, fadiga, baixa tolerância ao exercício e retenção de líquidos (ZIAEIN; FONAROW, 2016).

A terapia farmacológica para IC inclui uma combinação de medicamentos, cuja indicação clínica dependerá da sintomatologia do doente e das contraindicações de cada fármaco (REIS FILHO et al., 2015). A abordagem terapêutica objetiva melhorar os sintomas e a capacidade funcional, prevenir a dilatação da câmara cardíaca, proporcionar qualidade de vida, reduzir a frequência de hospitalizações e mortalidade associada (BERLINER; BAUERSACHS, 2017). Está descrito que tanto a ativação simpática contínua quanto os efeitos proliferativos da angiotensina II afetam adversamente o cardiomiócito e a função contrátil da câmara levando à deterioração da função cardíaca (BERLINER; BAUERSACHS, 2017). Neste contexto, o bloqueio farmacológico destas vias, ou seja, através da administração de betabloqueadores associados à Inibidores da Enzima Conversora de Angiotensina (IECA) ou Bloqueadores de Receptores da Angiotensina II (BRA) determinam benefícios diretos sobre o remodelamento cardíaco, visto que os IECA previnem a dilatação cardíaca e os betabloqueadores revertem, efeitos que melhoram o prognóstico dos indivíduos com IC (BERLINER; BAUERSACHS, 2017; REIS FILHO et al., 2015).

Segundo a Diretriz Brasileira de Insuficiência Cardíaca (2012) a estratégia de tratamento disponível integra os fármacos betabloqueadores, IECA ou BRA, Diuréticos, Nitratos, Anticoagulantes, Antiagregantes plaquetários, Antiarrítmicos, Bloqueadores de canais de cálcio, Ivabradina, ômega 3, Inibidores da Fosfodiesterase 5 e Moduladores do Metabolismo Energético Miocárdico (BOCHI et al., 2012). Apesar dos avanços significativos em terapias e prevenção, a mortalidade e a morbidade ainda são extremamente altas (SAVARESE, 2017).

O remodelamento ventricular (ou cardíaco) precede o desenvolvimento e progressão da IC, no qual as câmaras cardíacas aumentam progressivamente e a função contrátil se deteriora. (COHN et al., 2000). Esse evento é caracterizado por alterações moleculares, celulares e intersticiais que se manifestam clinicamente através da alteração de tamanho, massa, geometria e função da câmara cardíaca em resposta à sobrecarga hemodinâmica (pressão/volume) e/ou lesão cardíaca, associada à ativação neuro-hormonal (AZEVEDO et al., 2016). Essas alterações levam à dilatação da câmara, redução da contratilidade, aumento do estresse de parede, hipertensão, morte de cardiomiócitos (COHN et al., 2000). De fato, diferentes mecanismos contribuem para o desenvolvimento e progressão do remodelamento cardíaco, incluindo alterações no metabolismo energético cardíaco, indução de morte celular, disfunção de proteínas contráteis e colágeno, alteração no transporte de cálcio, inflamação e estresse oxidativo

(SCHIRONE et al., 2017; AZEVEDO et al., 2016). Esses fatores podem ter vários efeitos sobrepostos prejudiciais resultando em IC e mau prognóstico (SCHIRONE et al., 2017; AZEVEDO et al., 2016).

O remodelamento ventricular pode ocorrer por meio de mecanismo adaptativo (fisiológico) ou mal adaptativo (adverso/patológico). No primeiro caso, ocorrem alterações estruturais a fim de se adaptar e manter a função cardíaca normal. Porém, quando submetido ao estresse sustentado, o remodelamento cardíaco leva a uma disfunção progressiva e irreversível do coração (COHN et al., 2000).

Interessantemente, o remodelamento mal adaptativo pode provocar disfunção tanto no ventrículo direito (VD) quanto no ventrículo esquerdo (VE) (KONSTAM et al., 2011; NORTON et al., 2002). O remodelamento ventricular direito pode ser decorrente de vasoconstrição e hipertensão arterial pulmonar, que provocam aumento de pós-carga ao ventrículo direito, gerando hipertrofia excêntrica, dilatação da câmara, redução da fração de ejeção e débito cardíaco (MCLAUGHLIN et al., 2011).

No ventrículo esquerdo, o remodelamento adverso deriva de eventos isquêmicos como o infarto agudo do miocárdio (IAM), dentre outros fatores, prejudicando a função tanto de cardiomiócitos infartados quanto de remanescentes (GAJARSA; KLONER, 2011). Para manter a função cardíaca pós-infarto, os cardiomiócitos remanescentes são expostos a uma carga de trabalho maior, resultando inicialmente em hipertrofia compensatória e posterior redução da espessura da parede, dilatação da câmara esquerda, fibrose e morte celular por ativação de apoptose e/ou autofagia (KONSTAM et al., 2011). A taxa de morte de cardiomiócitos no tecido remanescente é um preditor da gravidade do remodelamento e determinará a taxa de progressão para IC (ABBATE; NARULA, 2012).

O diagnóstico clínico de remodelamento ventricular é baseado na sintomatologia e na detecção de alterações morfológicas, incluindo alterações no diâmetro da cavidade, massa (hipertrofia e atrofia), geometria (espessura e forma da parede de ambos ventrículos), áreas de cicatriz após infarto e fibrose (COHN et al., 2000). Os métodos mais utilizados para detectar essas alterações são exames de imagem, como ecocardiografia, ventriculografia e ressonância magnética nuclear (ANAND et al., 2002).

1.4 Remodelamento ventricular: mecanismos fisiopatológicos

Diversas vias moleculares convergem no remodelamento cardíaco adverso. Por exemplo, evidências descrevem que a perda de cardiomiócitos pode ocorrer por necrose, apoptose ou autofagia, enquanto fibrose ocorre por meio da proliferação de fibroblastos

e reorganização da matriz extracelular (BURCHfiELD et al., 2013; WHELAN et al., 2010).

Apoptose ou morte celular programada de células cardíacas promove perda de tecido viável que pode ter um efeito desastroso sobre a geometria e a função ventricular, processo fundamental na progressão para IC (VAN et al., 2005). Ocorre degeneração e fragmentação da célula e formação de corpos apoptóticos que são fagocitados por macrófagos, mecanismo que não gera estímulos inflamatórios (WHELAN et al., 2010). A apoptose pode ser controlada e ativada por via de sinalização intrínseca (mitocondrial) e extrínseca (disparada por receptores de membrana plasmática), sendo que ambas vias culminam na ativação de caspases, família de proteínas que induz apoptose (WHELAN et al., 2010). Os estímulos pró-apoptóticos são transmitidos, em sua maioria, através da via intrínseca, que pode ser ativada por mecanismos de dano oxidativo celular, toxinas, ativação de vias de sinalização de sobrevivência, dentre outros (TRACHOOTAM et al., 2008). A formação de poros e a permeabilidade mitocondrial é regulada pelo balanço entre proteínas Bcl-2 (anti-apoptótica) e Bax (pró-apoptótica) (TRACHOOTAM et al., 2008). Abbate e cols (2003) evidenciaram que a apoptose de cardiomiócitos foi determinante para o remodelamento desfavorável do VE e insuficiência sintomática precoce em humanos, com causa morte atribuída ao IAM.

Outro importante contribuinte para o remodelamento ventricular é a autofagia sustentada, um processo catabólico que ocorre em resposta ao estresse, direcionando proteínas, macromoléculas e organelas para a degradação lisossomal (GURUSAMY et al., 2009). Durante a autofagia, diversos constituintes citosólicos são envolvidos por vesículas de membrana dupla, autofagossomos, que depois se fundem com os lisossomos ou com o vacúolo para degradar sua carga (GATICA et al., 2015). A falha em mecanismos de controle fino da autofagia, causa ativação de morte celular, contribui para o processo de transição no remodelamento cardíaco patológico (ZHU et al., 2007). A ativação exacerbada de mecanismos autofágicos em fibroblastos cardíacos produz degradação aumentada do colágeno Tipo 1, prejudicando a integridade da matriz extracelular (LAVANDERO et al., 2015). Em cardiomiócitos de camundongos transgênicos, a elevada rexpressão de beclina 1, proteína necessária para formação do autofagossomo, amplifica substancialmente o remodelamento adverso (MOSCAT; DIAZ-MECO, 2009).

Além disso, redução de contratilidade pode ser causada por disfunção mitocondrial e anormalidades metabólicas, contribuindo para a disfunção cardíaca

(BURCHfiELD et al., 2013; WHELAN et al., 2010). A homeostase do cálcio e consequentemente, prejuízo no acoplamento excitação-contração em cardiomiócitos também pode ser prejudicado durante o remodelamento ventricular. Ocorre deficiência na liberação e captação do íon cálcio (Ca^{2+}) pelo retículo sarcoplasmático, mecanismos mediados por proteínas como Cálcio ATPase SERCA, fosfolamban e receptores rianodina (LEHNART et al., 2009). Portanto, o mecanismo de remodelamento reduz a oferta de cálcio durante a sístole e aumenta a concentração de cálcio na diástole, prejudicando tanto o mecanismo de contração quanto o de relaxamento (AZEVEDO et al., 2016). O desequilíbrio do Ca^{2+}, além promover disfunção sistólica e arritmias, pode interferir em processos como o crescimento hipertrófico, metabolismo energético, função mitocondrial e sobrevivência celular (BURCHfiELD et al., 2013**)**. Essas alterações são manifestadas por mudanças na geometria do coração (passando de uma forma elíptica para esférica) e hipertrofia, podendo comprometera fração de ejeção (SCHIRONE et al., 2017).

A inflamação e a oxidação também afetam diretamente a contratilidade cardíaca e o relaxamento. A resposta inflamatória exacerbada induzida por macrófagos e monócitos pós-infarto prejudica o processo de cicatrização do tecido danificado, induzindo fibrose, remodelamento adverso e redução da contratilidade (ANZAI, 2018). Além disso, a ativação neuro-hormonal, induzida pelo sistema renina-angiotensina-aldosterona, aumenta a síntese de proteínas envolvidas na inflamação, estimulando o estresse oxidativo e a morte celular de cardiomiócitos (SCHIRONE et al., 2017). Pós injúria cardíaca, a sinalização inflamatória é sustentada através da regulação positiva da liberação de citocinas, levando à proliferação de fibroblastos e ativação de metaloproteinases (FRANGOGIANNIS 2012).

Evidências sugerem que mecanismos de dano oxidativo e desequilíbrio na sinalização redox, associados à alteração no metabolismo energético ventricular desencadeiam cascatas de sinalização hipertrófica e dilatação da câmara cardíaca, resultando em perda progressiva de cardiomiócitos, fatores que conjuntamente prejudicam progressivamente a função cardíaca (TAKIMOTO et al., 2007; TSUTSUI et al., 2011).

1.5 O impacto do estresse oxidativo no remodelamento ventricular

O desequilíbrio redox intracelular também contribui para o remodelamento ventricular adverso, sendo este mecanismo ativado pelo aumento da produção de espécies

reativas de oxigênio (EROs) e/ou nitrogênio (ERNs), subprodutos do metabolismo aeróbico (SANTOS et al., 2011; SHAH; CHANNON, 2004). Estas espécies incluem o ânion superóxido ($O^{2\bullet-}$), peróxido de hidrogênio (H_2O_2), radical hidroxil ($^{\bullet}OH$), óxido nítrico (NO) e peroxinitrito ($ONOO^-$), dentre outros, que são capazes de oxidar proteínas, lipídios e ácido desoxirribonucleico prejudicando a homeostase das células cardíacas (BELLÓ-KLEIN et al., 2014; KEMP; CONTE, 2012). Neste contexto, as altas concentrações de oxidantes em relação a sistema de defesa antioxidante intrínseco promovem dano e perturbam a homeostase celular, caracterizando o estresse oxidativo e/ou nitrosativo (SIES, 1985). Mais recentemente, o estresse oxidativo foi definido como uma situação em que a concentração de EROs no estado estacionário pode ser transitória ou cronicamente aumentada, perturbando o metabolismo celular e sua regulação, além de prejudicar os constituintes celulares (LUSHCHAK, 2011). Ainda, o termo “distress” tem sido relacionado a uma definição mais atual (NIKI, 2016). Interessantemente, uma classificação para o estresse oxidativo, baseada em níveis de intensidade, tem sido proposta em sistemas biológicos, sendo classificada em estresse oxidativo basal, estresse oxidativo de baixa intensidade, estresse oxidativo de intensidade intermediária e estresse oxidativo de alta intensidade, diferenças decorrentes a concentração de indutores (EROs) e parâmetros finais avaliados (LUSHCHAK, 2014).

O acúmulo de espécies oxidantes sobrecarrega o sistema antioxidante enzimático primário, gerenciado pelas enzimas superóxido dismutase (SOD; converte ânion superóxido em peróxido), catalase (CAT) e glutationa peroxidase (GPx), ambas conjuntamente reduzem H_2O_2 em água (WALLIWEL; GUTTERIDGE, 2007). Em relação as defesas antioxidantes não-enzimáticas, destaca-se o papel da glutationa na remoção de EROs. Composta por aminoácidos que contêm grupos tióis (glutamato, cisteína e glicina), a forma reduzida da glutationa (GSH) serve como doador de elétrons para a redução de peróxidos, reação catalisada pela enzima GPx, sendo convertida em glutationa oxidada (GSSG). A reciclagem da glutationa ocorre pela ação da glutationa redutase (GR), que catalisa a conversão de GSSG a GSH, pela transferência de elétrons do nicotinamida adenina dinucleotídeo fosfato (NADPH) mantendo íntegro o principal sistema de proteção antioxidante celular não enzimático (HALLIWELL; GUTTERIDGE, 1999; VASCONCELOS et al., 2007). Além disso, a GSH reage não enzimaticamente com radicais como $O^{2\bullet-}$, •OH e ONOO-, atuando como um varredor direto de EROs (JONES, 2002).

Adicionalmente, o sistema tiorredoxina, incluindo a tiorredoxina (TRx), tiorredoxina redutase (TRxR) e NADPH que formam um sistema integrado adicional às defesas antioxidantes das células, funcionando como poderosa proteína dissulfeto oxidorredutase (TSUTSUI et al., 2011).

Diversos trabalhos demonstram os efeitos deletérios do desequilíbrio redox sobre a função ventricular (BELHAJ et al., 2013; CASTRO et al., 2014; DAI et al., 2011). Por exemplo, em um modelo de hipertensão arterial pulmonar a falência do ventrículo direito foi associada à regulação negativa da proteína de resposta ao estresse heme oxigenasse-1 (HO-1) e a ativação de processos inflamatórios locais (BELHAJ et al., 2013). Shiomi e cols. (2004) demonstraram significativa redução no remodelamento do VE após IAM em camundongos transgênicos que superexpressam a glutationa peroxidase, uma importante enzima antioxidante. Tanto a modulação do balanço redox, quanto a administração exógena de tiorredoxina estão associados à cardioproteção e prevenção do remodelamento mal adaptativo, por redução de apoptose de cardiomiócitos em modelo de IAM (CASTRO et al., 2014; TÃO et al., 2006). Em outro trabalho, a superexpressão de catalase mitocondrial em camundongos transgênicos com cardiomiopatia hipertensiva, reduziu tanto a fibrose quanto a hipertrofia, sugerindo que terapias antioxidantes podem prevenir o remodelamento (DAI et al., 2011).

De fato, o acúmulo de EROs e o estresse oxidativo não só estão associados ao dano direto a biomoléculas e organelas, mas também se relacionam à modulação das vias de sinalização intracelular que regulam o remodelamento cardíaco (Figura 3) (ZHANG et al., 2016). Muitas proteínas que participam destas vias de sinalização sofrem regulação redox, podendo direcionar respostas de estresse adaptativas ou mal adaptativas, sendo o equilíbrio entre essas vias determinante para adaptação ou falha em condições patológicas de sobrecarga ventricular (SANTOS et al., 2011; TRACHOOTAM et al., 2008). Interessantemente, o $^{\bullet}OH$ é capaz de causar oxidação severa e disfunção cardíaca mal adaptativa, mesmo em concentrações muito baixas (escala subnanomolar), enquanto que as EROs menos oxidativas (como $O^{2\bullet-}$, H_2O_2 e NO) estão comumente envolvidas na transdução do sinal em cardiomiócitos, gerando impacto em respostas cardíacas tanto adaptativas, quanto mal adaptativas (SANTOS et al., 2011).

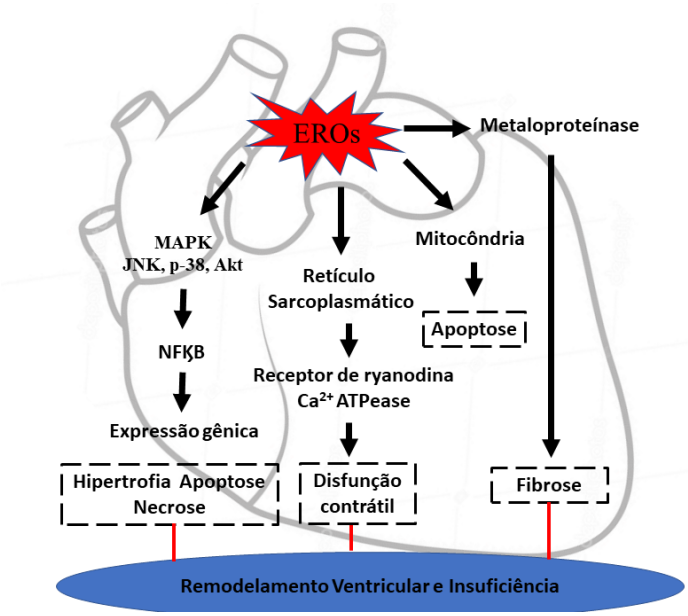


Figura 3: Potenciais alvos moleculares das EROs durante o remodelamento ventricular e insuficiência cardíaca. Adaptado de Tsutsui et al., 2011. EROs: espécies reativas de oxigênio; MAPK: proteínas quinases ativadas por mitógenos; JNK: quinase c-Jun N-terminal; p-38: p-38 MAP-quinase; AKT: serina/treonina quinase; NF-κB: fator nuclear kappa B; Ca^{2+}ATPease: bomba transportadora cálcio.

A sinalização redox envolve a oxidação dos resíduos de cisteína de proteínas mediada por H_2O_2. Esta espécie oxida o ânion tiolato, gerando a forma sulfênica (Cys-SOH) e causando alterações alostéricas dentro da proteína que alteram sua função. A forma sulfênica pode ser reduzida pelas enzimas dissulfeto redutases, como Trx e glutaredoxina (Grx), para retornar à função da proteína ao seu estado original (WINTERBOURN; HAMPTON, 2008). Assim, a oxidação de primeiro grau dos resíduos de cisteína em proteínas serve como um mecanismo de transdução de sinal reversível (SCHIEBER; CHANDEL, 2014).

Modificações redox pós-tradução para proteínas miocárdicas pode afetar a conformação, estabilidade e atividade de diversos receptores, transportadores iônicos (bombas/trocadores/canais), proteínas cinases, fosfatases, caspases, translocadores (GTPases), fatores de transcrição, assim como proteínas estruturais/contráteis, perturbando a homeostase cardíaca (SANTOS et al., 2011). As EROs exercem efeitos centrais sobre a fibrose e remodelação da matriz extracelular em cardiomiócitos, por estimular a proliferação de fibroblastos cardíacos e ativar as metaloproteinases da matriz mitocondrial (MMPs) (DUARTE et al., 2009). As espécies reativas também

desempenham um papel importante na estimulação hipertrófica acoplada à proteína G, por meio da estimulação α-adrenérgica e da angiotensina II (KASS; TAKIMOTO, 2007).

Dentre os principais mediadores de sinalização induzidos por EROs estão proteínas quinases como quinase regulada por sinal extracelular 1 e 2 (ERK1/2), quinase c-Jun N-terminal (JNK), p-38 MAP-quinase (p38) e serina/treonina quinase (AKT). A ativação das proteínas ERK1/2 e AKT é induzida por baixas concentrações de EROs e está associada a sobrevivência e citoproteção na adaptação cardíaca (KWON et al., 2003). Por outro lado, as proteínas ativação da JNK e p38 são estimuladas em resposta ao estresse oxidativo, desencadeando apoptose e contribuindo para a progressão da IC (LOU et al., 2005). Altas concentrações de EROs participam de mecanismos de apoptose através da ativação de cinases de sinalização pró-apoptóticas e por dano oxidativo mitocondrial, enquanto que efeitos opostos foram observados, quando as EROs se mantêm em níveis baixos **(**KASS; TAKIMOTO, 2007). Adicionalmente, outras proteínas redox sensíveis são particularmente importantes para a função e homeostase de cardiomiócitos, incluindo proteína cinase A (PKA) (BRENNAN et al., 2006), proteína cinase G (PKG) (BURGOYNE et al., 2007), receptor de rianodina (RyR) (XU et al., 1998), e histona desacetilase de classe II (AGO et al., 2008).

Finalmente, os EROs influenciam diretamente a função contrátil, modificando as proteínas centrais para o acoplamento excitação-contração. Isso inclui a modificação de grupos -SH no receptor de rianodina (aumentando o tempo de abertura) e interação oxidativa e nitrosativa com Ca^{2+} ATPase reticular sarcoplasmática para inibir a captação de Ca^{2+} (KASS; TAKIMOTO, 2007).

De fato, a compreensão dos mecanismos fisiopatológicos envolvidos no processo de hipertrofia e remodelação cardíaca é fundamental para o desenvolvimento de novos alvos terapêuticos, principalmente porque as taxas de mortalidade relacionadas ao remodelamento/disfunção cardíaca permanecem elevadas (RABABA'H et al., 2018). Neste contexto, a identificação de novos compostos com eficácia biológica e clínica é necessária para expandir as opções terapêuticas a fim de prevenir/tratar o remodelamento ventricular mal-adaptativo e progressão para IC (SCHIRONE, 2017).

2. Objetivos

2.1 Objetivo Geral

Avaliar a toxicidade, determinar a dose terapêutica, potencial antioxidante do pterostilbeno complexado à ciclodextrina (complexo PTS:HPβCD), bem como seu efeito sobre parâmetros funcionais e na modulação de vias redox sensíveis no ventrículo direito e esquerdo de ratos com insuficiência cardíaca.

2.2 Objetivos Específicos

Explorar aspectos tecnológicos farmacêuticos (a fim de melhorar solubilidade do PTS), assim como determinar um curva de dose efetiva e segura, mediante à:

- ✓ Complexação do pterostilbeno com hidroxipropil-β-ciclodextrina.
- ✓ Caracterização do complexo PTS:HPβCD formado sob o ponto de vista químico e físico.
- ✓ Avaliação de três diferentes doses do complexo PTS:HPβCD, sobre parâmetros morfométricos de órgãos, capacidade antioxidante, função hepática, metabolismo da glicose e apoptose no fígado de ratos.

Determinar ação do complexo PTS:HPβCD em ratos com insuficiência ventricular direita, mediante a:

- ✓ Indução de *Cor pulmonale* por meio do modelo de hipertensão pulmonar por administração de monocrotalina.
- ✓ Medida de parâmetros hemodinâmicos, morfométricos e ecocardiográfico do VD
- ✓ Identificação do impacto sobre a lipoperoxidação, carbonilação de proteínas, concentração de espécies reativas, concentração de sulfidrilas, e conteúdo de GSH e GSSG.
- ✓ Investigação do efeito sobre a atividade de enzimas antioxidantes, como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa-S-transferase (GST), glutationa redutase (GR) e glutarredoxina (GRx)

- ✓ Avaliar o efeito do tratamento com o complexo PTS:HPβCD sobre proteínas responsáveis pela contratilidade (serca, fosfolamban) no VD de ratos submetidos ao remodelamento maladaptativo causado por *Cor pulmonale*.

Identificar o efeito do complexo PTS:HPβCD em ratos com insuficiência ventricular esquerda, por meio da:

- ✓ Indução do infarto do miocárdio pela ligadura da coronária esquerda descendente.
- ✓ Avaliação da lipoperoxidação, conteúdo de GSH, GSSG e GSH/GSSG, bem como sobre a atividade da GR, GST, TRx e GRx.
- ✓ Determinação da expressão de Nrf2 no remodelamento do VE pós IAM.
- ✓ Avaliação da expressão de proteínas envolvidas na sinalização para a sobrevivência (AKT total e fosforilada), assim como, para apoptose (GSK-3β total e fosforilada).

3. Abordagem Metodológica

3.1 Reagentes

PTS foi adquirido da Changha Organic Herb (Changha, China). HPβCD fornecida pela Roquette Frères (Lestrem, França). Todos os reagentes utilizados foram de grau analítico ou grau HPLC.

3.2 Preparo do complexo PTS:HPβCD

A HPβCD foi utilizada para promover a solubilidade do PTS em meio aquoso, por meio de sua complexação. O complexo PTS:HPβCD foi obtido através da solubilização de 0,3 M de HPβCD em água, seguida da adição do PTS 0,6 M, sob agitação a 37°C por 72 horas (YEO et al., 2013). Posteriormente, a mistura foi filtrada e o teor de pterostilbeno determinado no filtrado utilizando cromatografia líquida de alta eficiência (CLAE), método adaptado de Lin e cols. (2009). O filtrado contendo o complexo PTS:HPβCD foi seco por liofilização (liofilizador modular Edwards MODULYO 4K), resultando num produto sólido.

3.3 Preparo da mistura física PTS:HPβCD

Para comparação, uma mistura física contendo PTS e HPβCD foi preparada em gral de vidro. PTS e HPβCD foram pesados com precisão na proporção molar de 1:1 (m/m) e cuidadosamente misturados durante 30 min.

3.4 Caracterização do complexo PTS:HPβCD

3.4.1 Análise térmica

A análise térmica foi realizada por meio de calorimetria diferencial de exploratória (DSC) com o intuito de registrar a variação de energia calorífica de uma substância em função da temperatura. Para este método foi utilizado o calorímetro Shimadzu DSC-60. Para esta metodologia foram utilizados cerca de 1 a 2 mg das amostras: pterostilbeno, HPβCD, mistura física e complexo (PTS:HPβCD) secos por liofilização. As seguintes condições foram empregadas na análise: atmosfera de nitrogênio de 50 $mL.min^{-1}$, e resolução de 10°$C.min^{-1}$ (25 a 400°C). Os dados foram obtidos utilizando TA Analysis Software.

3.4.2 Espectroscopia no infravermelho

Os espectros no infravermelho de transformada de Fourier foram obtidos em espectrômetro Spectrum BX FTIR equipado com um acessório ATR MIRacle. Foram acumuladas vinte varreduras para todas as amostras (PTS, HPBCD, complexo e mistura física PTS:HPBCD), no intervalo de 4000-400 cm^{-1} com taxa de varredura de 4 cm^{-1}.

3.4.3 Análise por ressonância magnética nuclear de prótons

A análise por ressonância magnética nuclear (^1H RMN) foi realizada utilizando-se espectrômetro Bruker ASCEND 400 MHz e D2O como solvente. Os espectros de ^1H RMN unidimensionais foram adquiridos sob condições padrão. Espectros homonucleares 2D-ROESY bidimensionais foram obtidos a fim de obter informações sobre as interações entre PTS: HPβCD, no complexo e mistura física.

3.4.4 Potencial antioxidante de captura radical total (TRAP)

O TRAP mede a capacidade antioxidante total, baseando-se na decomposição do dicloridrato de 2,2'-Azobis (2-amidinopropano), que gera radicais livres e emite luz. Estes radicais, por sua vez, reagem com o luminol, formando outro radical livre mais energético com maior emissão de luz, que é mais facilmente detectado pelo contador-beta (LKB Rack Beta Liquid Scintillation Spectrometer-1215; LKB Produkter AB, Brommma, Suécia) (LISSI; PASCUAL; CATILHO 1942).

3.5 Estudos *in vivo*

3.5.1 Animais

Para este estudo foram utilizados ratos Wistar, machos, pesando 250 g, provenientes do CREAL-UFRGS. Os animais foram mantidos no Biotério Setorial do Departamento de Farmacologia, agrupados (n = 4/caixa) em caixas de polipropileno (33 x 17 x 40 cm), sob condições de iluminação claro/escuro de 12 horas, temperatura (22 ± 2º C) e umidade (55%) controladas, com livre acesso à água e alimento.

3.5.2 Cálculo Amostral

Para a indução do modelo de remodelamento ventricular direito, o tamanho amostral foi calculado pelo do programa *Sigma Plot 11.0*. No cálculo do tamanho da

amostra, foram considerados probabilidade de erro α = 0.05 e poder do teste estatístico (1-β probabilidade de erro) = 0.95. Para o experimento envolvendo o modelo de *Cor pulmonale* (experimento 1), o n calculado foi 10 animais por grupo experimental (8 grupos). No entanto, de acordo com resultados encontrados na literatura, taxa de mortalidade nos animais tratados com monocrotalina para indução da insuficiência cardíaca direita (ICD) é de aproximadamente 25%. Assim, nos grupos de animais (MCT) induzidos a insuficiência cardíaca, é recomendado acrescentar mais 4 animais ao tamanho amostral calculado (SOUZA-RABBO et al., 2008). Portanto, para o experimento 1 foram necessários 96 animais.

Para indução do remodelamento ventricular esquerdo decorrente do infarto do miocárdio, o tamanho amostral foi calculado através do programa *Sigma Plot 11.0*, sendo considerados probabilidade de erro α = 0,05 e poder do teste estatístico = 0,80. O tamanho de efeito calculado foi = 0,7 baseando-se nos valores de médias e desvios padrões entre os grupos SHAM e IAM para o parâmetro fração de ejeção (%EF) apresentados no trabalho de Teixeira e cols. (2017). O n calculado, portanto, foi de 8 animais por grupo (3 grupos experimentais), totalizando 24 animais.

3.5.3 Desenho Experimental

Para determinar os efeitos do complexo PTS:HPβCD sobre o remodelamento do VD e VE foram utilizados dois diferentes modelos experimentais, como se segue.

3.5.3.1 Remodelamento do VD: indução da hipertensão arterial pulmonar e demais procedimentos

Hipertensão arterial pulmonar induz remodelamento do ventrículo direito e progressão para insuficiência cardíaca. Neste modelo experimental, foram utilizados 64 ratos Wistar machos, adultos, divididos em grupo controle (CTR; n=7) e grupo submetido ao modelo de hipertensão pulmonar (MCT; n=7-8). A hipertensão pulmonar foi induzida pela administração do alcaloide monocrotalina (Crotaline – C240 SIGMA) 60mg/kg via intraperitoneal (SINGAL et al., 2000). Após 7 dias da indução (período para o desenvolvimento da doença cardíaca direita), tanto os animais CTR como MCT receberam doses diárias do complexo PTS:HPβCD correspondentes a 25, 50 ou 100 mg/kg/dia de PTS (grupos: MCT25; MCT50; MCT100, respectivamente) ou veículo (solução aquosa com HPβCD) (grupos: CTR0 e MCT0), via gavagem, por um período de

14 dias. Ao final do período experimental, os animais foram anestesiados com xilazina (20 mg/kg i.p) e ketamina (90 mg/kg i.p.) e submetidos à avaliação da função ventricular direita por meio de ecocardiografia. Ainda sob o efeito da anestesia, foi realizado cateterismo da veia jugular para avaliação dos parâmetros hemodinâmicos. Posteriormente, os animais foram eutanasiados, sendo o fígado, o coração e os pulmões retirados e pesados. Os ventrículos foram separados e pesados, e o VD utilizado para as análises bioquímicas e moleculares.

Durante a avaliação ecocardiográfica, as imagens foram obtidas por modo bidimensional e modo-M (Philips HD7 Ultrasound System; Andover, MA, EUA), utilizando um transdutor S12-4 (Philips; Andover, MA,5USA). Para determinar a função sistólica e diastólica direita, os seguintes parâmetros foram avaliados: fração de encurtamento (FEC), mudança de área fracional (FAC), excursão sistólica do plano do anel da tricúspide (TAPSE) e razão E/A (RUDSKI et al., 2010).

Os parâmetros hemodinâmicos foram determinados para estimar os efeitos do complexo PTS:HPβCD sobre a função cardíaca. A monitorização da pressão sistólica (PSVD mmHg) e diastólica final do VD (PDFVD mmHg) foi mensurada pela canulação do VD, com auxílio de um cateter conectado a um transdutor e amplificador de pressão (SCHENKEL et al., 2010). A partir dos registros da onda de pressão ventricular direita e detecção de pontos máximos e mínimos de cada ciclo cardíaco, foram obtidos os valores positivos da derivada de contração (dP/dtmáx) e negativos da derivada de relaxamento (dP/dtmin) que foram em mmHg/s (SOUZA-RABBO et al., 2008).

O peso dos animais foi monitorado (2 vezes/semana) para correção de dose do complexo PTS:HPβCD e verificar o efeito do tratamento sobre a variação de peso corporal. A fim de determinar o aumento de massa muscular do coração, foi utilizado o índice de hipertrofia do VD, calculado pela razão do peso em grama do ventrículo direito e esquerdo pelo comprimento da tíbia multiplicado por cem e expresso em porcentagem (HU et al., 2003).

3.5.3.2 Remodelamento do VE: indução do infarto agudo do miocárdio e demais procedimentos

O infarto agudo do miocárdio foi produzido por método cirúrgico previamente descrito por Johns e Olson (1954). Anterior ao procedimento cirúrgico, os animais foram anestesiados (ketamina 90 mg/kg e xilazina 20 mg/kg, i.p.) e submetidos a uma ligadura cirúrgica dos ramos anteriores descendentes da artéria coronária esquerda (grupo AMI)

ou a uma operação simulada em que todos os procedimentos cirúrgicos foram realizados, exceto a sutura em torno da artéria coronária (grupo SHAM). Para minimizar a dor e o desconforto no pós-operatório, todos os animais foram tratados durante três dias com analgésicos tramadol (12,5 mg/kg, via subcutânea, de 12/12 horas) e dipirona (50 mg/kg, por gavagem, de 12/12 horas). Sete dias após o procedimento cirúrgico, os animais foram submetidos a uma avaliação ecocardiográfica para confirmar a eficácia da cirurgia de infarto e foram divididos em três grupos: animais simulados (grupo SHAM), animais infartados não tratados (grupo IAM) e animais infartados e tratados (Grupo IAM + PTS). Posteriormente, os ratos IAM + PTS começaram a receber o complexo PTS: HPβCD em dose correspondente a 100 mg/kg/dia de PTS (AMI + PTS) e os ratos SHAM e AMI receberam o veículo (solução aquosa com HPβCD), diariamente por administração oral, por 8 dias. A dose de 100 mg/kg/ dia foi selecionada com base em resultados anteriores do nosso grupo de pesquisa, evidenciados no VD (Lacerda et al., 2017). A mortalidade, avaliada 24 h após o procedimento cirúrgico, foi de aproximadamente 10%.

A função cardíaca esquerda foi analisada por ecocardiografia, 14 dias após a cirurgia. Os ratos foram anestesiados (ketamina 90 mg/kg e xilazina 20 mg/kg, i.p.) e colocados na posição de decúbito lateral esquerdo (45°) para obter imagens cardíacas. O sistema de ultra-som Philips HD7 XE com um transdutor L2-13 MHz foi utilizado. As áreas transversais sistólica e diastólica do ventrículo esquerdo (cm^2) foram obtidas pelo rastreamento da borda endocárdica em três níveis: basal, médio e apical. Os diâmetros sistólico e diastólico do ventrículo esquerdo (cm) foram medidos usando o Modo-M (NOZAWA et al., 2006). A variação da área fracionada (%), medida como FAC = (área diastólica, área sistólica/área diastólica) × 100, foi utilizada para determinar a função contrátil e a função ventricular esquerda (NOZAWA et al., 2006). Em cada plano transversal ecocardiográfico, o arco correspondente aos segmentos com infarto (I) e o perímetro endocardial total (EP) foram medidos na diástole final. O tamanho do infarto (IS) foi estimado como% IS = (I / EP) × 100 (TAVARES et al., 2010).

Após o período experimental, os animais foram eutanasiados, o VE foi separado, pesado e utilizado para as análises bioquímicas e moleculares.

3.5.4 Morfometria e índice de hipertrofia ventricular

O peso dos animais foi monitorado (2 vezes/semana) para correção de dose do complexo PTS:HPβCD e verificar o efeito do tratamento sobre a variação de peso corporal. A fim de determinar o aumento de massa muscular do coração, foi utilizado o

índice de hipertrofia do VD e do VE, calculado pela razão do peso em grama do ventrículo direito e esquerdo pelo comprimento da tíbia multiplicado por cem e expresso em porcentagem (HU et al., 2003). A diferença entre o peso úmido e seco do pulmão e fígado foi utilizado para estimar congestão hepática e pulmonar (SINGAL et al., 2010).

3.5.5 Preparo do homogeneizado

Amostras de VD e VE foram homogeneizadas (OMNI Tissue Homogeneizer, OMNI International, USA) durante 30 segundos em tampão KCl 1,15% (p/v) contendo 1% (v/v) de inibidor de proteases (fluoreto de fenil metil sulfonil; PMSF).Os homogeneizados foram centrifugados (1358 xg, 20 min, a 4°C; ALC Multispeed Refrigerated Centrifuge PK 121R, Thermo Electron Corporation, USA), sendo o sobrenadante coletado para a determinação dos parâmetros bioquímicos.

Para as análises moleculares de Western Blotting, ambos homogeneizados de VD e VE foram preparados com tampão Tris HCl 100mM + EDTA 5mM, pH=7,4 (Cell Lise) e cloreto de potássio (KCl) 1,15 % na proporção de 1:10 (v/v) + fluoreto de fenil metil sulfonil (PMSF) e homogeneizados durante 30 segundos (OMNI Tissue Homogeneizer, OMNI International, USA). Ambas amostras foram centrfugadas a 8000 xg por 10 minutos. A concentração de proteínas foi determinada pelo método de Lowry, utilizando-se albumina sérica bovina como padrão (LOWRY; ROSEBROUGH, 1951).

3.5.6 Ensaios bioquímicos e moleculares

A metodologia dos diferentes ensaios bioquímicos e moleculares apresentados nesta tese está detalhadamente descrita na seção material e método contida nos artigos. As técnicas utilizadas estão listadas abaixo:

- ✓ **Dosagens séricas de função hepática e renal:** proteínas totais, albumina, AST, ALT, GGT, ureia, creatinina, realizadas de acordo com metodologia descrita no kit comercial (LABTEST).
- ✓ **Avaliação de dano oxidativo tecidual:** substâncias reativas ao ácido tiobarbitúrico **(**TBARS) (OHKAWA et al., 1979); lipoperoxidação (LPO) (GONZALEZ et al., 1991); carbonilas proteicas (REZNICK; PACKER, 1994).
- ✓ **Avaliação de espécies reativas totais:** ROS total (LEBEL et al., 1992);
- ✓ **Avaliação de defesas antioxidantes:** Conteúdo de sulfidrilas totais **(**AKSENOV; MARKESBERY, 2001); GSH e GSSG (AKERBOOM; SIES, 1981).

- ✓ **Atividade de enzimas antioxidantes:** SOD (MARKLUND, 1995); CAT (AEBI, 1984); GPx (FLOHE, 1984); GR (ANDERSON, 1985); GST (MANNERVIK; GUTHENBERG, 1981); TRxR (HOLMGREN; BJORNSTEDT, 1995) GRx (HOLMGREN; ASLUND, 1995).
- ✓ **Expressão proteica por western blotting:** AKT total e fosforilada; glicogênio sintase cinase 3- β (GSK-3β) total e fosforilada; Nrf2; fosfolamban e cálcio ATPease SERCA (LAEMMLI, 1970).

3.5.7 Aspectos Éticos

Este projeto foi devidamente aprovado pela Comissão de Ética na Utilização de Animais (CEUA/UFRGS), sob número 28218. Todos os procedimentos desse estudo ocorreram de acordo com a Lei 11.794, de 08 de outubro de 2008, que estabelece normas para a Prática Didático-Científica da Vivissecção de animais; assim como àquelas contidas nos Princípios Internacionais Orientadores para a pesquisa Biomédica envolvendo Animais provenientes do Council for International Organizations of Medical Science (CIOMS) (GOLDIM, 1997).

4. Resultados

Os resultados obtidos na presente tese estão apresentados no formato de Artigos. Para facilitar a organização e o entendimento, os resultados foram compilados e descritos em capítulos, como se segue:

4.1 Capítulo I: Efeito do pterostilbeno complexado com ciclodextrina no fígado de ratos: potencial redução do estresse oxidativo e modulação de proteínas redox sensíveis

4.2 Capítulo II: Pterostilbeno reduz estresse oxidativo, previne hipertrofia e preserva a função sistólica do ventrículo direito em modelo de *Cor pulmonale*

4.3 Capítulo III: Pterostilbeno melhora o volume sistólico e o débito cardíaco em um modelo rato com insuficiência cardíaca direita através da modulação de proteínas do manejo de cálcio e estresse oxidativo

4.4 Capítulo VI: Estilbenoide pterostilbeno preserva a função ventricular esquerda após infarto do miocárdio em ratos: possível envolvimento de proteínas tiois e modulação de GSK-3β fosforilada

Capítulo I

4.1 Efeito do pterostilbeno complexado com ciclodextrina no fígado de ratos: potencial redução do estresse oxidativo e modulação de proteínas redox sensíveis

Este capítulo aborda a metodologia utilizada na complexação do pterostilbeno à ciclodextrina para melhorar sua solubilidade e viabilizar sua administração pela via oral em ratos. Adicionalmente, descreve os resultados da caracterização do complexo obtido sob aspectos físicos e investiga seu potencial antioxidante e a modulação de proteínas redox sensíveis.

Artigo Original intitulado:

Effect of pterostilbene complexed with cyclodextrin on rat liver: potential reduction of oxidative damage and modulation redox sensitive proteins

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ORIGINAL RESEARCH

Effect of pterostilbene complexed with cyclodextrin on rat liver: potential reduction of oxidative damage and modulation redox-sensitive proteins

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Abstract
The objectives of this study were to promote the aqueous solubility of pterostilbene (PTS) by complexation with hydroxypropyl-β-cyclodextrin (HPβCD), characterize the complex under physical aspects, to make its oral administration feasible in biological tests, and to investigate their pharmacological properties. For 14 days, rats received daily PTS:HPBCD complex at doses of 25, 50, or 100 mg kg^{-1} per day orally. The results showed no kidney or liver damage, nor any induction of apoptosis by the administered doses. Also, the complex showed dose-dependent antioxidant effects in the rat liver, as evidenced by a reduction in lipid peroxidation and reactive oxygen species, as well as an increase in non-enzymatic antioxidant. PTS:HPBCD complex also increased the expression of sensitive redox proteins such as AKT and GSK-3β related to the insulin signaling pathway in the liver. Thus, the complexation demonstrated to be able to increase the apparent solubility of PTS making feasible dose curve administration and could be a food alternative complementary to antioxidant therapeutic. Therefore, the PTS:HPβCD complex can be used for prevention of diseases related to oxidative damage and insulin signaling.

Keywords Cyclodextrins · Stilbene · Natural products · Antioxidant · Functional food

Introduction

Bioactive phytochemicals found in fruits and vegetables have functional properties and a positive effects on human health (Yang and Xiao 2013; Lacerda et al. 2016). The stilbenoid pterostilbene (PTS), for example, has attracted great scientific interest due to the many therapeutic properties, including antioxidant, anti-inflammatory, anti-diabetic, and anticarcinogenic activities (Pari and Satheesh 2006; Remsberg et al. 2008; Chakraborty et al. 2010; McCormack and McFadden 2013). PTS is found naturally in a wide variety of berries, such as blueberries (*Vaccinium* spp) and grapes (*Vitis* spp) (Suh et al. 2007; Schmidlin et al. 2008). Chemically, PTS (trans-3,5-dimethoxy-4'-hydroxy stilbene) correspond to the dimethylated resveratrol, differing from the parent, especially by its higher lipophilicity (2010). These PTS characteristics has been related to its differentiated biological effect as the stronger modulation of cellular stress in a dementia model, compared with equivalent doses of resveratrol (Chan and Stewart 1996). It looks promising, but has significantly less research than its predecessor.

Oxidative stress mechanisms alter homeostasis cellular, redox signaling, and apoptosis processes (Sies and Cadenas 1985; Clutton 1997; Dröge 2002). These effects occur through the oxidative modification of macromolecules that

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inhibit protein function, as well modulate sensitive redox proteins, including bax and bcl2, promoting cellular damage and apoptose (Circu and Aw 2010). In addition, ROS can modify the phosphorylation levels of AKT and GSK kinases and impair insulin signaling, among other effects (Rains and Jain 2011). Thus, the use of the antioxidant PTS may represent a therapeutic strategy against oxidative imbalance in different tissues (Kosuru et al. 2016). The antioxidant mechanism of action of PTS is related to reduced production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet-}$) as well as increased intracellular availability of enzymatic and non-enzymatic antioxidants, which are able to restore the intracellular redox balance (McCormack and McFadden 2013). Concomitantly to the potential scavenger, PTS can activate the nuclear factor erythroid 2-related factor 2 (Nrf2), a key regulator of the antioxidant response (Elango et al. 2016). Also, other pharmacological mechanisms have been described for PTS, such as antidiabetic and anti-inflammatory effects (Paul et al. 2009).

PTS is highly hydrophobic, a feature that limits the bioactive potential of PTS, especially when administered orally, resulting in a low bioavailability profile, a pattern observed in the administration of most phenolic compounds (Hu et al. 2003; Bethune et al. 2011). Thus, technological strategies that improve the aqueous solubility and bioavailability of biologically active phenols are of potential interest both for use in the market of food supplements, cosmetological, and pharmaceutical industry (Fang and Bhandari 2010; Rubió et al. 2014).

Cyclodextrins has been widely used for improving the aqueous solubility of food additives and drugs, especially when the poor aqueous solubility represent the main limitation for absorption (Munin and Edwards-Lévy 2011; Fenyvesi et al. 2016). Regarding drug candidates, cyclodextrins seems also to be useful for making feasible biological tests, avoiding the use of solvents, which are forbidden for oral formulations as is the case of dimethylsulfoxide (DMSO). Cyclodextrins are most known for be able to form inclusion complex with lipophilic drugs into their hydrophobic cavity. Its hydrophilic outside surface is responsible for the aqueous solubility of the complexes (Loftsson and Brewster 1996; Pinho et al. 2014). Thus, the inclusion of a lipophilic molecule promotes changes in drug physicochemical properties, increasing their apparent aqueous solubility and also other drug properties (Szejtli 1988). In particular, a derivative of natural cyclodextrin, hydroxypropyl-β-cyclodextrin (HPβCD), has been used in both in vitro and in vivo studies due to its low toxicity and high water solubility (Loftsson and Brewster 1996; López-Nicolás et al. 2009). The aim of the present work was to promote the water solubility of PTS via the formation of a complex with HPβCD, in order to test, in vitro and in vivo, the antioxidant properties. In addition, another objective was to test, in vivo, whether the mechanism of action of the PTS complex involves the modulation of survival and cell death proteins which can be redox-sensitive.

Materials and methods

Chemistry

PTS ($C_{16}H_{16}O_3$, 98% purity) was purchased from Changsha Organic Herb (Changha, China). HPβCD (average molecular weight 1380–1480 Da) was supplied by Roquette Frères (Lestrem, France). All reagents used were of analytical grade or high-performance liquid chromatography (HPLC) grade.

Preparation of a PTS:HPβCD complex

The complexation method was carried out according to our previous study (dos Santos Lacerda et al. 2017). Briefly, the PTS:HPβCD complex was prepared by dissolving 0.3 M of HPβCD in water with an excess of PTS (0.6 M). After shaking (for 72 h at a temperature of 37 ± 0.1) (MULTIST Velp®, Usmate Velate, Italy), the mixture was filtered and lyophilized. Subsequently, HPLC (Shimadzu LC-20A system, Kyoto, Japan) was used to quantify the lyophilized content of PTS in the complex.

Preparation of a PTS:HPβCD physical mixture

For comparison, a physical mixture of PTS and HPβCD was prepared in a glass mortar. PTS and HPβCD were accurately weighed at a molar ratio of 1:1 and carefully mixed for 30 min.

Thermal analysis

Differential scanning calorimetry (DSC) analysis of PTS, HPβCD, their complex and their physical mixture were performed using a Shimadzu DSC-60 calorimeter. The samples were accurately weighed (approximately 2 mg) in aluminum pan and crimped. The measurements were performed under a dynamic nitrogen atmosphere of 50 mL min $^{-1}$, with a heating rate of 10 °C per min^{-1} (25 to 350 °C). The thermograms were evaluated by TA Analysis software using an empty sealed aluminum pan as reference.

Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectra were obtained for samples using a Spectrum BX FTIR spectrometer equipped with a MIRacle ATR accessory. Twenty scans for all samples (PTS,

HPβCD, complex, and physical mixture) were obtained at a resolution of 4 cm^{-1}, from 4000–600 cm^{-1}.

Nuclear magnetic resonance spectroscopy

^1H nuclear magnetic resonance (^1H NMR) analysis was recorded on a Bruker ASCEND 400 spectrometer operating at 400 MHz, using D_2O as solvent. One-dimensional ^1H NMR spectra were acquired under standard conditions. Two-dimensional ^1H homonuclear 2D-ROESY spectra were obtained in order to gain insights into the supramolecular geometry of the PTS:HPβCD complex.

Animals

Male Wistar adult rats (250–300 g) from the *Centro de Reprodução de Animais de Laboratório* (CREAL) of the Universidade Federal do Rio Grande do Sul (UFRGS) were housed in polypropylene cages (40 × 33 × 17 cm), three or four to a cage, under standard environmental conditions (room temperature, 22 ± 2 °C; 12-h light–dark cycle, 7 a.m.–7 p.m.). All rats had free access to food and water. Our experimental protocol was carried out in accordance with the International Guidelines for Use and Care of Laboratory Animals and with Brazilian laws for the scientific use of animals. The protocol began after approval by the Ethical Committee for Animal Experimentation at UFRGS (CEUA-UFRGS #28218). All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable data. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny et al. 2011).

Experimental groups and procedures

Thirty-two male Wistar rats were randomized into four experimental groups (eight animals per group) to receive either the PTS:HPβCD complex, in doses corresponding to 25, 50, and 100 mg kg^{-1} per day of pterostilbene (PTS25, PTS50, PTS100) or vehicle (HPβCD) (PTS0), via gavage for 14 days. At the end of the experimental protocol, animals were anesthetized (intraperitoneal ketamine and xylazine, 90 mg kg^{-1} and 10 mg kg^{-1}, respectively) and euthanised by decapitation. Troncular blood and liver were harvested. Blood samples were centrifuged (1358 × *g* for 15 min), and stored (5 °C) for subsequent renal and hepatic evaluation. Livers were immediately weighed, dissected, stored (−80 °C) and was used for biochemical and molecular analysis.

Evaluation of morphometric parameters

Animals were weighed twice a week for dose correction and to assess the effect of the treatment on the body weight. The total liver weight and relative liver weight (grams of tissue per 100 g body weight) were also measured for inference of toxicity due to the administration of different doses of the PTS:HPβCD complex (Mukinda and Eagles 2010).

Serum biochemical parameters: evaluation of liver and kidney function

Levels of total proteins, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), urea, and creatinine in serum samples were determined using commercial test kits (LABTEST, São Paulo, SP, Brazil) employing an enzymatic quantitative colorimetric method. Endpoint optical densities were measured using a microplate reader (Anthos Zenyth 200 rt, Biochrom, UK).

Tissue preparation

The homogenization of the samples from livers was performed for 40 s with Ultra-Turrax (OMNI Tissue Homogeneizer, OMNI International, USA) in the presence of 1.15% KCl (5 mL g^{-1} tissue) and 100 mM phenyl methyl sulfonyl fluoride (PMSF). Then the homogenates were centrifuged for 20 min at 10,000 × *g* at 4 °C (ALC Multi-speed Refrigerated Centrifuge PK 121R, Thermo Electron Corporation, USA). The supernatant was collected and stored (−80 °C) for determination of oxidative parameters. Protein concentrations in samples were determined by the Lowry method using bovine serum albumin as a standard (Lowry et al. 1951).

Evaluation of oxidative damage in liver

Lipid oxidative damage was determined in liver homogenates by the thiobarbituric acid reactive substances (TBARS), method as described by Ohkawa and colleagues (1979) (Ohkawa et al. 1979). The absorbance (535 nm) was measured using a spectrophotometer (Anthos Zenyth 200 rt, Biochrom, UK). Malondialdehyde was used as standard and the results are expressed as nmol mg^{-1} protein.

Protein oxidative damage in liver samples was determined by the carbonyl assay according to Reznick and Packer (1994) (Reznick and Packer 1994). Absorbance was determined (360 nm) (Anthos Zenyth 200 rt, Biochrom, UK) and results are expressed as nmol mg^{-1} protein.

Total reactive species

In this assay, total levels of intracellular reactive species were measured by the reaction of these species with 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) generating a fluorescence emission (collected at 525 nm)

(Sigma-Aldrich, USA). The results are expressed as pmols of DFC mg^{-1} protein (LeBel et al. 1992).

Antioxidant defense assay

Total radical trapping antioxidant potential (TRAP)

The technique used to measure the total antioxidant capacity is based on the decomposition of 2,2′-Azobis (2-amidinopropane) dihydrochloride, which generates free radicals and emits light. These radicals, in turn, react with luminol, forming another more energetic free radical with higher light emission, which is thus more readily detected by the beta-counter (LKB Rack Beta Liquid Scintillation Spectrometer-1215; LKB Produkter AB, Brommma, Sweden) (Lissi et al. 1992).

Sulfhydryl content

In the liver samples, the sulfhydryl content was measured at 412 nm (Anthos Zenyth 200 rt, Biochrom, UK). This technique evaluates the capacity for non-enzymatic antioxidant defense. The results are expressed as nmol mg^{-1} protein (Aksenov and Markesbery 2001).

GST activity

Gluthatione-S-tranferase activity was evaluated according to Mannervik and Guthenberg (1981). Formation of dinitrophenyl-S-glutathione (DNP-SG) was quantified spectrophotometrically at 340 nm (Anthos Zenyth 200 rt, Biochrom, UK). Results are expressed as μmol mg^{-1} protein.

Western blot evaluation

Liver samples (one-hundred micrograms of protein), electrophoresis (gels of 8–12%, w/v), and protein transfer were performed as previously described (Laemmli 1970). The immunodetection was processed using the following primary antibodies: p-AKT (60 kDa) and AKT (60 kDa), p-GSK-3β (47 kDa) and GSK-3β (47 kDa), Bax (20 kDa) and Bcl-2 (28 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA or Cell Signaling Technology, Beverly, MA). Primary antibodies were detected using anti-mouse or anti-rabbit horseradish peroxidase-conjugate secondary antibodies, and the membranes were revealed by chemiluminescence. The autoradiographic films were quantitatively analyzed in the image densitometer (Imagemaster VDS CI, Amersham Biosciences, Europe, IT). The molecular weights of the protein bands were determined using a molecular weight marker (RPN 800 Rainbow Full Range Bio-Rad, CA, USA) as reference. The results of each membrane were normalized by the method of Ponceau (Klein et al. 1995).

Statistical analysis

The normal distribution of results was evaluated using Shapiro–Wilk tests. Parametric results were analyzed using one-way analysis of variance (ANOVA) and treatment with different doses of PTS:HPβCD complex (25, 50, or 100 mg kg^{-1} per day) as independent variables. Tukey's test was performed to detect differences between groups. Differences were considered significant when $P < 0.05$. The results are expressed as the mean ± S.E.M. The data were analyzed using the Sigma Stat Program (Jandel Scientific Co., v. 11.0, San Jose, USA).

Results

In this study, the PTS:HPβCD complex showed a 1:1 stoichiometry. The complexation constants (K_F) value found was 13,085.24 ± 481 M^{-1}, similarly to the value reported by López-Nicolás et al. (2009).

Thermal analysis

DSC thermograms obtained for pure PTS and HPβCD, their physical mixture, and the corresponding inclusion complex are shown in Fig. 1. The thermogram obtained for PTS (Fig. 1a) exhibited a sharp endothermic peak at 94.55 °C corresponding to PTS melting point (Bethune et al. 2011). In the HPβCD thermogram (Fig. 1b) a broad endothermic band is observed at around 100 °C, which correspond to its dehydration. No melting endothermic peaks are observed in the HPβCD thermogram, corroborating the amorphous structure of the cyclodextrin; above 300 °C a band related to the

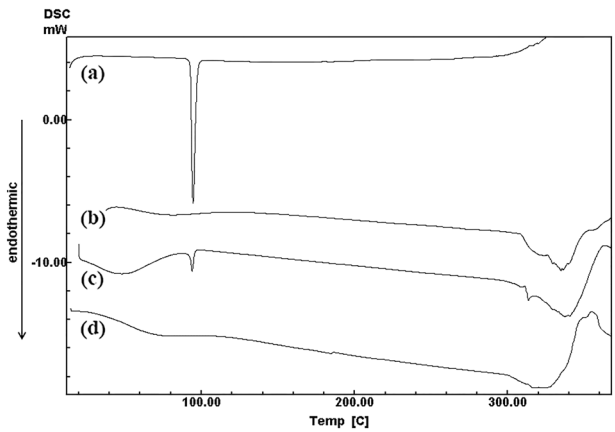

Fig. 1 Differential scanning calorimetry (DSC) analysis. Curves of pterostilbene **a**, HPβCD **b**, pterostilbene:HPβCD physical mixture (1:1) **c** and PTS:HPβCD complex **d**

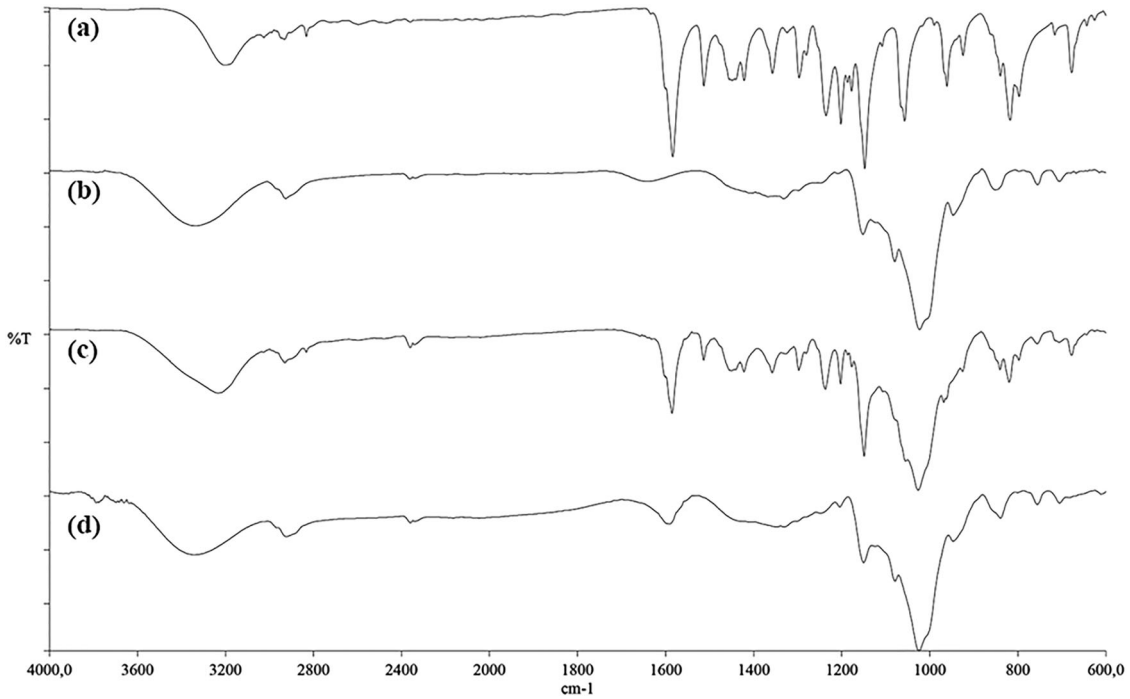

Fig. 2 Fourier transform infrared (FTIR) spectroscopy. FTIR spectra of pterostilbene **a**, HPβCD **b**, pterostilbene:HPβCD physical mixture (1:1) **c**, and PTS:HPβCD complex **d**

HPβCD decomposition is observed. For the PTS: HPβCD physical mixture (Fig. 1c), a superposition of the thermograms of both components is observed. The DSC curves of the PTS:HPβCD complex (Fig. 1d) show the complete disappearance of the PTS fusion peak, indicating the formation of amorphous aggregates and interaction between the two components. Decomposition of the PTS:HPβCD complex is observed above 300 °C.

Fourier transform infrared (FTIR) spectroscopy

The investigation of the functional groups of PTS that are involved in cyclodextrin complexation is described in Fig. 2. The PTS spectrum exhibited characteristic intense bands at 3204 cm^{-1} (O–H stretching), 2932–2832 cm^{-1} (aromatic groups), 1583 cm^{-1} (C–C aromatic double bond), and 817 cm^{-1} (C–H stretching) as observed by Silva et al. (2014). The HPβCD spectrum showed absorption bands at 3339.81 cm^{-1} (O–H stretching), 2925.99 cm^{-1} (C–H stretching), and 1331.87 cm^{-1} (H–O–H bending) (Yatsu et al. 2013). The FTIR spectra of the PTS:HPBCD complex were compared to the physical mixtures and pure PTS. Bands of similar wavelength as in the HPβCD spectrum were observed in the physical mixture. The PTS band in the region of 1583 cm^{-1} was found in both, inclusion complex and physical mixture FTIR spectra, but the intensity was reduced. In the PTS:HPβCD solid complexes spectra, the characteristic absorption bands of PTS (3204 cm^{-1}, 2932–2832 cm^{-1}, and 1583 cm^{-1}) decreased in intensity. The C–C aromatic double bond stretching (1591 cm^{-1}), the C–C olefinic stretching (1513 cm^{-1}), and the *trans* C–H olefinic stretching (961 cm^{-1}) peaks also appear bumped and diminished in intensity in the complex spectra.

Nuclear magnetic resonance spectroscopy

One-dimensional ^1H NMR spectra showed characteristic signs for HPβCD, corresponding to the hydrogens of the cavity, H_3 and H_5, at, respectively, 3.40 to 4.00 ppm (Morales Moraes et al. 2007). The most characteristic signs for PTS, according to Liu et al (2015), hydrogens H_a (6.67 ppm) and H_b (7.40 ppm) also were observed. Figure 3 presents ^1H homonuclear 2D-ROESY contour maps of the PTS:HPβCD complex. The expansion of the interaction region in the spectrum of PTS:HPβCD complex reveals intermolecular cross-peaks between the hydrogen H_a (6.67 ppm) of PTS and the hydrogen H_5 (3.80 ppm) of HPβCD, demonstrating that the interaction between the phenol moiety of PTS and the hydrogens inside of cyclodextrin cavity yield an inclusion complex.

Morphometrical parameters

Oral treatment with the PTS:HPβCD complex in doses corresponding to 25, 50, and 100 mg kg^{-1} per day of pterostilbene did not significantly change the final body weight,

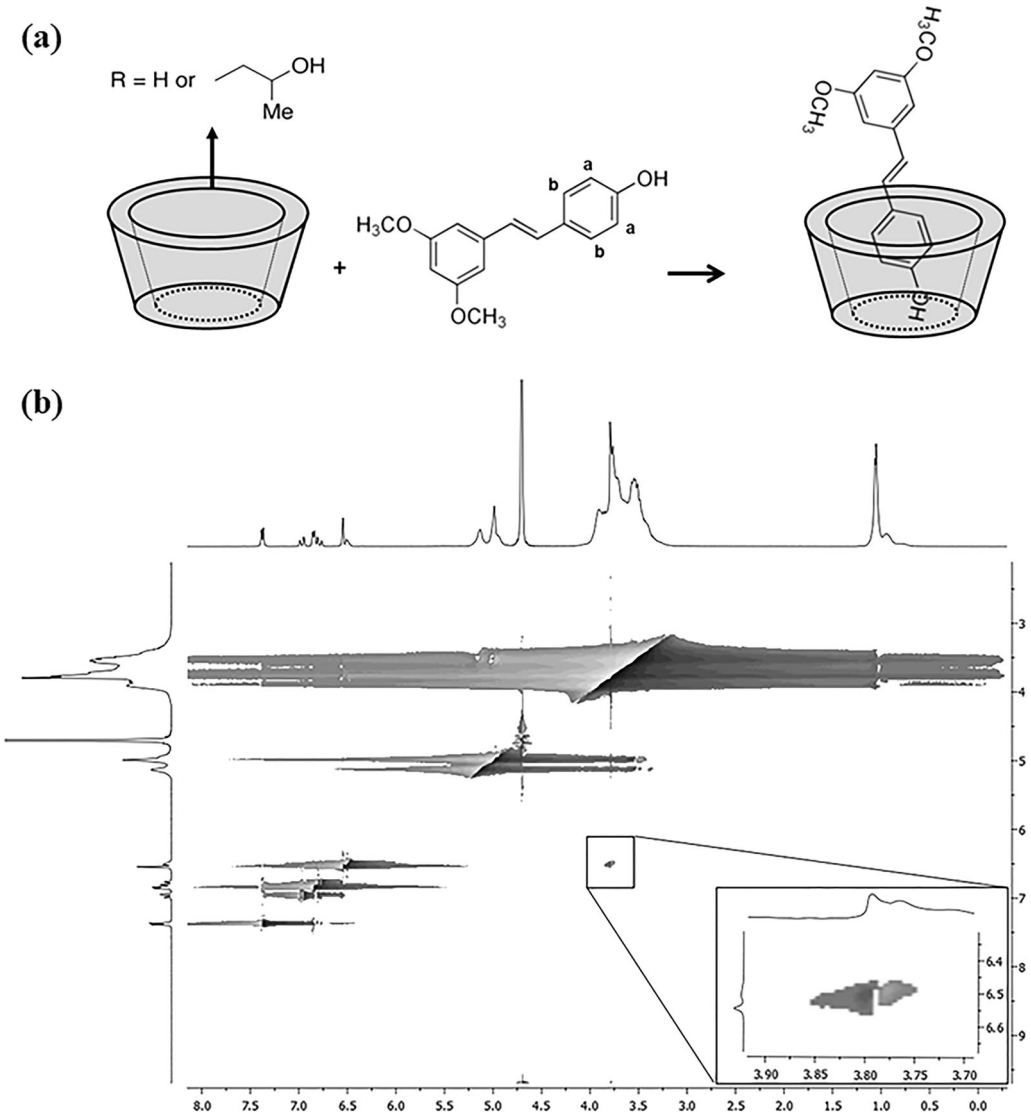

Fig. 3 Nuclear magnetic resonance spectroscopy. **a** Chemical structures of pterostilbene, HPβCD, and PTS:HPβCD complex. **b** ^{1}H homonuclear 2D-ROESY contour map containing PTS:HPβCD complex (400 MHz, D2O)

or cause variations in the relative liver weight after 14 days or the final liver weight, after 14 days (Table 1).

Hepatic and renal function

The PTS:HPβCD complex at all doses (25, 50, and 100 mg kg^{-1} per day of PTS) reduced the creatinine and urea concentrations in serum. Total proteins, albumin, AST, ALT, and GGT levels in serum were not altered by the treatment (Table 1).

Oxidative parameters

TBARS and proteins carbonyl content were measured in the liver to estimate the effects of the PTS:HPβCD complex on lipid and protein oxidation. Oral administration of the PTS:HPβCD complex reduced levels of TBARS compared to the control at all doses tested (25, 50, and 100 mg kg^{-1}) ($P < 0.001$). The highest doses (50 and 100 mg kg^{-1}) were able to reduce this parameter with higher efficiency (57% and 60%, respectively) compared with the 25 mg kg^{-1} dose (45.4%) ($P < 0.05$) (Fig. 4a). Protein carbonyl content did not show significant difference between the treatment groups and the control group (Fig. 4b). The levels of total reactive species were reduced by all tested doses of the PTS:HPβCD complex (25, 50, and 100 mg kg^{-1}) (28.4%, 33% and 25.3%, respectively) ($P < 0.05$) (Fig. 4c).

The non-enzymatic antioxidant potential was also assessed by quantification of total sulfhydryl groups. For this parameter, we found that oral administration of PTS:

Table 1 Morphometric measurements and serum parameters after 14 days of oral administration of the PTS complex: HPβCD in doses of 25, 50, or 100 mg kg^{-1} per day

Parameter	PTS0	PTS25	PTS50	PTS100	*P*-value
Final BW(g)	305 ± 14.49	313 ± 20.16	336 ± 23.94	333 ± 16.02	0.27
BW change (g)	68.83 ± 5.60	71.28 ± 5.31	70.42 ± 6.616	69.14 ± 5.17	0.83
Liver weight (g)	10.68 ± 0.72	10.78 ± 1.20	11.70 ± 0.72	11.80 ± 1.06	0.53
Albumin (mg dL^{-1})	7.41 ± 1.59	7.86 ± 0.44	7.91 ± 0.54	7.87 ± 1.15	0.72
AST (U L^{-1})	82.64 ± 20.04	92.66 ± 17.20	86.86 ± 15.12	88.29 ± 15.79	0.76
ALT (U L^{-1})	38.12 ± 10.74	31.17 ± 10.87	36.16 ± 10.74	45.27 ± 10.31	0.17
GGT (U L^{-1})	10.71 ± 1.45	8.92 ± 2.01	10.20 ± 0.90	7.96 ± 4.95	0.45
Creatinine (mg dL^{-1})	0.94 ± 0.20	0.52 ± 0.11[a]	0.51 ± 0.06[a]	0.48 ± 0.08[a]	<0.001
Urea (mg dL^{-1})	55.60 ± 7.96	38.25 ± 7.87[a]	42.25 ± 10.56[a]	38.75 ± 10.47[a]	<0.05

Values represented as mean ± standard deviation, $n = 6$–8/group. One-way ANOVA and Tukey's post-hoc tests were performed

BW body weight, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma-glutamyl transferase, *UI* units per liter, *mg* dL^{-1} milligrams per deciliter

[a]Different compared to PTS0 group. PTS0: received the vehicle solution.

HPβCD complex only in the dose of 100 mg kg^{-1} increased sulfhydryl levels in liver tissue (65%) ($P < 0.05$) (Fig. 4d). Moreover, GST activity was not affected by the treatments ($P = 0.17$) (Fig. 4e).

The TRAP in vitro antioxidant activity of the PTS: HPβCD complex (1:1 M) had the same behavior as PTS dissolved in dimethylsulfoxide (DMSO) when using equimolar concentrations (195.05 µmolar) (Fig. 5). HPβCD (231.4 µmolar) tested alone did not exhibit antioxidant activity (Fig. 5). In summary, TRAP in vitro test showed that PTS exhibited high capacity for scavenging peroxyl radicals, in both, DMSO solution or as HPβCD complex.

Western blot evaluation

The expression of Bax and Bcl-2 proteins was determined in order to estimate the effect of administration of PTS on hepatic apoptosis. Our results show that there was no change in the expression of these proteins in the liver of rats after oral administration of the HPβCD complex in our experiment (Fig. 6).

When evaluating the hepatic expression of Total AKT, we observed that PTS: HPβCD complex (100 mg kg^{-1} dose) increased the expression of this protein ($P < 0.05$) (Fig. 7a), but did not alter the expression of p-AKT (Fig. 7b), nor did the relation between them (P-AKT/AKT) (Fig. 7c).

Regarding the expression of total GSK-3β, we observed that the PTS: HPβCD complex increased the expression of this protein only by the dose of 50 mg kg^{-1} ($P < 0.05$) (Fig. 8a). However, there was no change in p-GSK-3β and p-GSK-3β/GSK-3β rate in any of the doses tested (Fig. 8b, c).

Discussion

Functional foods, such as pterostilbene, have demonstrated therapeutic potential for the treatment and/or prevention of several diseases (Rimando et al. 2002; Saw et al. 2014; Elango et al. 2016). However, the bioactive potential of PTS may be limited by its very low solubility in aqueous media, resulting in a low oral bioavailability profile (Bethune et al. 2011). In the present study, the HPβCD was used to enhance the aqueous solubility of PTS for oral administration in rats. This study is pioneering to demonstrate that the formation of the inclusion complex between PTS and HPβCD can be confirmed using ^{1}H homonuclear 2D-ROESY. Additionally, we show here that complexation does not alter antioxidant activity in vitro and in vivo (rat liver). Still, we detected the administration of PTS:HPβCD complex reduced lipoperoxidation and ROS levels, increased non-enzymatic antioxidants, as well as modulated the expression of AKT and GSK-3-β proteins in the liver of rats. Further, we demonstrated the safety profile of the administered doses and its possible protective effect in renal function.

Due to its very low aqueous solubility (approximately 21 µg mL^{-1}), most of the reported tests have been carried out using DMSO to dissolve the PTS. However, the use of this kind of solvent represents a strong limitation for pharmaceutical preparations due to its low physiological tolerance and adverse effects (Hameroff et al. 1981; Santos et al. 2003). Thus, the use of cyclodextrin complexes is a strategy with potential application to the aqueous solubilization of PTS for in vivo administration.

The PTS:HPβCD complex was prepared in aqueous media followed by lyophilization and characterized using the techniques of DSC, FTIR, and ^{1}H NMR. The PTS:

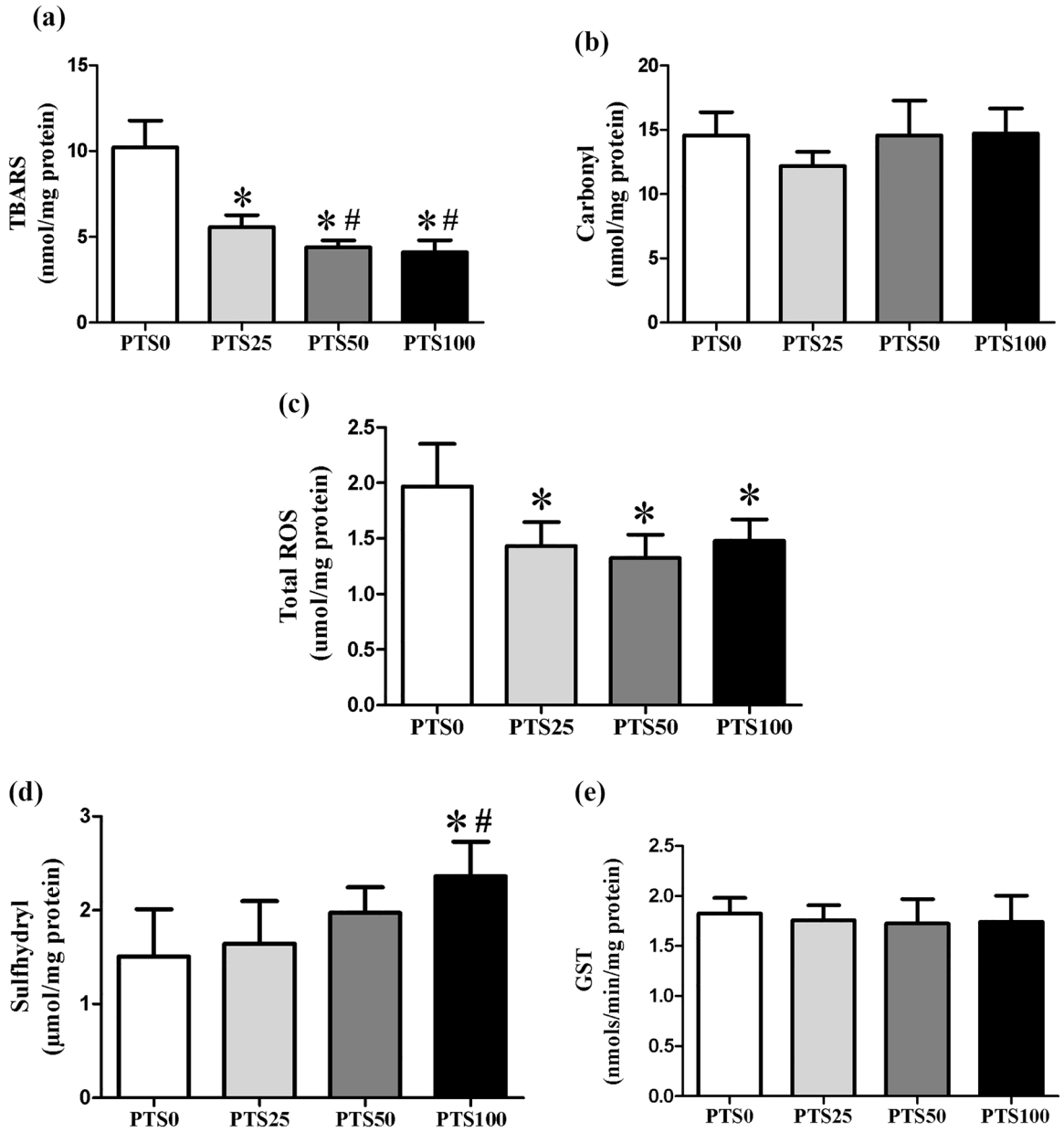

Fig. 4 Evaluation of oxidative damage. Effect of oral administration of the PTS:HPβCD complex at doses of 25, 50, and 100 mg kg^{-1} per day (PTS25, PTS50, PTS100 groups) or vehicle solution (PTS0) on thiobarbituric acid reactive species (TBARS) **a**, proteins carbonyl content **b**, total ROS **c**, sulfhydryl content (total of thiol groups) **d**, and glutathione-S-tranferase (GST) activity **e** in the liver of rats treated via gavage for 14 days. Values are expressed as mean ± standard deviation; $n = 8$ per group; One-way ANOVA and Tukey's post-hoc tests were performed. *Different compared to PTS0 ($P < 0.001$); #Different compared to PTS25 ($P < 0.05$)

HPβCD complex resulted in PTS apparent solubility of 150 mg mL^{-1} in aqueous media, about 7.000 times more soluble than the non-complex PTS (0.021 mg mL^{-1}) (Bethune et al. 2011). Analysis of thermograms obtained for free PTS, and the PTS:HPβCD physical mixture showed the melting point of PTS in both samples. This is indicative that there is probably no interaction between the PTS and HPβCD in a physical mixture. On the other hand, in the PTS:HPβCD complex the peak corresponding to the melting point of PTS disappeared. This effect could be ascribed to a PTS:HPβCD interaction. FTIR spectrum of the PTS:HPβCD physical mixture showed that all the intense bands derived from PTS are present in the spectrum, but are diminished in intensity. This indicates that there is no interaction in the physical mixture. On the other hand, in the inclusion complex obtained by lyophilisation, slight deviations were observed in the wave number of PTS spectral features corresponding to aromatic groups and C–C aromatic double bonds. These observations are compatible with those made by Silva et al. (2014), suggest the presence

of interactions between PTS and HPβCD molecules, and open the possibility of inclusion complex formation (Silva et al. 2014).

The one-dimensional ^{1}H NMR spectrum of PTS showed a hydrogen H_a signal 6.67 ppm that appears displaced from 6.49 ppm in the PTS:HPβCD complex spectrum. In addition, an interaction between PTS hydrogen H_a (6.49 ppm) with the hydrogen of the cavity, probably H_5 at 3.78 ppm, was observed in a ^{1}H homonuclear 2D-ROESY spectrum. Therefore, the 2D-ROESY spectrum suggests that the PTS:HPβCD inclusion complexes have been formed through the insertion of the aromatic ring containing hydrogen H_a into the cyclodextrin cavity, since the hydrogen H_5 of HPβCD is located inside the cyclodextrin cavity. The inclusion complex formation between PTS and HPβCD was confirmed for the first time using ^{1}H homonuclear 2D-ROESY. Traditionally inclusion complexes obtained by solution complexation methods are formed by establishing a hydrodynamic equilibrium between free and complexed PTS molecules to HPβCD. During inclusion complex formation, the water molecules present in the cyclodextrin cavity are replaced by the PTS molecules with less polar characteristics. This process is thermodynamically favored, decreasing the total energy of the system (Rekharsky and Inoue 1998).

In addition to enabling the solubilization of poorly soluble compounds in pharmaceutical preparations, the complexation with cyclodextrin allows the incorporation bioactive compound in food production allowing the development of new functional foods, increasing its

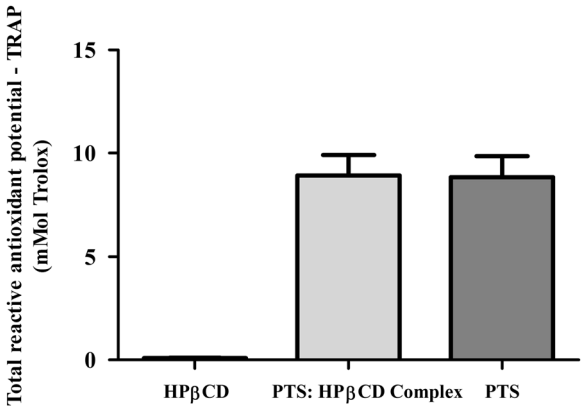

Fig. 5 Total antioxidant capacity (TRAP). TRAP (mmol Trolox) of pterostilbene (PTS), PTS:HPβCD in equimolar concentrations (195.05 μmolar) and HPβCD in vitro

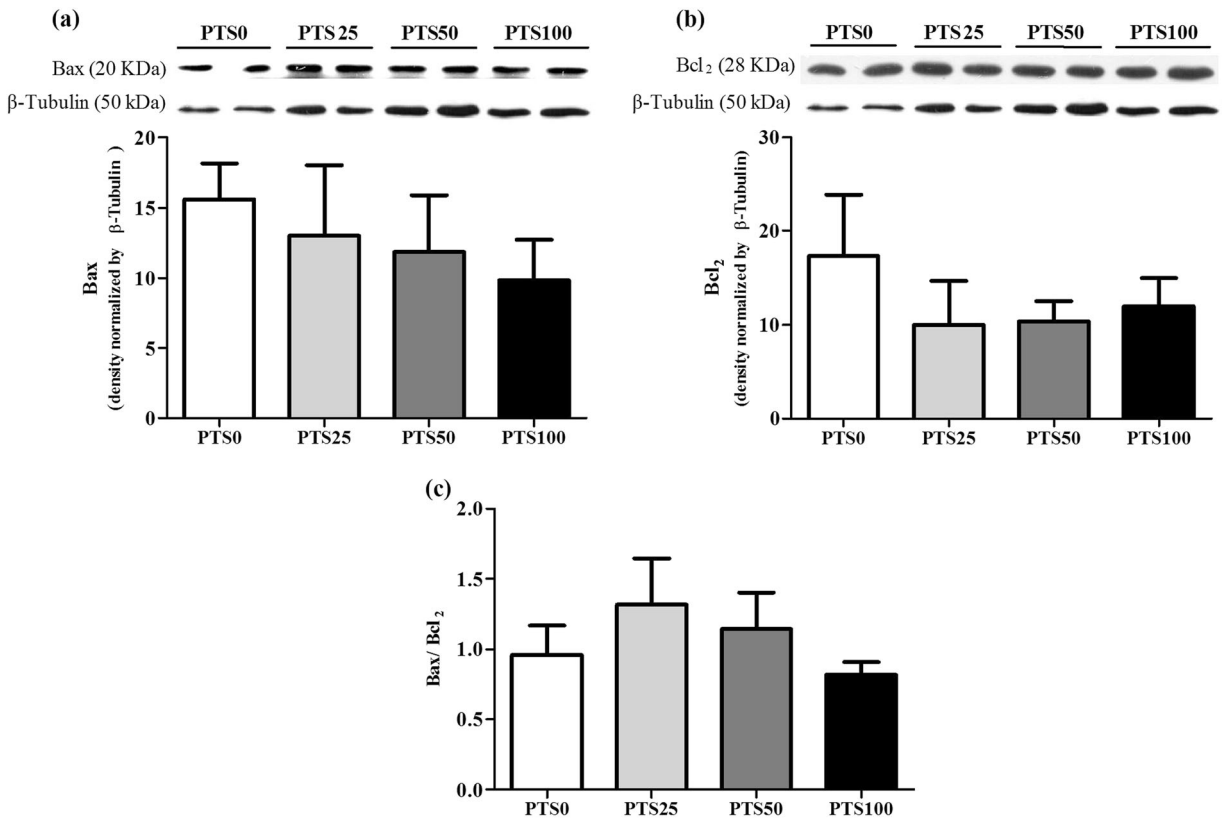

Fig. 6 Expression of apoptosis-related proteins. Effect of oral administration of the PTS:HPβCD complex at of 25, 50, and 100 mg kg^{-1} per day (PTS25, PTS50, PTS100 groups) or vehicle solution (PTS0) on Bax **a** and Bcl-2 **b** protein expression and on Bax/Bcl-2 ratio (**c**) in liver of rats treated via gavage for 14 days. Values are expressed as mean ± standard deviation; $n = 8$ per group; One-way ANOVA

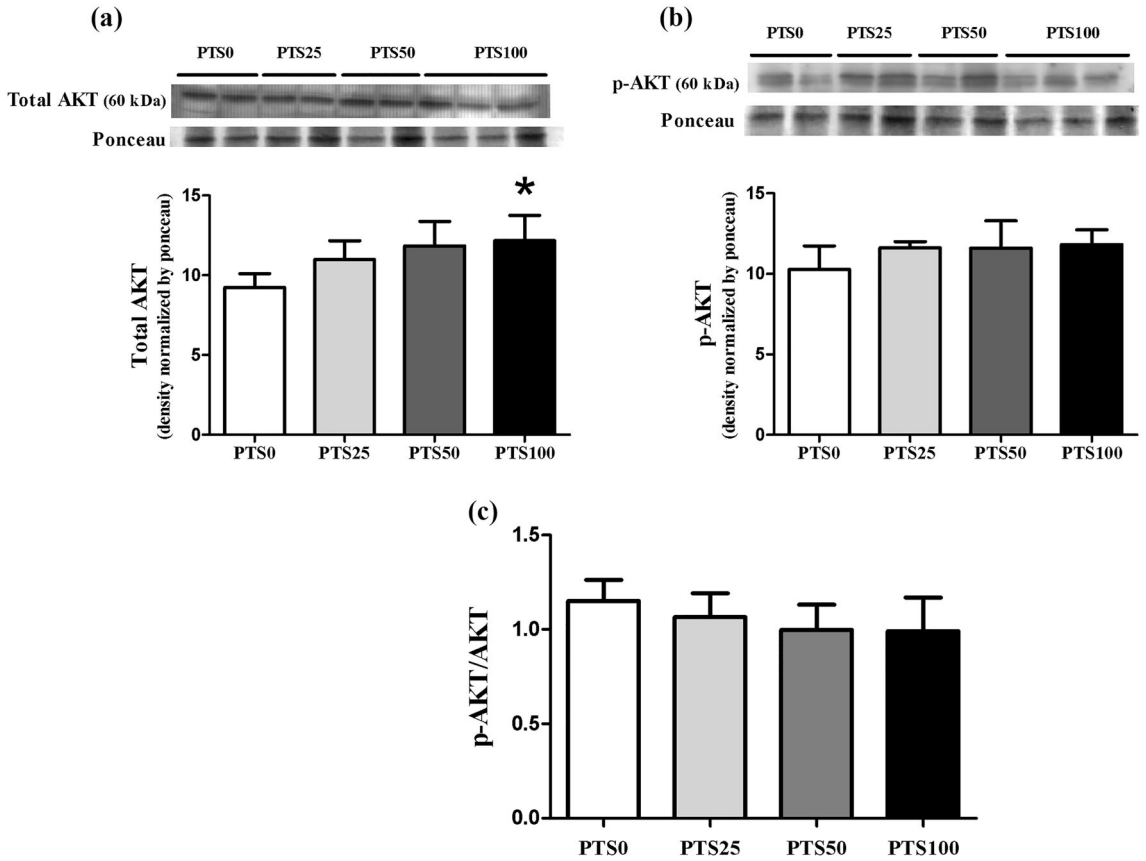

Fig. 7 Expression of redox-sensitive proteins: AKT signaling. Effect of oral administration of the PTS:HPβCD complex at of 25, 50, and 100 mg kg^{-1} per day (PTS25, PTS50, PTS100 groups) or vehicle solution (PTS0) on Total AKT **a** and p-AKT **b** protein expression and on p-AKT/AKT ratio **c** in liver of rats treated via gavage for 14 days. Values are expressed as mean ± standard deviation; $n = 8$ per group; One-way ANOVA and Tukey's post-hoc tests were performed. *Different compared to PTS0 ($P < 0.05$)

functionality (Pasrija et al. 2015). Moreover, the complexation protects bioactive molecules from degradation (against changes in temperature, pH, exposure to light), and increases by four times, the oral bioavailability of PTS compared to PTS in suspension/non-complexed (Yeo et al. 2013; Pinho et al. 2014). Studies have demonstrated the in vitro free-radical scavenging ability of non-complexed PTS using different methodologies (Rimando et al. 2002; Acharya and Ghaskadbi 2013). However, little is known about how this complexation influences the antioxidant effects of PTS.

After complexation and characterization, the PTS:HPβCD complex was used to determine the effects therapeutics potential, in vitro and in vivo, administered orally to rats at three different doses (corresponding to 25, 50, and 100 mg kg^{-1} per day of pterostilbene) during 14 days. When evaluating the effect of administration of the PTS:HPβCD complex on oxidative parameters in liver tissue, we found a reduction in the total concentration of ROS and lipid peroxidation by all three doses, with a more pronounced reduction of TBARS levels at the higher doses (50 and 100 mg kg^{-1} per day). The non-enzymatic antioxidant levels (such as total sulfhydryl groups) have been increased in liver tissue only at the 100 mg kg^{-1} per day dose. Thiol groups (SH), presented on cysteine residues and on glutathione, are non-enzymatic defense systems, which play an important role in maintaining the intracellular redox state (Prakash et al. 2009). Phenolic compound can modulate enzymes related to glutathione synthesis increasing the availability of non-enzymatic antioxidants (Moskaug et al. 2005). Increased levels of glutathione have been found in human erythrocytes after PTS administration (Mikstacka et al. 2010). Other studies highlight the ability of PTS to activate the Nuclear Factor (Erythroid-Derived 2)-Like 2 (Nfr2), a regulator of genes involved in antioxidant responses. These effects are important, because ROS in high concentration participate in the pathophysiology of liver diseases, promoting cellular damage through the oxidation of lipids and proteins, as well as the reduction in levels of enzymatic and non-enzymatic antioxidants (Valko et al. 2007; Marí et al. 2010; Ayala et al. 2014).

The reduction in total ROS in liver tissue caused by administration of the PTS:HPβCD complex can be related to the reducing capacity of the phenolic hydroxyl of PTS,

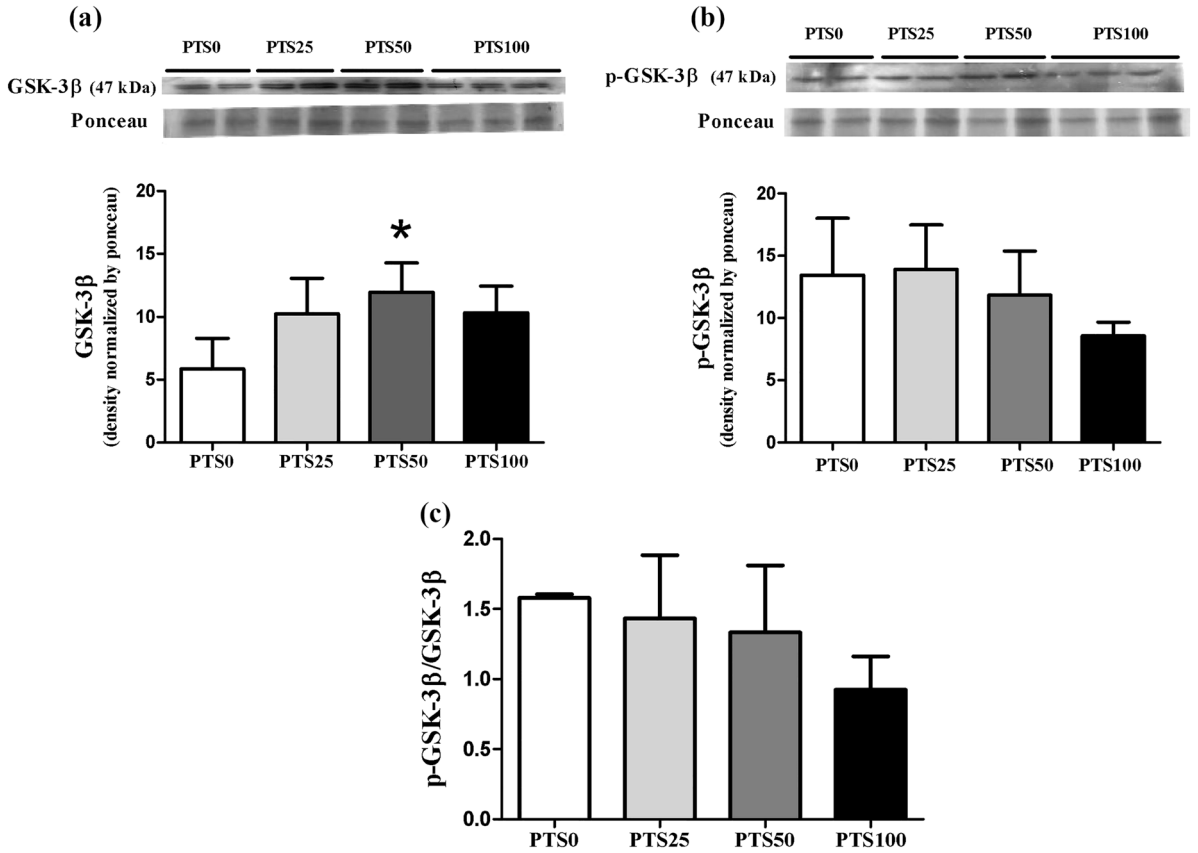

Fig. 8 Expression of redox-sensitive proteins: GSK-3β signaling. Effect of oral administration of the PTS:HPβCD complex at of 25, 50, and 100 mg kg^{-1} per day (PTS25, PTS50, PTS100 groups) or vehicle solution (PTS0) on GSK-3β **a** and p-GSK-3β **b** protein expression and on p-GSK-3β/GSK-3β ratio (**c**) in liver of rats treated via gavage for 14 days. Values are expressed as mean ± standard deviation; $n = 8$ per group; One-way ANOVA and Tukey's post-hoc tests were performed. *Different compared to PTS0 ($P < 0.05$)

which donates an electron to radical species, stabilizing them (Ross and Kasum 2002; Perron and Brumaghim 2009). The results have shown this antioxidant action, since PTS:HPβCD complex exhibits a superior capacity for the elimination of peroxyl radicals compared with Trolox (a well-known reference antioxidant) and that the PTS:HPβCD complex is as effective as non-complexed PTS dissolved in DMSO in terms of its antioxidant activity, indicating that complexation with HPβCD is able to avoid the use of DMSO in this biological test. Phenolic compound can also act as chelating agents for metals such as iron (Fe^{2+}) and copper (Cu^{2+}), inhibiting the Fenton and Haber-Weiss reaction and consequently the production of ROS, contributing to protection against lipoperoxidation (Perron and Brumaghim 2009; Rodrigo et al. 2011). Corroborating our results, other authors also highlight the antioxidant effect of PTS in rat liver (Pari and Satheesh 2006; El-Sayed et al. 2015). In right heart failure, the oral administration of PTS + HPβCD complex is able to reduce ROS production associated to the inductor effect of antioxidant response, resulting in protection of systolic function (dos Santos Lacerda et al. 2017). Acharya and Ghaskadbi (2013) highlighted the ability of PTS to inhibit $HO^{\bullet}$ in vitro, as well as its ability to protect mitochondrial lipids from oxidative damage in hepatocytes (Acharya and Ghaskadbi 2013).

Sensitive redox protein kinases, such as AKT (or protein kinase B) and GSK-3-β, can be modulated by intracellular ROS levels by altering signaling pathways that can induce pathological processes (Zhang et al. 2016). These proteins regulates glucose metabolism and signals to cell survival, among other cellular functions (Hanada et al. 2004). In this context, we also evaluated the effects of PTS complex on AKT/GSK-3β signaling in rat liver. PTS:HPβCD complex (dose 100 and 50 mg kg^{-1}, respectively) induced increased expression of total AKT and total GSK-3β in the liver of rats. This increase the intracellular availability of this protein (AKT) for phosphorylation in the event of signaling for activation of PI3K/AKT, demonstrating that PTS complex may consolidate redox-sensitive signaling pathway mediate by insulin action. However, an opposite effect seems to have been induced by PTS complex at dose of 50 mg kg^{-1}, since there was increased expression of GSK-3β, an enzyme that inhibits insulin-stimulated hepatic glycogen synthesis. It has been described that ROS activate nuclear factor-κB

(NFKB) and mitogen-activated protein kinases (MAPKs), that increase phosphorylation in serine/threonine residues of key components in the insulin signaling pathway, culminating negative effects on signal transduction and glucose homeostase impairment (Rains and Jain 2011). Literature data have reported that phenolic antioxidant may elevate the total AKT level, as well as levels of total GSK-3 in the liver rats (Cao et al. 2007). Pari and Satheesh (2006) report that PTS increases insulin levels and modulate enzymes of the hepatic glycolysis pathway, regulating glucose metabolism.

The complexation significantly increases the bioavailability of PTS, a compound that has a non-linear pharmacokinetics, elevating its plasmatic levels. In this context, the elimination kinetics of PTS could be saturated, leading to increased tissue exposure (Yeo et al. 2013; Choo et al. 2014). Moreover, using a solution formulated with HPβCD, Choo and colleagues showed that PTS is extensively distributed in the liver. Also, high doses of phenolics can have pro-oxidant effects, overloading the cytochrome P-450 enzyme that is involved in the detoxification of these compounds, which can lead to irreversible liver damage (Babich et al. 2011; Bhattacharyya et al. 2014). Toxic effects lead to tissue necrosis and apoptosis, especially in liver, an organ central role in biotransformation, metabolism of these compounds (Sturgill and Lambert 1997; Lee 2003; Kim and Moon 2012). Considering these factors, we evaluated some parameters of liver toxicity to ensure that the repeated doses would be potentially secure, although other studies have found no toxic effects by administration (oral or intravenous) of PTS (non-complexed) in animals and humans (Ruiz et al. 2009; Riche et al. 2013; Choo et al. 2014; El-Sayed et al. 2015). We detected that the body weight, morphometric parameters, as well as seric parameters of hepatic function and GST activity were unchanged. Similarly, we found that administration of the PTS:HPβCD complex not affect the hepatic expression of Bax and Bcl-2, proteins involved in promoting or blocking apoptosis, respectively (Elmore 2007). Administration of the PTS:HPβCD compound reduced levels of creatinine and urea, indicating a possible improvement of renal function. Studies show that the effects of PTS over renal function may be mediated by the regulation of renal transporters, reduced oxidative stress, and inflammation (Shi et al. 2012; Wang et al. 2015).

Given the results obtained in this study, we conclude that the HPβCD complexation allowed us to carry out several biological tests of PTS, a molecule presenting very low aqueous solubility (PTS), in the absence of organic solvents such as DMSO. The PTS:HPβCD complexation does not change the antioxidant capacity of PTS in vitro and promotes its antioxidant effect in vivo, as evidenced by a reduction in total ROS and lipid peroxidation, and an increase in antioxidants in rat liver, especially at the highest dose. Additionally, we show that the PTS:HPβCD complex modulate of expression of sensitive redox proteins related to the insulin signaling pathway. These pharmacological effects showed to be dose-dependent regarding to the PTS:HPβCD complex oral administration. In addition, our findings showed neither renal and hepatic damage, nor any induction of apoptosis by the administered doses during the experimental protocol. Therefore, our results suggest that the PTS:HPβCD complex can be used for health maintenance and prevention of diseases related to oxidative damage and insulin signaling. Moreover, this complexation could be a food alternative complementary to antioxidant therapeutic.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Capítulo II

4.2 Pterostilbeno reduz estresse oxidativo, previne hipertrofia e preserva a função sistólica do ventrículo direito em modelo de *Cor pulmonale*

Neste capítulo reportamos os efeitos cardioprotetores do pterostilbeno sobre o ventrículo direito (VD) de ratos com hipertensão pulmonar. Neste trabalho sugerimos que o mecanismo terapêutico deste fitofenol pode estar relacionado a redução na produção de ânion superóxido dependente de NADPH oxidase, redução do estresse oxidativo, assim como prevenção da remodelação mal adaptativa resultando em proteção da função sistólica do VD.

Artigo Original intitulado:

Pterostilbene reduces oxidative stress, prevents hypertrophy and preserves systolic function of right ventricle in *cor pulmonale* model

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RESEARCH PAPER

Pterostilbene reduces oxidative stress, prevents hypertrophy and preserves systolic function of right ventricle in *cor pulmonale* model

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BACKGROUND AND PURPOSE

In *cor pulmonale*, the increased afterload imposed on the right ventricle (RV) generates a maladaptive response, impairing the contractile cardiac function. Oxidative mechanisms play an important role in the pathophysiology and progression of this disease. The administration of pterostilbene (PTS), a phytophenol with antioxidant potential, may represent a therapeutic option. In the present study, we evaluated the effect of PTS complexed with hydroxypropyl-β-cyclodextrin (HPβCD) on hypertrophy, contractile function and oxidative parameters in the RV of rats with pulmonary hypertension, induced by the administration of monocrotaline (MCT).

EXPERIMENTAL APPROACH

The rats received daily doses of the PTS : HPβCD complex at 25, 50 or 100 mg·kg^{-1}, p.o., for 14 days. The diastolic function, E/A ratio, and systolic function, shortening fraction, fractional area change (FAC) and tricuspid annular plane systolic excursion (TAPSE) of the RV were determined by echocardiography.

KEY RESULTS

The PTS : HPβCD complex reduced the production of NADPH oxidase-dependent superoxide anions and oxidative stress in the RV of MCT-treated rats in a dose-dependent manner. At higher doses it prevented the reduction in FAC and TAPSE in MCT-treated animals.

CONCLUSIONS AND IMPLICATIONS

The PTS : HPβCD complex prevented the maladaptative remodelling and protected systolic function in the RV of rats with pulmonary hypertension. These cardioprotective mechanisms may be related, in part, to the antioxidant potential of PTS, favoured by the increased p.o. bioavailability promoted by the presence of HPβCD in the complex.

Abbreviations

CAT, catalase; DCFH-DA, 2′,7′-dichloro-dihydro-fluorescein diacetate; FAC, fractional area change; H_2O_2, hydrogen peroxide; HPβCD, hydroxypropyl-β-cyclodextrin; MCT, monocrotaline; oxLDL, oxidized LDL; PTS, pterostilbene; RV, right ventricle; TAPSE, tricuspid annular plane systolic excursion

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Introduction

Pulmonary arterial hypertension is a progressive disease characterized by pulmonary vascular remodelling, which gives rise to increased pulmonary vascular resistance and pressure, with a prevalence of 10–52 cases per million people Peacock *et al*. (2007). This condition leads to an increase in afterload imposed on the right ventricle (RV) and progressive deterioration of right heart function, leading to *cor pulmonale* (Weitzenblum and Chaouat, 2009). Initially, the RV adapts to the increased afterload, increasing its wall thickness and contractility (compensatory hypertrophy). However, these compensatory mechanisms are insufficient, eventually generating a maladaptive response characterized by contractile deficiency, hypertrophy and/or dilation and, later, right heart failure (Greyson, 2008; Vonk-Noordegraaf *et al*., 2013).

Among the pathological mechanisms involved in maladaptive RV remodelling are increased protein synthesis, activation of neurohormonal signalling (adrenergic and angiotensin pathways) and activation of apoptotic, inflammatory and oxidative pathways (Bogaard *et al*., 2009; Wrigley *et al*., 2011; Vonk-Noordegraaf *et al*., 2013).

Evidence suggests that ROS and nitrogen species (RNS) and oxidative stress contribute to the transition from hypertrophy to RV dilation and right heart failure (Bogaard *et al*., 2009). In cardiomyocytes, the reactive species can be produced from mitochondrial oxidative phosphorylation, as well as by the enzymes NADPH oxidases, xanthine oxidase and decoupled NO synthase (Tsutsui *et al*., 2011). Thus, an increase in oxidants associated with an insufficient response of the primary antioxidant system facilitates the oxidation of lipids and cellular proteins, resulting in cardiomyocyte dysfunction and death (Bello-Klein *et al*., 2014). In addition to direct oxidative effects, the ROS generated modify central proteins in the excitation–contraction coupling, impairing the contractile function, as well as activating protein kinases and transcription factors that increase signalling for hypertrophy (Tsutsui *et al*., 2011).

Cor pulmonale is a very debilitating disease with a poor prognosis and very limited therapeutic options; there are no specific guidelines for its prevention and/or treatment (Greyson, 2008). Few studies have been dedicated to exploring new therapies for the prevention of RV insufficiency due to an overload of pressure and volume (Greyson, 2008), which makes the search for therapeutic alternatives that maintain the function of the right heart quite relevant.

Pterostilbene (PTS), 4-(3,5 dimethoxystyryl)phenol – a dimethylated analogue of **resveratrol** naturally found in grapes, blackberries, blueberries, among others – has been found to have many beneficial biological effects: antitumour, as it stimulates apoptosis signalling; anti-inflammatory, given its inhibitory effect on **COX2** and **iNOS** activities; antidiabetic, due to its negative effect on gluconeogenesis; insulinotropic and antioxidant (Acharya and Ghaskadbi, 2013; McCormack and McFadden, 2013; Lv *et al*., 2014). This latter effect of PTS may involve the scavenger mechanism and the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2), a key regulator of the antioxidant response. This mechanism is related to inhibition of the interaction of the **Kelch-like ECH-associated protein-1 (Keap1)** with **Nrf2** (Keap1–Nrf2), with consequent activation of Nrf2, resulting in its translocation to the nucleus, which culminates in transcriptional activation of phase II antioxidants genes and reducers such as **glutathione** (GSH) (Bhakkiyalakshmi *et al*., 2016).

Studies have shown the superior beneficial properties of PTS compared with resveratrol, effects that are attributed to increased permeation of cell membranes, due to its superior lipophilicity, as well as its extensive distribution level in cardiac and pulmonary tissues due the favourable pharmacokinetics (Acharya and Ghaskadbi, 2013; Choo *et al*., 2014). However, this apolar characteristic reduces the water solubility of PTS in the gastrointestinal environment, impairing its dissolution, and consequently, its absorption and biostability, which limits its use as an oral treatment (Helen Chan and Stewart, 1996). In view of this, to improve the p.o. uptake of PTS and potentially enhance its biological effects, the water solubility of PTS can be increased by forming inclusion complexes with hydroxypropyl-β-cyclodextrin (HPβCD), a solubility promoting agent that accommodates lipophilic substances in its internal cavity while the hydrophilic outer surface promotes aqueous solubility (López-Nicolás *et al*., 2009). Previous studies from our research group have demonstrated the *in vitro* and *in vivo* antioxidant capacity of different doses of PTS administered p.o. when complexed with HPβCD (PTS : HPβCD complex), as well as its lack of toxicity to the hepatic and renal function of rats.

Little is known about the effects of PTS on the heart, especially in pathological conditions such as RV failure. Therefore, the aim of the present study was to evaluate the effects of the PTS : HPβCD complex at three different daily doses (25, 50 and 100 mg·kg^{-1}) on RV hypertrophy, as well as haemodynamic, functional and oxidative parameters in rats with pulmonary hypertension induced by monocrotaline (MCT) administration.

Methods

PTS : HPβCD complex

To increase the hydrosolubility of the PTS, it was complexed to HPβCD. The complex was prepared using 0.3 M HPβCD dissolved in water with an excess of PTS (Yeo *et al*., 2013). The resultant suspension (PTS : HPβCD complex) was stirred for 72 h, at a temperature of 37 ± 0.1°C using a magnetic stirrer (MULTIST Velp®, Usmate Velate, Italy) and a temperature-controlled bath (IKA). The formulation was filtered, frozen at −18°C and freeze-dried (Edwards Modulyo EF4) for 48 h. The content of PTS in this lyophilized sample was quantified by HPLC (Shimadzu LC-20A system; Kyoto, Japan).

Animals

Male Wistar adult rats (250–300 g) from the Laboratory Animal Reproduction Centre of the Universidade Federal do Rio Grande do Sul were housed in polypropylene cages (40 × 33 × 17 cm), four per cage, under standard environmental conditions (room temperature, 22 ± 2°C; 12 h light–dark cycle, 07:00–19:00 h.). All rats had free access to food and water. Our experimental protocol was carried out in accordance with the International Guidelines for Use and Care of Laboratory Animals of the National Institutes of Health and with

Brazilian Laws for the Scientific Use of Animals. The protocol began after it had been approved by the Ethical Committee for Animal Experimentation at UFRGS (CEUA-UFRGS # 28218). All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable data. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Experimental groups and procedures

Initially, the rats were randomized and divided into equal-sized groups, called control (CTR) and pulmonary hypertension (MCT) groups. Pulmonary hypertension was induced by administration of monocrotaline (MCT; crotaline – C240 Sigma) 60 mg·kg^{-1} i.p. (Singal *et al.*, 2000). After 7 days of induction, both CTR and MCT animals received daily the PTS : HPβCD complex in doses corresponding to 25, 50 or 100 mg·kg^{-1} (CTR25, CTR50 and CTR100; MCT25, MCT50 and MCT100; n = 8 per group) or vehicle (aqueous solution with HPβCD) (CTR0 and MCT0; n = 8 per group) *via* gavage for a period of 14 days. At the end of the experimental period, the animals were anaesthetized (ketamine 90 mg·kg^{-1} and xylazine 20 mg·kg^{-1}, i.p.) and submitted to echocardiographic analyses, followed by catheterization of the right jugular vein for evaluation of haemodynamic parameters. The animals were killed by decapitation while still anaesthetized. The anaesthetic methods efficiency was evaluated by verification of decrease of motor reflex, reduction of muscle contraction, and absence of inferior limb movement, according to Laboratory Animal Anaesthesia (Third Edition) (Flecknell, 2009). The liver, heart and lungs were removed and weighed. The ventricles were separated and weighed, and the RV was used for biochemical analysis.

Echocardiographic evaluation

The images were obtained using two-dimensional mode and M-mode (Philips HD7 Ultrasound System; Andover, MA, USA) using a S12-4 transducer (Philips). To determine the right systolic and diastolic function, the following parameters were evaluated: tricuspid flow E/A ratio, RV shortening fraction (FEC), fractional area change (FAC = 100 × end-diastolic area – end-systolic area/end-diastolic area) and tricuspid annular plane systolic excursion (TAPSE) (Rudski *et al.*, 2010).

Evaluation of haemodynamic parameters

Haemodynamic parameters were determined to estimate the effects of the PTS : HPβCD complex on cardiac function. Systolic (right ventricular systolic pressure, mmHg) and final diastolic BP (right ventricular end diastolic pressure, mmHg) was measured by cannulation of the RV, using a catheter connected to a transducer (Strain-Gauge, Narco Biosystem Miniature Pulse Transducer PR-155; Houston, TX, USA) and pressure amplifier (Pressure Amplifier HP 8850C). The positive values of the contraction derivative (dP/dt_{min} and dP/dt_{max}, mmHg·s^{-1}) and negative values of the relaxation derivative (dP/dt_{min}, mmHg·s^{-1}) were obtained from the records of the right ventricular pressure wave by determining the maximum and minimum points of each cardiac cycle (Schenkel *et al.*, 2010).

Evaluation of morphometric parameters

Animals were weighed twice a week for dose correction and to evaluate the effect of the treatments on the body weight. The cardiac hypertrophy index infers the increase in heart muscle mass, which is an important characteristic in heart failure, and was calculated as the ratio of the right and left ventricle (LV) weight (mg), corrected by the tibia length (mm) and expressed as a percentage (Hu *et al.*, 2003).

Pulmonary and hepatic congestion

Pulmonary and hepatic congestion are secondary to the development of right heart failure. Lung and liver were conditioned at 65°C and weighed daily until they had a constant mass value. Pulmonary and hepatic congestion was estimated using the wet weight/dry weight ratio (g) (Farahmand *et al.*, 2004).

Tissue preparation

Samples from RV were homogenized (OMNI Tissue Homogenizer, OMNI International, Georgia, USA) for 30 s in 1.15% KCl buffer containing 1% PMSF. The homogenates were centrifuged (1358 × *g*, 20 min, at 4°C; ALC Multispeed Refrigerated Centrifuge PK 121R, Thermo Electron Corporation, Massachusetts , USA), and the supernatant was collected for subsequent determination of oxidative parameters. The protein concentrations in the samples were determined by the Lowry method using BSA as a standard (Lowry *et al.*, 1951).

Total reactive species

Total reactive species levels were measured using 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescence emission (Sigma-Aldrich, Saint Louis, USA). DCFH-DA is membrane permeable and is rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS. The samples were excited at 488 nm, and emission was collected with a 525 nm long pass filter. The results are expressed as pmol of DCF mg^{-1} protein (LeBel *et al.*, 1992).

Activity of NADPH oxidase

NADPH oxidase generates superoxide anions through the transfer of electrons from NADPH to molecular oxygen. The activity of the enzyme was determined in RV homogenate by measuring the consumption of NADPH at 340 nm, its activity being directly proportional to the production of the superoxide anion. The results are expressed as nmol·min^{-1}·mg^{-1} protein (Wei *et al.*, 2006).

Non-enzymatic antioxidant defence assay

The sulphydryl content represents a non-enzymatic antioxidant defence. For the sulphydryl assay, we added 0.1 mM of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) to 120 μL of RV samples, which were incubated for 30 min at ambient temperature in a dark environment as described by Aksenov and Markesbery (2001). Absorbance was measured at 412 nm (Anthos Zenyth 200rt, Biochrom, Cambridge, UK), and the results are expressed as nmol·mg^{-1} protein.

Determination of antioxidant enzyme activity

SOD activity was evaluated on the basis of the inhibition of a superoxide radical reaction with pyrogallol, measured at 420 nm. It is expressed as U·mg^{-1} of protein (Marklund, 1985). Catalase (CAT) activity was measured by following the decrease in **hydrogen peroxide (H_2O_2)** absorbance at 240 nm. It was expressed as pmol of H_2O_2 reduced

min^{-1}·mg^{-1} protein (Aebi, 1984). The balance between SOD and CAT activity was estimated by the SOD/CAT ratio.

Glutathione peroxidase (GPx) activity

GPx is an enzyme that catalyses the reaction of hydroperoxides with reduced glutathione (GSH). In this test, the GPx activity was determined by measuring the consumption of NADPH in the oxidation reaction of GSH. The decrease of absorbance of NADPH at 340 nm was observed. The results were expressed as nmol·min^{-1}·mg^{-1} protein (Flohé and Günzler, 1984).

Western blot evaluation

Tissue homogenization, electrophoresis and protein transfer were performed as previously described (Laemmli, 1970). Fifty micrograms of protein from RV homogenates were submitted to one-dimensional SDS-PAGE in a discontinuous system using an 8–12% (wv^{-1}) separating gel and a stacking gel (Laemmli, 1970). The immunodetection was processed using the following primary antibodies: SOD (23 kDa), CAT (64 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, or Cell Signalling Technology, Beverly, MA, USA). The bound primary antibodies were detected using anti-rabbit or anti-mouse HRP-conjugated secondary antibodies, and the membranes were developed using chemiluminescence detection reagents. The autoradiographs generated were quantitatively measured with an image densitometer (ImageMaster VDS CI, Amersham Biosciences Europe, IT). The MWs of the bands were determined by reference to a standard MW marker (RPN 800 rainbow full range Bio-Rad, California, USA). The results were normalized using the Ponceau method (Klein *et al.*, 1995).

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). The normal distribution of results was evaluated using the Shapiro–Wilk test. Parametric results were analysed using two-way ANOVA and treatment with different doses of PTS : HPβCD complex (25, 50 or 100 mg·kg^{-1}) as independent variables. Tukey's test was performed to detect differences between groups. Differences were considered significant when $P < 0.05$. The results are expressed as the mean ± SD. The data were analysed using the Sigma Stat Programme (Jandel Scientific Co., v. 11.0, San Jose, CA, USA).

Materials

PTS was purchased from Changsha Organic Herb (Changsha, China). HPβCD was supplied by Roquette Frères (Lestrem, France). MCT (Crotaline – C240 from Sigma-Aldrich, Saint Louis, USA). All reagents used were of analytical or HPLC grade.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

Results

Echocardiographic evaluation

When evaluating the functional echocardiographic measurements, a reduction in tricuspid flow (E/A ratio) and FEC in MCT animals was observed, and these parameters were not altered by the administration of the PTS : HPβCD complex at any of the doses tested (Figure 1A, B). However, we found a reduction in FAC (Figure 1C) and TAPSE (Figure 1D) in MCT0 animals in relation to CTR0. Administration of the PTS : HPβCD complex increased the FAC after the administration of the 100 mg·kg^{-1} dose (Figures 1C and 2A), whereas the TAPSE was increased by both the 100 and 50 mg·kg^{-1} doses (Figures 1D and 2B).

Evaluation of haemodynamic parameters

Pressure recordings in the RV during systole (SPRV) and diastole (DPRV) showed that MCT animals presented an elevation in SPRV and DPRV in relation to CTR (Table 1). Interestingly, the PTS : HPβCD complex at the dose of 100 mg·kg^{-1} increased the DPRV in the MCT100 group compared with the MCT0 group. Also, the dP/dt_{max} (contractility) and dP/dt_{min} (relaxation) derivatives were higher in the MCT groups compared with the CTR groups; however, these parameters were not altered by the PTS : HPβCD complex at any of the doses tested (Table 1).

Weight measurements and morphometry

The results show that there was no difference in the initial body weight of the animals. However, there was a reduction in weight gain in the MCT group in relation to the CTR group ($P < 0.05$), and this parameter was not affected by the PTS : HPβCD complex at any of the doses tested (Table 2). However, we observed that MCT animals had an increase in RV weight, RV/tibia index and RV/LV weight in relation to CTR, parameters that were reduced by the PTS : HPβCD complex at a dose of 100 mg·kg^{-1} (Table 2).

Pulmonary and hepatic congestion

According to the results, there was an increase in the pulmonary congestion index in MCT animals, as evidenced by increased wet/dry weight (g) and wet lung/body weight (mg·g^{-1}), parameters that were not altered by administration of the PTS : HPβCD complex (Table 3). However, in relation to pulmonary congestion, no significant differences were found between groups (Table 3).

Oxidative parameters

The results show that there was no reduction in the concentration of total ROS in the RV of rats in this experimental protocol (Figure 3A). We observed an increase in NADPH oxidase activity in the MCT0 group in relation to CTR0 (Figure 3B). Administration of the PTS : HPβCD complex at the dose of 100 mg·kg^{-1} reduced NADPH oxidase activity in the animals with heart failure induced by pulmonary hypertension; however, this same dose increased NADPH oxidase activity in the CTR animals (Figure 3B). There was also a negative correlation of NADPH oxidase activity with TAPSE (correlation coefficient: −0.967; $P = 0.0331$) and FAC (correlation coefficient: −0.592; $P = 0.00229$) of the RV, indicating an association

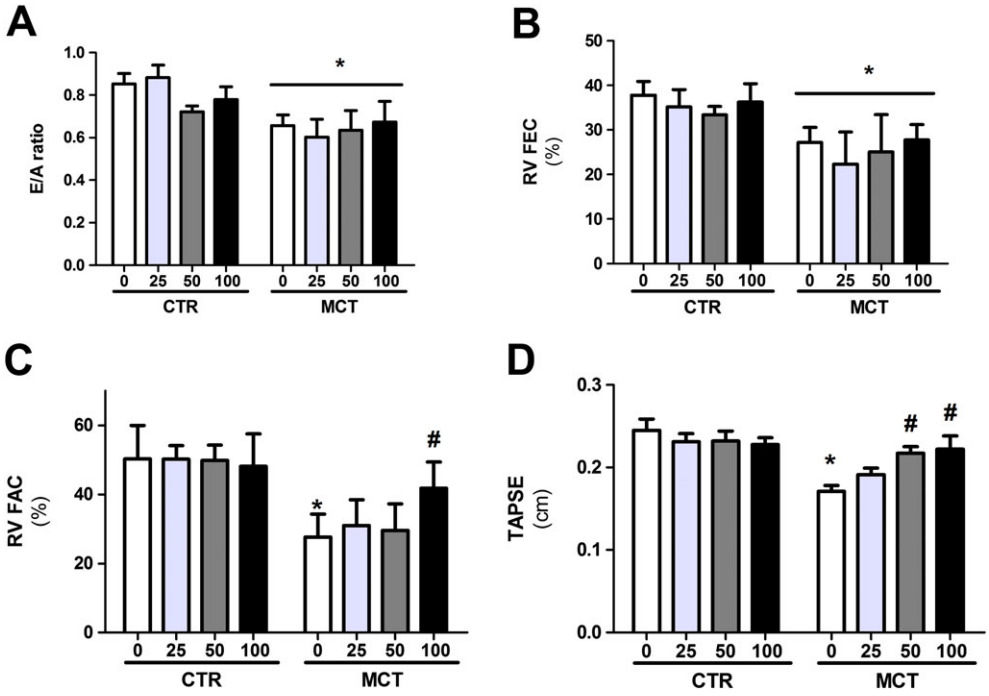

Figure 1

Effect of p.o. administration of PTS : HPβCD complex at different doses (25, 50 and 100 mg·kg^{-1}) on functional echocardiographic measurements. Tricuspid flow (E/A ratio) (A), FEC (B), FAC (C) and TAPSE (D) of control (CTR0, CTR25, CTR50 and CTR100) and MCT (MCT0, MCT25, MCT50 and MCT100) rats treated *via* gavage for 14 days. Values are expressed as mean ± SD, $n = 8$ per group; two-way ANOVA with Tukey's *post hoc* test was performed. *Significantly different compared with CTR0; #significantly different compared with MCT0.

between these parameters. In contrast, the levels of non-enzymatic antioxidants were not altered in our experimental protocol (Figure 3C).

When evaluating the activity of antioxidant enzymes, we detected that heart failure induced by pulmonary hypertension reduced SOD activity (Figure 4A) and increased CAT activity (Figure 4C). The PTS : HPβCD complex restored the SOD activity at the dose of 100 mg·kg^{-1} (Figure 4A), while the CAT activity was reduced at the three doses tested (Figure 4C). We observed a reduction in the SOD/CAT ratio in the RV of the MCT group in relation to CTR (Figure 4E). Administration of the PTS : HPβCD complex did not change this parameter.

In relation to GPx activity, we observed a decrease in its activity in the MCT0 group in relation to the CTR0 group (Figure 4F). Administration of the PTS : HPβCD complex at the dose of 100 mg·kg^{-1} restored the activity in the animals with heart failure induced by pulmonary hypertension (Figure 4F).

Western blot evaluation

The expression of antioxidant enzymes was also measured in the heart tissue. We observed an increase in the immunocontent of the SOD enzyme in the RV of the MCT25 animals compared with the other MCT groups (Figure 4B), as well as a reduction in the immunocontent of the SOD enzyme in the MCT50 and MCT100 groups in relation to MCT0 (Figure 4B). CAT expression was decreased in the MCT groups in relation to CTR (Figure 4D). Administration of the PTS : HPβCD complex at doses of 50 and 100 mg·kg^{-1} reduced CAT expression compared with the MCT0 and MCT25 groups, and this effect was even more pronounced at the dose of 100 mg·kg^{-1} (Figure 3D). Additionally, the PTS : HPβCD complex increased CAT expression in the control group (CTR25 and CTR50) (Figure 4D).

Pterostilbene modulates NADPH oxidase activity in right ventricle

NADPH oxidases are transmembrane proteins, composed of two catalytic subunits (gp91phox and p22phox) and four regulatory subunits (p47phox, p40phox, p67phox and Rac1). NADPH oxidases transfer electrons, reducing oxygen to superoxide anion (Bedard and Krause, 2007). In the heart, the activation of NADPH oxidase is due to hypertrophic stimuli, through angiotensin II, adrenaline and mechanical overload, and its overactivation is related to cardiac injury and failure (Bedard and Krause, 2007; Borchi *et al*., 2010). Our findings suggest that administration of PTS is able to reduce NADPH oxidase activity, diminishing anion superoxide production (Figure 2B), associated to the inductor effect of antioxidant response in RV, which can be observed by its effect on GPx and SOD activities (Figure 4A, F). This results in the prevention of maladaptative remodelling and protection of systolic function.

Discussion

In *cor pulmonale*, the increase in ROS associated with a defective antioxidant system is considered a determinant for the

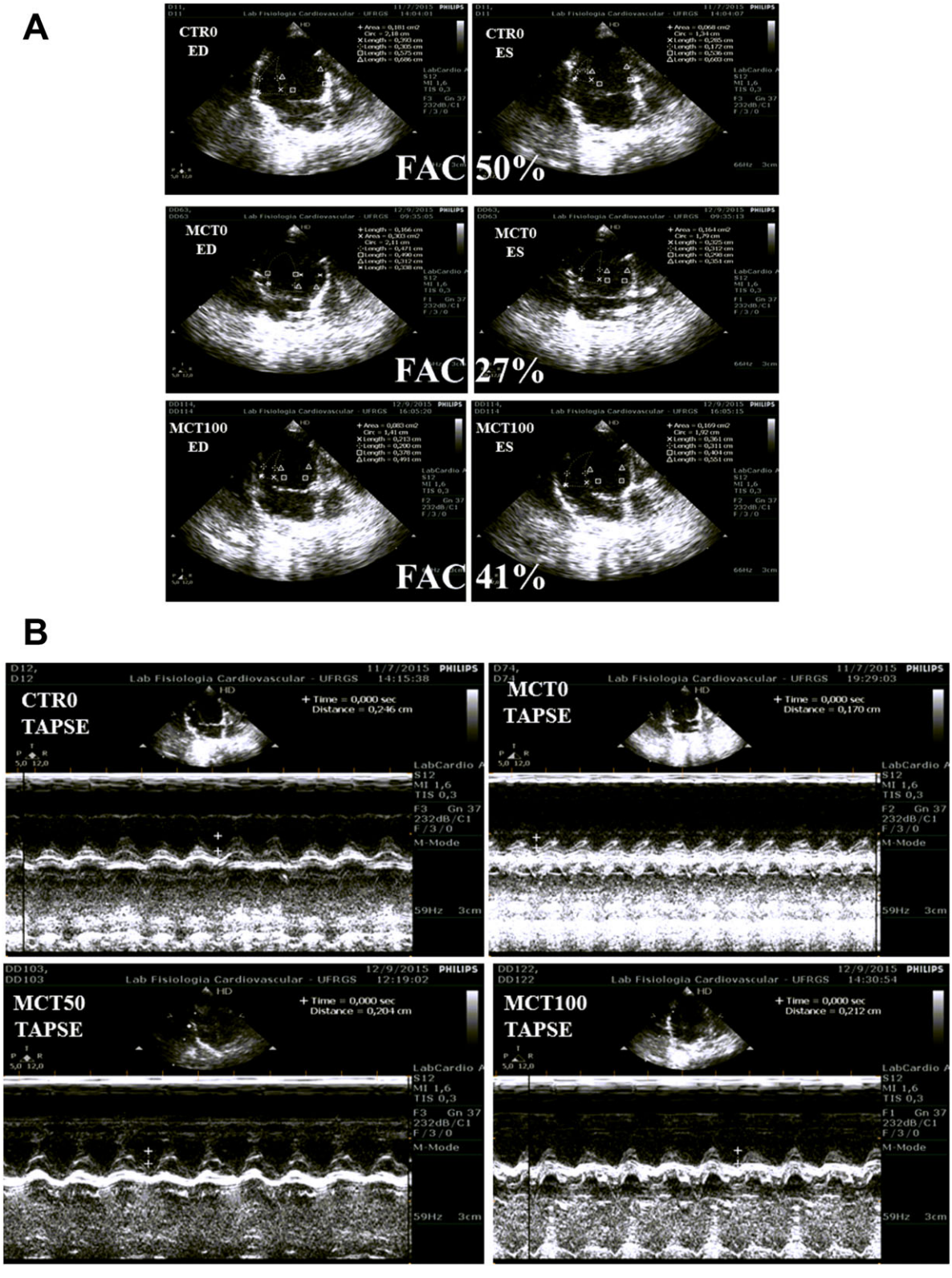

Figure 2

Representative image of echocardiographic data in the RV of control and MCT-treated rats. (A) FAC is used to demonstrate changes in the FAC of the control, MCT0 and MCT100 groups. (B) The TAPSE is marked for the groups CTR0, MCT0, MCT50 and MCT100. The endocardial border is traced in apical four-chamber views from the tricuspid annulus along the free wall to the apex, then back to the annulus, along the interventricular septum at end diastole (ED) and end systole (ES). RV FAC 50%: normal subject; (middle) moderately dilated RV, RV FAC 40%: (middle) moderately dilated; and RV FAC 21%: markedly dilated.

Table 1

Haemodynamic parameters of control (CTR) rats and rats with heart failure (MCT) after 14 days of p.o. administration with the PTS : HPβCD complex at different doses (25, 50 and 100 mg·kg^{-1})

Groups	RVSP (mmHg)	RVEDP (mmHg)	dP/dt_{max} (mmHg)	dP/dt_{min} (mmHg)	HR (beat·min^{-1})
CTR0	30.36 ± 5.25	2.12 ± 1.13	1607.42 ± 318.13	−893.25 ± 194.34	230.89 ± 17.13
CTR25	30.79 ± 4.30	1.81 ± 1.67	1969.42 ± 503.01	−1101.29 ± 307.29	235.00 ± 27.09
CTR50	34.33 ± 5.85	3.15 ± 1.38	1552.84 ± 355.68	−997.97 ± 217.28	236.89 ± 19.15
CTR100	32.91 ± 4.44	3.25 ± 1.37	1347.95 ± 268.73	−893.56 ± 164.25	287.57 ± 14.48
MCT0	71.06 ± 5.8[a]	5.31 ± 1.16[a]	3022.37 ± 355.68[a]	−1771.91 ± 217.28[a]	256.25 ± 19.15
MCT25	62.20 ± 5.65[a]	4.10 ± 1.18[a]	2382.91 ± 355.52[a]	−1403.51 ± 217.28[a]	252.33 ± 15.64
MCT50	63.04 ± 3.9[a]	3.45 ± 1.03[a]	2749.65 ± 290.41[a]	−1475.51 ± 117.41[a]	237.33 ± 18.55
MCT100	67.56 ± 6.49[a]	8.77 ± 1.26[b,c]	3557.38 ± 355.68[a]	−2361.22 ± 217.28[a]	258.25 ± 19.15

Values are expressed as mean ± SD, $n = 8$ animals per group. Two-way ANOVA and Tukey's *post hoc* tests were performed. dP/dt_{max}, contractility index; dP/dt_{min}, relaxation index; HR, heart rate; RVSP, right ventricular systolic pressure; RVEDP, right ventricular end diastolic pressure.
[a]Significantly different compared with CTR ($P < 0.05$).
[b]Significantly different compared with MCT0 ($P < 0.05$).
[c]Significantly different compared with MCT25 and MCT50 ($P < 0.05$).

Table 2

Body weight and morphometric measurements of control rats (CTR) and rats with heart failure (MCT) after 14 days of p.o. administration with the PTS : HPβCD complex at 25, 50 or 100 mg·kg^{-1} dose

Groups	Initial BW (g)	BW variation (%)	RV gross weight (mg)	RV weight/tíbia (mg·mm^{-1})	RV weight/LV weight (mg·mg^{-1})
CTR0	238 ± 9.2	53.3 ± 3.0	200 ± 13.3	5.9 ± 0.4	0.31 ± 0.02
CTR25	243 ± 9.2	49.6 ± 3.0	197 ± 13.0	5.4 ± 0.3	0.28 ± 0.02
CTR50	231 ± 10.6	55.9 ± 3.5	188 ± 15.2	5.0 ± 0.5	0.27 ± 0.02
CTR100	250 ± 9.21	54.8 ± 3.0	192 ± 13.2	5.3 ± 0.4	0.29 ± 0.02
MCT0	238 ± 9.5	35.6 ± 2.3[a]	291 ± 9.9[a]	8.0 ± 0.3[a]	0.48 ± 0.01[a]
MCT25	248 ± 6.9	37.0 ± 2.1[a]	259 ± 10.0[a]	7.2 ± 0.4[a]	0.42 ± 0.02[a]
MCT50	236 ± 6.5	33.0 ± 2.3[a]	262 ± 9.9[a]	7.3 ± 0.4[a]	0.43 ± 0.01[a]
MCT100	254 ± 6.9	30.1 ± 3.0[a]	222 ± 10.0[a,b]	6.2 ± 0.30[a,b]	0.34 ± 0.01[b,c]

Values are expressed as mean ± SD, $n = 8$ animals per group. Two-way ANOVA and Tukey's *post hoc* tests were performed. BW, body weight.
[a]Significantly different compared with CTR ($P < 0.05$).
[b]Significantly different compared with MCT0 ($P < 0.05$).
[c]Significantly different compared with MCT25 and MCT50 ($P < 0.05$).

progression and severity of the disease (Bogaard *et al.*, 2009). Thus, the use of natural antioxidant products, such as the phytophenol PTS, may represent a therapeutic strategy against oxidative/nitrosative imbalance in cardiovascular diseases (Kosuru *et al.*, 2016). Studies that focus on exploring the effects of PTS on the heart are scarce. This is the first study to evaluate the effects of PTS complexed to HPβCD on cardiac function. In the present study, p.o. administration of a PTS : HPβCD complex reduced oxidative stress, prevented RV maladaptive remodelling and improved right systolic function in rats with *cor pulmonale*.

Complexation with cyclodextrin promotes the hydrosolubility, protects bioactive molecules from degradation (against changes in temperature, pH and exposure to light) and greatly increases the p.o. bioavailability of stilbenes, as observed for PTS (>59%) and resveratrol (>45%), increasing its potential pharmacological effects (Lin and Ho, 2011; Yeo *et al.*, 2013).

In this study, the morphometric and haemodynamic changes in the RV caused by pulmonary hypertension agree with the findings of other authors (Mosele *et al.*, 2012; Colombo *et al.*, 2015). Increased pressure in systole and diastole as well as increased RV contractility and relaxation may be considered as adaptive mechanisms for pulmonary arterial hypertension and sustained afterload imposed on the RV (Vonk-Noordegraaf *et al.*, 2013). In this context, the change in geometry (more rounded shape) and increase in right ventricular mass (hypertrophy) are due to an increase in protein synthesis induced by stretching and by the presence of additional sarcomeres in cardiomyocytes (Bogaard *et al.*,

Table 3

Lungs and liver congestion indexes of control rats (CTR) and rats with heart failure (MCT) after 14 days of p.o. administration with PTS : HPβCD complex at 25, 50 or 100 mg·kg^{-1} dose

Groups	Lungs wet weight/ dry weight (g)	Lungs wet weight/ BW (mg·g^{-1})	Liver wet weight/ dry weight (g)	Liver wet weight/ BW (mg·g^{-1})
CTR0	2.4 ± 0.12	2.97 ± 0.35	2.85 ± 0.04	31.64 ± 2.43
CTR25	2.2 ± 0.13	2.69 ± 0.14	2.86 ± 0.04	31.86 ± 2.43
CTR50	2.43 ± 0.14	3.11 ± 0.41	2.84 ± 0.05	32.89 ± 2.80
CTR100	2.31 ± 0.12	2.31 ± 0.35	2.86 ± 0.04	32.87 ± 2.43
MCT0	2.7 ± 0.10[a]	4.31 ± 0.27[a]	2.95 ± 0.03	35.56 ± 1.43
MCT25	2.57 ± 0.10[a]	4.37 ± 0.26[a]	2.86 ± 0.03	34.37 ± 1.83
MCT50	2.36 ± 0.11[a]	3.85 ± 0.27[a]	2.85 ± 0.03	34.10 ± 1.85
MCT100	2.68 ± 0.10[a]	3.62 ± 0.29[a]	2.88 ± 0.04	36.56 ± 1.98

Values are expressed as mean ± SD, n = 8 animals per group. Two-way ANOVA and Tukey's *post hoc* tests were performed. BW, body weight.
[a]Significantly different compared with CTR group.

2009). In our study, the RV hypertrophy index results showed that administration of the PTS : HPβCD complex at a dose of 100 mg·kg^{-1} inhibited this increase, demonstrating the beneficial effect of this treatment on ventricular remodelling. Evidence shows that PTS inhibits the proliferation and growth of muscle cells (Park *et al.*, 2010; McCormack and McFadden, 2013), events that are also related to cardiac hypertrophy (Han *et al.*, 2009). Similarly, resveratrol produces anti-hypertrophic effects in rat cardiomyocytes, both *in vitro* and *in vivo*, by activating AMP-activated protein kinase and inhibiting PKB (Akt), reducing the content and rate of protein synthesis (Chan *et al.*, 2008). The flavonoid quercetin administered p.o. also prevents cardiac hypertrophy induced by pressure overload in rats by inhibiting the MAPK signalling pathway, which plays an important role in cardiac hypertrophy (Han *et al.*, 2009).

The size of the chamber as well as the thickness of the ventricular free wall are determinants of the demand and consumption of oxygen by the myocardium, contributing to the production of mitochondrial ROS (Giordano, 2005). Another important source of ROS in the RV is *via* the activation of the enzyme NADPH oxidase, increasing the concentration of the superoxide anion, which can stimulate the production of other reactive species that are capable of damaging cellular macromolecules and can result in the dysfunction and death of cardiac cells (Bedard and Krause, 2007; Redout *et al.*, 2007). In parallel, the superoxide anion increase participates in the regulation of cell signalling in cardiomyocytes under afterload stress conditions, favouring maladaptive RV remodelling (Seddon *et al.*, 2007). In the present study, rats that received MCT showed increased NADPH oxidase activity, similar to that described by Redout *et al.* (2007). Increased NADPH oxidase activation and expression in cardiomyocytes may occur in response to pressure overload, generating oxidative stress and cardiac failure (Bedard and Krause, 2007). We observed that the PTS: HPβCD complex at a dose of 100 mg·kg^{-1} reduced the production of NADPH oxidase-dependent superoxide anions in the RV by modulating the activity of this enzyme. In contrast, the PTS: HPβCD complex stimulated the activity of NADPH oxidase in the heart of control animals. Chakraborty *et al.* (2010) reported that the production of ROS increased in cells in culture after incubation with PTS. In parallel, this ROS may lead to the rupture of the Keap1-Nrf2 complex, activating Nrf2 and inducing an adaptation of the antioxidant system, preparing the cell to respond more efficiently to challenges, similar to the induction of a hormesis effect. Phytochemicals have been described as modulators of NADPH oxidase (Maraldi, 2013). In this context, extracts enriched with anthocyanins or catechins reduced the expression of NADPH oxidase and prevented cardiac hypertrophy in rats (Al-Awwadi *et al.*, 2005).

In addition, the intracellular increase of superoxide anion may lead to the generation of other reactive species such as H_2O_2, hydroxyl and peroxy nitrite radicals, which may make the redox environment more oxidized. This could impair the functioning of antioxidant enzymes such as SOD and CAT, compromising the primary antioxidant defence system (Halliwell and Gutteridge, 2007). It has been reported that H_2O_2, the product of SOD, oxidizes the histidine residue of cytosolic SOD, inactivating it (Uchida and Kawakishi, 1994). These mechanisms could be determinants of the impairment in RV SOD activity of the MCT rats in this study, compromising the intracellular elimination of superoxide anions. In contrast, the improvement in SOD activity induced by the administration of the PTS : HPβCD complex (100 mg·kg^{-1} dose) may be a result of the scavenging potential of PTS phenolic hydroxyl groups, which stabilize free radicals through their reducing ability (Perron and Brumaghim, 2009). This results in lower superoxide anion concentrations and, consequently, reduces the need for the expression of SOD. Other authors also noted that PTS modulates the activity of antioxidant enzymes (SOD and CAT) in vascular endothelial cells exposed to high levels of oxidized LDLs (oxLDLs), as well as in acetaminophen-injured hepatocytes (Zhang *et al.*, 2012; El-Sayed *et al.*, 2015).

Measurements of CAT activity in the RV presented a different activity pattern compared with SOD. The increase in CAT activity in MCT-treated rats may reflect a

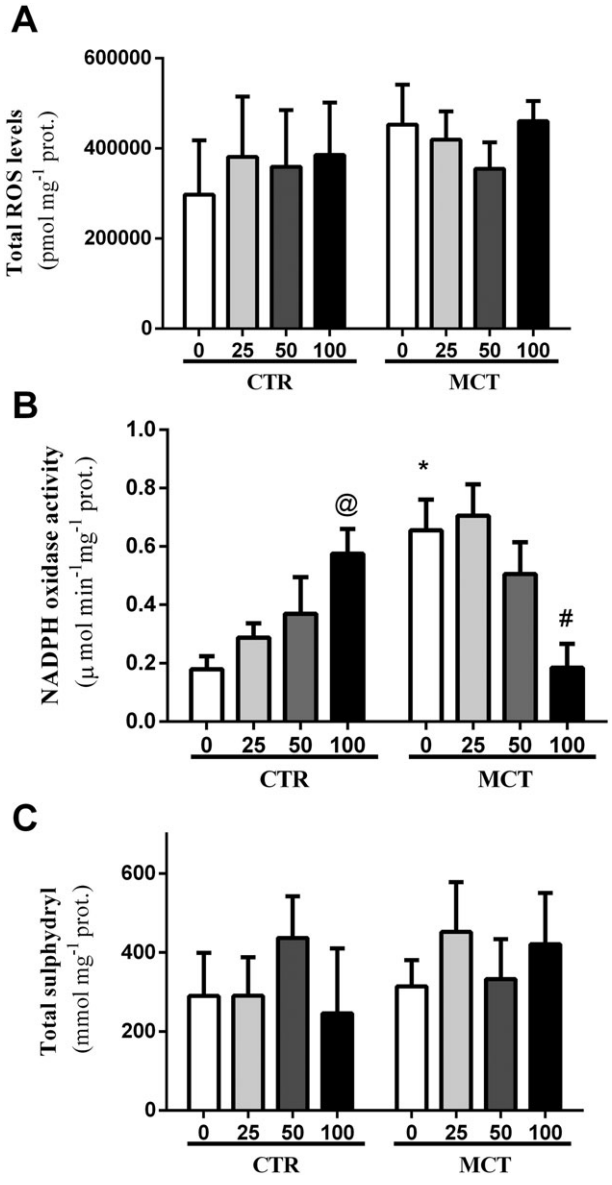

Figure 3

Effect of p.o. administration of PTS : HPβCD complex at different doses (25, 50 and 100 mg·kg^{-1}) on oxidative parameters. Total ROS levels (A), NADPH oxidase activity (B) and total sulphydryl content (C) in the RV of control (CTR0 and CTR25: CTR50 and CTR100) and MCT (MCT0, MCT25, MCT50 and MCT100) rats treated *via* gavage for 14 days. Values are expressed as mean ± SD, $n = 8$ per group; two-way ANOVA with Tukey's *post hoc* test was performed. * Significantly different compared with CTR0; # significantly different compared with MCT0; and @ different compared with CTR.

compensatory mechanism against the high availability of H_2O_2 in cardiomyocytes that protects the RV against oxidative damage (Dieterich *et al.*, 2000). The PTS : HPβCD complex reduced CAT activity at the three doses administered, preserving the antioxidant potential of the enzyme. Still, there was a reduction in the immunocontent of CAT at the higher doses. Similar effects have also been described by other authors. A reduction in the activity and expression of CAT induced by polyphenols in cardiac and muscular cells, respectively, occurs through the induction of non-enzymatic antioxidants and the neutralization of reactive species (Babu *et al.*, 2006; Goutzourelas *et al.*, 2015). In contrast, the PTS : HPβCD complex increased CAT protein expression in the control groups. This could be the result of a PTS-induced pro-oxidant action that provides adaptation of antioxidant system, in order to activate mechanisms of cellular protection (Chakraborty *et al.*, 2010). The SOD/CAT ratio reflects the balance between the activity of the two enzymes, since the SOD product is a CAT substrate (Halliwell and Gutteridge, 2007). In contrast, it was observed that the PTS : HPβCD complex did not change the SOD/CAT ratio reduced by MCT. In addition, the PTS : HPβCD complex (100 mg·kg^{-1} dose) treatment also elevated GPx activity in the RV of rats with pulmonary arterial hypertension. This enzyme plays an important role in the control of redox homeostasis, and an increase in its levels will reduce oxidative stress, compensating for the reduced CAT levels. However, the cardio-protective effects of PTS are not limited to its antioxidant action. Consistently, studies have shown that PTS has anti-inflammatory properties (in addition to its antioxidant effects) in the heart mediated through various mechanisms, such as inhibition of COX2 and inducible NOS activities of NF-κB signalling, decreasing myeloperoxidase activity, inhibition of angiotensin-converting enzyme, elevation of endothelial NOS and a reduction in the levels of TNF-α and IL-1 (Lv *et al.*, 2014; Yu *et al.*, 2017). Therefore, the PTS-induced cardio-protective action may depend on several of these effects.

In *cor pulmonale*, increased oxidative stress is correlated with a decrease in right ventricular function, which is considered to be the main determinant of functional capacity and survival of patients (van de Veerdonk *et al.*, 2011). In this study, RV dysfunction was confirmed using classical echocardiographic parameters of diastolic function (E/A ratio) and systolic function (FEC, FAC and TAPSE) (Rudski *et al.*, 2010; Kimura *et al.*, 2015). FAC expresses the percentage variation in the RV area between diastole and final systole, with values below 35% indicating systolic dysfunction (Rudski *et al.*, 2010). In addition, TAPSE is a parameter of the overall RV function that describes longitudinal apex shortening to the base (Rudski *et al.*, 2010). We observed that pulmonary hypertension (MCT animals) impaired both diastolic and systolic function. Administration of the PTS : HPβCD complex at higher doses (50 and 100 mg·kg^{-1}) prevented the reduction in FAC and TAPSE, indicating protection of systolic function. These results were correlated with the decrease in NADPH oxidase activity observed in our study, since there is a negative correlation of this enzyme activity with the functional parameters of the RV. Resveratrol has also been shown to improve systolic function and reduce hypertrophy in hypertensive rats and these effects were attributed to a reduction in oxidative stress (Thandapilly *et al.*, 2010). In another study, improvement of the cardiac dysfunction induced by pressure overload in rodents was also observed after treatment with flavonoids extracted from propolis and also after treatment with the isoflavonoid pueranine (Yuan *et al.*, 2014; Sun *et al.*, 2016).

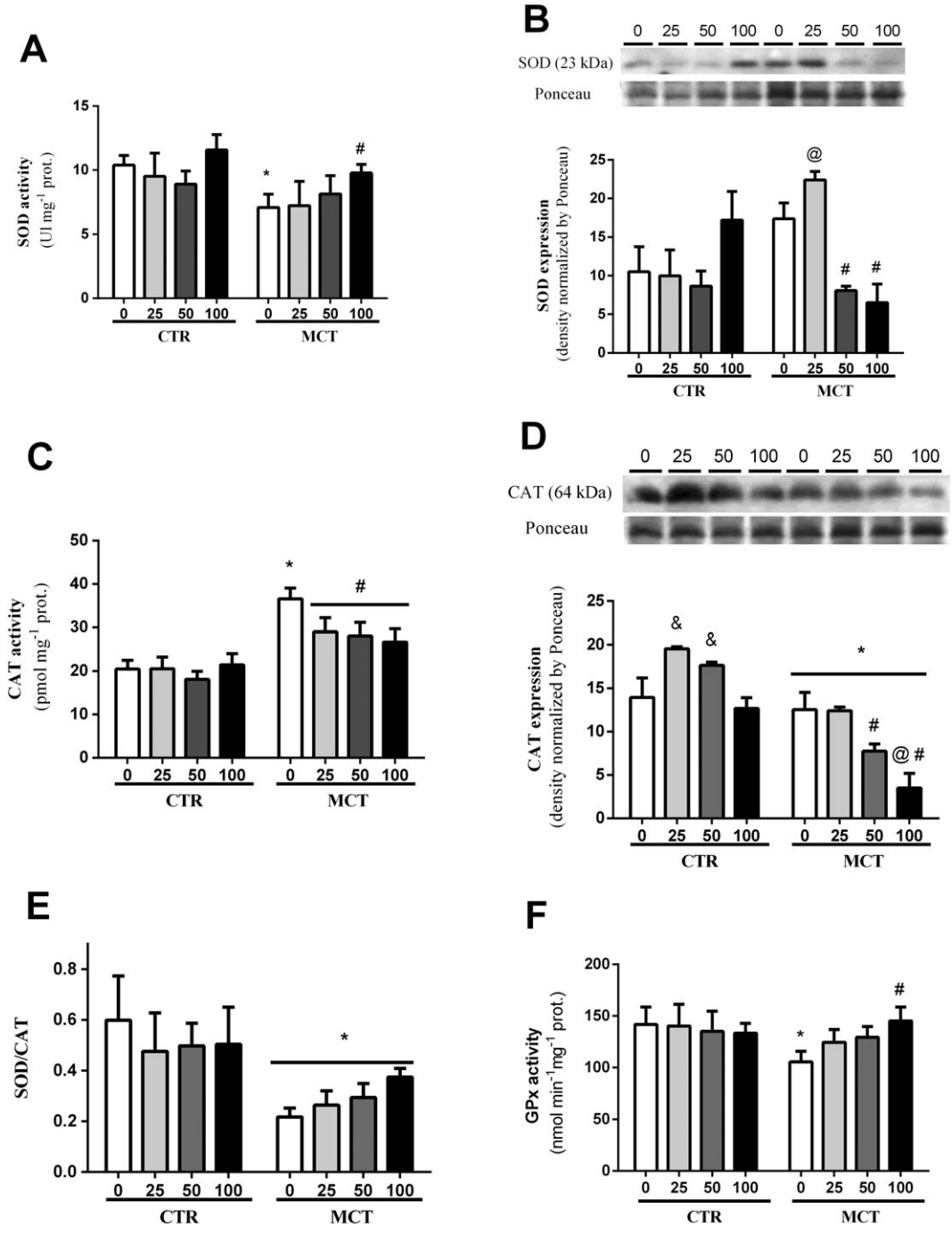

Figure 4

Activity and expression of antioxidant enzymes. Effect of p.o. administration of PTS : HPβCD complex at different doses (25, 50 and 100 mg·kg^{-1}) on SOD activity (A), SOD expression (B), CAT activity (C), CAT expression (D), SOD/CAT ratio (E) and GPx activity (F) in RV of control (CTR0, CTR25, CTR50 and CTR100) and MCT (MCT0, MCT25, MCT50 and MCT100) rats treated *via* gavage for 14 days. Values are expressed as mean ± SD, n = 8 per group; two-way ANOVA with Tukey's *post hoc* test was performed. * Significantly different compared with CTR0; # significantly different to MCT0; @ significantly different to CTR; and & significantly different to CTR0 and CTR100.

In summary, our findings suggest that administration of the PTS : HPβCD complex reduces NADPH oxidase-dependent superoxide anion concentration and oxidative stress in RV in a dose-dependent manner, resulting in the prevention of maladaptative remodelling and protection of systolic function. These cardioprotective mechanisms may be related, in part, to the antioxidant effects of PTS, favoured by the increased p.o. bioavailability promoted by the presence of HPβCD in the complex. These cardioprotective effects of PTS may represent an adjuvant therapy for the pathological changes that occur in *cor pulmonale*.

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Author contributions

D.S.L. participated in all phases (experimental and writing) of this manuscript. P.T. did the biochemical analysis and statistics; B.G.D.L.S. the haemodynamic analysis; R.C. the echocardiography analysis and V.D.O. the Western Blot analysis. J.H.P.B. took care of the animals. C.C.-C. was involved in the biochemical analysis and S.E.B. in the pterostisbene–cyclodextrin complexation. A.B.-K. provided intellectual contributions and V.L.B. and A.S.D.R.A. created the project and wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Capítulo III

4.3 Pterostilbeno, melhora o volume sistólico e o débito cardíaco em ratos: o papel da proteína SERCA e dos sistemas glutationa e glutarredoxina na insuficiência cardíaca direita

No terceiro capítulo da presente tese mostramos que o pterostilbeno produz efeitos cardioprotetores em modelo de insuficiência cardíaca direita secundária a hipertensão arterial pulmonar, através da ativação do sistema da glutationa e enzimas antioxidantes tióis. Estes efeitos denotam a habilidade antioxidante multialvo do PTS associado à modulação da expressão da proteína ATPase SERCA, resultando em proteção da função cardíaca direita, evidenciado pela melhora do volume sistólico e débito cardíaco.

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Pterostilbene improves cardiac function in a rat model of right heart failure through modulation of calcium handling proteins and oxidative stress

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ABSTRACT

This study explored the effect of pterostilbene (PTS) complexed with hydroxypropyl-β-cyclodextrin (HPβCD) on right heart function, glutathione and glutaredoxin systems and the expression of intracellular calcium handling redox-sensitive proteins in the experimental model of pulmonary arterial hypertension (PAH) induced by monocrotaline (MCT). After 7 days of PAH induction, rats received daily doses of the PTS:HPβCD complex (corresponding to 25, 50 or 100 mg kg^{-1} of PTS) or vehicle (control group, CTR0) (an aqueous solution containing HPβCD, CTR0 and MCT0) via oral administration during two weeks. The results showed that the PTS:HPβCD complex increased the content of reduced glutathione and the activity of glutathione-S-transferase and glutaredoxin in the right ventricle (RV) of MCT-treated rats in a dose-dependent manner. Additionally, at higher doses, it also prevented the reduction of stroke volume and cardiac output, prevented MPI increase, reduced lipoperoxidation, reduced total phospholamban, and increased the expression of SERCA in the RV of MCT-treated rats. These results demonstrate that the PTS:HPβCD complex has a dose-dependent antioxidant mechanism that results in improved cardiac function in experimental right heart failure. Our results open a field of possibilities to PTS administration as new therapeutic approach to conventional therapy for right ventricular dysfunction.

Novelty bullets: - Pterostilbene complexed with hydroxypropyl-β-cyclodextrin could be a new therapeutic approach; - Pterostilbene complexed with hydroxypropyl-β-cyclodextrin re-establishes redox homeostasis, through glutathione metabolism modulation, leading to an improved myocardial performance index in pulmonary arterial hypertension-provoked right heart failure.

Key words: Cyclodextrins; stilbene; functional food; glutathione; glutaredoxin; cardiac contractility; monocrotaline; oxidative stress; antioxidant; right heart failure

Introduction

Pterostilbene [4-(3,5 dimethoxystyryl)phenol] is a phytophenol naturally found in fruits such as blueberries (*Vaccinium* spp.) and grapes (Schmidlin et al. 2008; Suh et al. 2007). The pharmacological potential of this compound includes antitumor, antidiabetic, anti-inflammatory, antiapoptotic, and antioxidant activity (Yang et al. 2013; Bhakkiyalakshmi et al. 2016; Liu et al. 2015; Guo et al. 2016; Pari and Satheesh 2006; Wang et al. 2016). These effects have increased the scientific interest in identifying new pharmacological targets of pterostilbene and making it a candidate for use as an adjuvant therapy (Kosuru et al. 2016). Poor water solubility in the intestinal tract may be considered a barrier to oral bioavailability of pterostilbene (Yeo et al. 2013). Complexing with hydroxypropyl-β-cyclodextrin (HPβCD) is considered a strategy to increase the aqueous solubility of pterostilbene and, thus, making its oral administration feasible (Choo et al. 2014; Pinho et al. 2014).

Studies from our research group have been dedicated to explore the effects of pterostilbene complexed with HPβCD (PTS:HPβCD) in a model of right heart failure secondary to pulmonary arterial hypertension (*Cor pulmonale*). These investigations are relevant, as this disease is highly debilitating, has a poor prognosis, and few therapeutic options are available (Greyson 2008). In this cardiopathy, the afterload to the right ventricle (RV) increases, which induces a maladaptive response characterized by RV hypertrophy, right chamber dilatation (a more rounded shape), contractile deficiency, and ventricular failure (Vonk-Noordegraaf et al. 2013). In this context, the proteins responsible for intracellular calcium (Ca^{2+}) handling, such as the ryanodine channel and the sarcoplasmic reticulum calcium ATPase (SERCA), are determinant for cardiac function and are influenced by oxidative stress (Santos at al. 2011). For example, increased oxidation of the SERCA protein reduces Ca^{2+} uptake, impairing

cardiomyocyte relaxation (Santos at al. 2011). Proteins involved in cardiac contractility may also be modulated by the redox state and oxidative stress, contributing to the progression and severity of *Cor pulmonale* (Bogaard et al. 2009).

Oxidative stress leads to the oxidation of protein thiol groups (SH) and impairs cellular redox homeostasis in the heart (Farahmand et al. 2004). In this sense, the integrity of antioxidants, as glutathione, glutaredoxin (GRx) and thioredoxin systems, is fundamental, since such antioxidants may eliminate ROS and reduce oxidized proteins (Hanschmann et al. 2013; Nagarajan et al. 2017). To protect proteins from oxidation, reduced glutathione (GSH) forms disulphides with the thiol group of proteins in a mechanism called S-glutathionylation, which is particularly reversible through redox regulation (Giustarini et al. 2004). S-glutathione protects proteins from proteasomal degradation during oxidative stress (Lushchak et al. 2012).

A previous study showed that pterostilbene had cardioprotective effects in the *Cor pulmonale model*, as evidenced by the reduction of NADPH-dependent superoxide anion production, preserving RV systolic function (dos Santos Lacerda et al. 2017). However, to our knowledge, there are no studies evaluating whether this compound could be beneficial in pulmonary arterial hypertension, through the modulation of glutathione metabolism and its impact on calcium handling proteins, in order to restore cardiac function. Based on this, the present study aimed to verify whether the modulation of antioxidant systems such as glutathione, involving non-classical mechanisms such as glutarredoxin, could affect calcium-handling proteins and could improve cardiac function.

Material and Methods

Animals

Male Wistar adult rats (250-300 g) came from the Laboratory Animal Reproduction Center (CREAL) of the Universidade Federal do Rio Grande do Sul (UFRGS). Our experimental protocol was carried out in accordance with the International Guidelines for Use and Care of Laboratory Animals and with Brazilian laws for the Scientific Use of Animals. The protocol began after it had been approved by the Ethical Committee for Animal Experimentation at UFRGS (CEUA-UFRGS # 28218).

Experimental groups and procedures

Rats were divided into control (CTR0; n=7) and monocrotaline (MCT; n=28) groups. Pulmonary hypertension was induced by administration of monocrotaline (Crotaline - C240 SIGMA) 60mg kg^{-1} intraperitoneally (Singal et al. 2000). After 7 days of induction, the MCT animals received daily of PTS: HPβCD complex at doses corresponding to 25, 50 or 100 mg kg^{-1} (MCT25, MCT50 and MCT100, respectively) or vehicle (aqueous solution with HPβCD; CTR0 and MCT0) via gavage for a period of 14 days. The selected doses (corresponding to 25, 50, and 100 mg kg−1 per day of pterostilbene) during 14 days were based in previous reports, in which PTS showed superior oral bioavailability than equimolar oral dosing of resveratrol (Kapetanovic et al, 2011). Besides that, these three doses showed to be able to reduce lipid peroxidation and reactive oxygen species levels (dos Santos Lacerda et al, 2018). Since the three doses improved cellular redox status in treated animals, we chose them, considering as pharmacological doses, to study their cardioprotective effects in a pulmonary arterial

hypertension model. To increase hydrosolubility of the pterostilbene (PTS), it was complexed to hydroxypropyl-β-cyclodextrin (HP-β-CD). The methodology of complexation and chromatography analyses are described in dos Santos Lacerda et al (2017). At the end of the experimental period, animals were anesthetized (ketamine 90 mg kg^{-1} and xylazine 20 mg kg^{-1}, intraperitoneal), and submitted to echocardiographic analyses. Still under the effect of anesthesia, animals were euthanized by decapitation. Heart was removed, the ventricles were separated, and RV was used for biochemical analysis.

Echocardiographic evaluation

The images were obtained through the two-dimensional (2D) mode and pulsed wave Doppler (Philips HD7 Ultrasound System, Andover, MA, USA) using an S12-4 transducer (Philips, Andover, MA, USA). The following parameters were evaluated to determine the RV function: systolic output (SO, measured as the cross-sectional area of the pulmonary artery (2D) x velocity-time integral of pulmonary flow), heart rate (HR), cardiac output (ESV x HR), and RV myocardial performance index (MPI, measured as the tricuspid valve closure to opening time – pulmonary artery ejection time/pulmonary artery ejection time) (Lawrence et al, 2010). The chamber diameters were also measured at systole (RVSD) and diastole (RVDD) to measure the RV dimensions.

Tissue preparation

Samples from RV were homogenized (OMNI Tissue Homogeneizer, OMNI International, USA) during 30 seconds in 1.15% KCl buffer containing 1% phenyl methyl sulfonyl fluoride (PMSF). The homogenates were centrifuged (1358 x *g*, 20 min, at 4ºC; ALC Multispeed Refrigerated Centrifuge PK 121R, Thermo Electron

Corporation, USA) and the supernatant was collected for subsequent determination of biochemical and molecular parameters. The protein concentrations in samples were determined by the Lowry method using bovine serum albumin as standard (Lowry et al. 1951).

Determination of oxidised and reduced glutathione concentration

In order to evaluate oxidized (GSSG) and reduced total glutathione (total GSH) levels, the RV tissue was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 and min at 1000 x *g*. The supernatant was neutralized with 2 M potassium hydroxide. The determination of total GSH was based on the reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and catalyzed by glutathione reductase; the absorbance values were measured at 420 nm. To measure GSSG, n-ethylmaleimide was added to the neutralized supernatant to perform the reaction described above. From these data measured (total GSH and GSSG), we calculated GSH, according this stoichiometric formula (total GSH = GSH + 2GSSG). The GSH/GSSG ratio was calculated to estimate cellular redox state (Akerboom and Sies 1981).

Glutathione reductase activity

Glutathione reductase (GSR) uses the NADPH to reduce GSSG. In this assay, GSH (1 µM) was added in Tris-HCl buffer (100 mM, pH 7.8) containing EDTA (1 mM) and albumin (1 mg/ml). The amount of GSSG produced was measured from the addition of NADPH (0.2 mM) and GSR (6 µg/ml). Absorbance was read at 340 nm and results were expressed as mmol/mg of protein (Anderson 1985).

Determination of Glutathione-S-transferase activity

Glutathione-S-transferase (GST) catalyses the conjugation of GSH with various substrates and plays an important role in the detoxification of alkylating agents. GST activity was evaluated according to the work of Mannervik and Guthenberg (1981). The formation of dinitrophenyl-S-glutathione was quantified spectrophotometrically at 340 nm (Anthos Zenyth 200 rt, Biochrom, UK), and the results were expressed as μmol/mg of protein.

Determination of Glutaredoxin activity

In order to evaluate glutaredoxin (GRx) activity, the simple was added on a mixed containing glutathione reductase (100 unit), GSH (10 μM), Tris-base buffer (100 mM, pH 7.0), EDTA (200 mmol/L), NADPH (0.2 mM), albumin (1 mg/ml), and bis (2 hydroxyetil disulfid) (15 mM). The activity of GSR is proportional to the consumption of NADPH and can be spectrophotometrically monitored at 340 nm (Holmgren and Aslund 1995).

Evaluation of lipid oxidative damage

Lipid oxidative damage was determined in RV homogenates using the thiobarbituric acid reactive substances (TBARS) method, described by Ohkawa et al. (1979). Briefly, a reactive medium containing the homogenate, sodium dodecyl sulphate (8.1% w/v), acetic acid (20%, pH 3.5), and 375 μl of TBA (0.8% w/v) was incubated at 100°C in a water bath for 60 min. The supernatant was then read at 535 nm (Anthos Zenyth 200 rt). Malondialdehyde was used as the standard (Ohkawa et al. 1979), and the results were expressed as nmol TBARS/mg of protein.

Western blot evaluation

Tissue homogenisation, electrophoresis, and protein transference were performed as previously described (Laemmli 1970). Immunodetection was performed using primary antibodies against p-phospholamban (serine 16/threonine 17, 25 kDa), total phospholamban (25 kDa) and SERCA (97 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA or Cell Signaling Technology, Beverly, MA, USA). The secondary antibodies were antirabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies, and the membranes were developed using chemiluminescence detection reagents. The results were normalized using the Ponceau method (Klein et al. 1995). Ponceau staining of proteins has long been applied to quality control of membrane transfer in Western blotting; it is equally useful for this purpose, has similar or improved dynamic range and was previously validated to assess equal loading gel (Romero-Calvo et al. 2010). Membranes were transferred to an appropriate container and the Ponceau solution (0.1% Ponceau S in 1% acetic acid) was added, being stirred on a rocking platform for 2 minutes. Finally, successive washes with distilled water were made to remove the coloured background. Membranes were scanned and later analyzed by the software Image J (Klein et al. 1995). Ponceau was used given the interference that cardiac remodelling exerts in the structural proteins commonly used as loading controls, such as alpha-actinin, beta-tubulin, and GAPDH. The image of Ponceau staining is right below the image of each protein of interest for all graphs.

Statistical analysis

The normal distribution of data was evaluated using the Shapiro–Wilk test. Parametric data were analysed using a one-way ANOVA with Tukey's post hoc test. Differences were considered significant when $P < 0.05$. Data were expressed as mean ± SD. Data were analysed using the Sigma Stat Program (Jandel Scientific Co., v. 11.0, San Jose, USA).

Results

Echocardiographic evaluation

The echocardiographic evaluation showed that pulmonary arterial hypertension induced an increase in RV systolic and diastolic diameters in the monocrotaline (MCT group) ($P < 0.05$) (Table 1). However, the administration of the PTS:HPβCD complex at a dose of 100 mg kg^{-1} promoted an improvement in RV systolic and diastolic diameters. In relation to MPI, which is a global index of cardiac function, this parameter was increased in MCT group, indicating a worsening in the right heart function ($P < 0.05$). However, a partial improvement in the RV MPI was observed following PTS:HPβCD complex administration (100 mg kg^{-1}) (Table 1). A reduction in systolic output and cardiac output was also observed in MCT animals; however, this change was prevented by the administration of the PTS:HPβCD complex at 100 mg kg^{-1} ($P < 0.05$) (Figure 1). No change in HR was observed among any group (Table 1).

Glutathione redox cycle

Regarding the content of GSH and the redox state in the RV, the administration of the PTS:HPβCD complex at 100 mg kg-1 was shown to increase the concentration of GSH and the GSH-to-GSSG ratio when compared to the other groups (CTR0, MCT0,

MCT25 and MCT50) ($P < 0.001$) (Figure 2A and C); however, there was no change in the GSSG concentration (Figure 2B).

The GSR activity in the RV was reduced in the MCT0 and MCT25 groups compared to the CTR0 group ($P < 0.001$). At doses of 50 and 100 mg kg^{-1}, the PTS:HPβCD complex restored GSR activity in the MCT groups (Figure 2D). Additionally, GST activity was reduced in the MCT0 and MCT25 groups compared to the CTR0 group ($P < 0.05$) but was reversed by the PTS:HPβCD complex at doses of 50 and 100 mg kg^{-1} ($P < 0.05$) (Figure 3A).

GRx activity was reduced in the MCT0 and MCT50 groups compared to the CTR0 group ($P < 0.05$); however, activity was restored by 100 mg kg^{-1} of PTS:HPβCD ($P < 0.05$) (Figure 3B). GST and GRx activities were negatively correlated with TBARS levels (r = -0.697, $P = 0.001$ and r = -0.669, $P = 0.017$, respectively). In addition, GST and GRx activities were positively correlated with cardiac output (r = 0.632, $P = 0.004$ and r = 0.911, $P < 0.001$, respectively).

Lipid peroxidation levels

Lipid peroxidation results from RV homogenates revealed increased TBARS levels in the MCT groups compared to the CTR0 group ($P < 0.001$) (Figure 4A); however, 100 mg kg^{-1} of the PTS:HPβCD complex reduced the TBARS levels ($P < 0.05$) (Figure 4A).

Phospholamban (PLB) and SERCA expression

The quantification of p-phospholamban, total phospholamban and SERCA proteins was performed to estimate the effect of the PTS:HPβCD complex on calcium handling process in the right heart. The expression of p-phospholamban was not altered

(Figure 5A), whereas total phospholamban showed decreased expression in MCT50 and MCT100 groups compared to MCT0 (Figure 5B). The p-phospholamban/total phospholamban ratio was not different between the groups. On the other hand, SERCA expression was increased by the PTS:HPβCD complex at doses of 25 and 100 mg kg-1 in MCT animals ($P < 0.05$) (Figure 5C). The SERCA2a/PLB ratio was not different between the groups.

Discussion

Recent studies have reported that PTS exhibits various pharmacological effects that potentially confer cardioprotection, especially for the left side of the heart (Liu et al. 2017; Yu et al. 2016; Wu et al. 2017). The present study differs from the others as it was dedicated to exploring the pharmacological potential of the PTS:HPβCD complex on pulmonary hypertension-induced RV remodelling through the analysis of the calcium handling proteins expression, glutathione system and the action of antioxidant thiol enzymes. In this context, the antioxidant ability of PTS protects the cardiac function of the right side of the heart, as evidenced by the improvement in systolic and cardiac output. In addition, this study is the first to report that the PTS:HPβCD complex modulates the expression of the SERCA protein, which is responsible for the reuptake of cytosolic calcium, and improves RV function during the cardiac cycle.

The echocardiographic parameters evaluated in this study show that the administration of the alkaloid MCT increased the RV diameter, indicating right ventricle dilatation, and increased MPI, which is an index of cardiac function that provides an evaluation of systolic and diastolic functions at the same time. There is a paucity of data evaluating MPI in the right heart and there is no study in the literature evaluating pterostilbene effects in this parameter in an animal model of pulmonary

hypertension. Augmented MPI values represent a relevant prognostic date of cardiac dysfunction, being an important predictive signal for progression of heart failure, independently of other echocardiographic data (Basar et al. 2016). Besides that, MPI is linearly and directly correlated to cardiac oxygen consumption rate (Cury et al. 2005, Willis et al 2015). The increase in right ventricle MPI is an indicative of an increase of cardiac work due to a worsening in systolic and diastolic function. Besides that, MCT animals also showed a decrease in systolic and cardiac output, which are functional changes inherent to *Cor pulmonale* (Greyson et al. 2008). However, the administration of the PTS:HPβCD complex at a dose of 100 mg kg^{-1} promoted an improvement in RV systolic and diastolic diameters, indicating a decrease in right ventricle dilatation. Besides that, PTS:HPβCD protected RV systolic function, as evidenced by the maintenance of systolic and cardiac output, and also the RV global function (systolic and diastolic), as shown by the maintenance of MPI. In view of that, these data demonstrate a positive effect of pterostilbene both in morphologic and functional parameters of the right ventricle. It was recently shown that the PTS:HPβCD complex prevented a reduction in the RV fractional area and longitudinal function and thus protected the systolic function (dos Santos Lacerda et al, 2017). Similarly, Yu et al. (2016) reported that PTS improved ventricular function after ischemic injury followed by reperfusion (according to contractility and relaxation data), as well as left ventricular systolic and diastolic pressures. On the other hand, Akinwumi et al. (2017) showed an important anti-hypertrophic role of uncomplexed pterostilbene in cardiomyocyte's culture. However, the same authors, using an *in vivo* protocol of spontaneously hypertensive heart failure rat model, found that uncomplexed pterostilbene had no significative anti-hypertrophic and anti-hypertensive effects. These data reinforce the

benefit for oral administration of complexed form. These effects were related to the altered calcium handling in cardiac tissue.

Indeed, afterload increase induced by pulmonary hypertension may provoke structural and biochemical aspects of RV remodelling (Campos et al. 2017). In this sense, a detrimental state of calcium handling seems to be involved in the progression from RV hypertrophy to heart failure. Therefore, molecules with the ability to inhibit the development of cardiac injury are promising therapeutic alternatives. Our study showed that the PTS:HPβCD complex (25 and 100 mg kg^{-1}) increased the expression of the calcium ATPase protein SERCA and reduced total phospholamban, and thus increasing the RV relaxation signalling. This data may also be associated with the partially improved MPI. This pharmacological mechanism is fundamental, since calcium transport may be decreased in heart failure and may, subsequently, impair myocardial contractility (Santos et al. 2011). In addition, upregulated SERCA expression in cardiac myocytes increased calcium reuptake and improved contractility in transgenic mice (Belke et al. 2007). In relation to the effect of pterostilbene causing the increase in SERCA expression, more evaluations are needed to understand the exact mechanism of this modulation. In this sense, it would be important to assess SERCA2a activity to complement SERCA2a protein levels to understand enzymatic function in this context; however, evaluation of this point is a limitation of our study. It is possible that PTS:HPβCD complex could be directing modulating the gene expression of this protein, since previous studies shown that this compound can interact with transcription factors (Bhakkiyalakshmi et al. 2016), but since this mechanism was not evaluated, it is not possible to confirm it.

A limitation of western blot technique, in our study, was the use of red ponceau stain. Red ponceau was used given the interference that cardiac remodelling exerts in

the structural proteins commonly used as loading controls, such as alpha-actinin, beta-tubulin, and GAPDH. The image of red ponceau staining is right below the image of each protein of interest for all graphs (Romero-Calvo et al 2010).

Oxidative stress is a determinant of the progression and severity of *Cor pulmonary* (Bogaard et al. 2009). When the RV is under post-stress conditions, there is an increase in the production of ROS that is capable of oxidising macromolecules, including lipid molecules (Campos-Carraro et al. 2018). In addition to altering the cell membrane functionality, products of lipid oxidation are able to form complexes and inactivate proteins, to trigger intrinsic mitochondrial apoptosis, and to mediate inflammatory signalling (McIntyre and Hazen 2010). The present study showed that the PTS:HPβCD complex reduced the oxidation of lipids in the RV of MCT rats. Similar effects on the left ventricle of rats were found following PTS administration (Liu et al. 2017). Satheesh and Pari (2006) showed that PTS administration effectively reduced lipid oxidation in the liver and kidney of diabetic rats. This significant antioxidant activity was related to the free hydroxyl group in the 4′ position of the PTS.

Inactivation of ROS and an improved intracellular redox balance can be achieved by the reductive action of GSH, which is considered to be the most abundant nonenzymatic intracellular antioxidant (Lu et al. 2009). This tripeptide is involved in many important biochemical processes, such as the recycling of ascorbate which seems to be relevant to recovery of alfa tocopherol, reducing the lipid peroxidation of cellular membranes. Besides that, GSH can also scavenge reactive species, reducing the initiation of lipid peroxidation process, and maintaining redox homeostasis, avoiding oxidative damage of redox-sensitive proteins (Halliwell and Gutteridge, 1999). In our study, a 100 mg kg^{-1} dose of the PTS:HPβCD complex increased the GSH content and the GSH-to-GSSG ratio in the RV of rats with pulmonary artery hypertension and this

date was associated with reduced TBARS levels. The increase of GSH in the RV may be partly related to the induction of GSR activity by the PTS:HPβCD complex (at 100 mg kg^{-1} doses) in MCT rats, as this enzyme is responsible for the conversion of GSSG to GSH (Lushchak et al. 2012). Nevertheless, GSH levels was not increased in the MCT50 (receiving 50 mg/kg PTS:HPβCD), even though glutathione reductase (GSR) activity was increased in this group. GSH may react with lipid peroxidation products non-enzymatic or enzymatically, through a reaction catalysed by glutathione-s-transferase (GST). An example is hydroxyalkenals (4-hydroxy-2nonenal) that show relevant ability to react rapidly with GSH, forming a thioether linkage and consuming GSH (Halliwell and Gutteridge, 1999). Our results show that the dose of 50 mg/kg pterostilbene was not able to reduce lipid peroxidation (as observed by augmented TBARS levels). Moreover, the elevated lipid peroxidation levels in MCT50 group was associated with augmented GST activity, which can contribute to reduce the GSH levels in an oxidized environment. Taken together, these results would explain the absence of elevation on GSH levels, despite increased GSR activity. These findings denote the dose-dependent cardioprotective effects of the PTS:HPβCD complex in maintaining the intracellular content and recycling of GSH in *Cor pulmonale*. In a rodent model of isoproterenol-induced cardiac hypertrophy, treatment with the stilbene, resveratrol, also elevated GSH levels (Chakraborty et al. 2015).

The antioxidant action of GSH also occurs through enzymatic reactions, shuc as GST transferases, which catalyses the conjugation of GSH to a variety of electrophilic compounds and toxic substrates to promote cellular detoxification and reduce the redox stress (Wu et al. 2004). It is important to note that the inhibition of GST (with ethacrynic acid) in oxidatively-injured cardiomyocytes results in apoptosis via the activation of mitogen-activated protein kinase proteins (Röth et al. 2011). This study

found reduced GST activity in MCT rats; however, this effect was reversed by the administration of the highest doses (50 and 100 mg kg^{-1}) of the PTS:HPβCD complex. This result may be partly due to the increase of GSH levels promoted by the PTS:HPβCD complex in the RV, as GST activity is directly related to the intracellular availability of GSH (Lushchak 2012). Similarly, PTS-induced upregulation of GST activity contributed to the reduction of oxidative stress in different rat tissues (Pari et al. 2006).

In addition, the intracellular concentrations of GSH and GST are also known to be regulated by transcriptional activation mechanisms via the interaction of the Nrf2/Keap-1 system with antioxidant response elements. Recent evidence suggests that PTS mediates the activation of Nrf2 (Elango et al. 2016; Xue et al. 2017); therefore, it is also possible that this pharmacological mechanism also contributes to the activation of antioxidant systems in the RV via PTS; however, this was not the focus of this study.

The GRx system, aided by GSH, contributes to the antioxidant efficiency of thiol systems and redox buffering in the myocardium (Hanschmann et al. 2013). The inefficiency of this system results in ventricular remodelling and dilatation, reduced contractility, fibrosis, and apoptosis (Farahmand et al. 2004). Gallogly et al. (2010) showed increased apoptosis susceptibility in both GRx-deficient cardiac tissue and GRx knockdown H9c2 cardiomyocytes. These results highlight the importance of the integrity of this system for the survival of cardiac cells. This study found reduced GRx activity in the RV of animals with *Cor pulmonale*; however, this activity was restored following treatment with the highest does of the PTS:HPβCD complex. As far as the authors are aware, this is the first study to describe the activation of GRx in the RV of rats with C*or pulmonary* following PTS administration. Such a response reinforces the ability of PTS to induce the antioxidant potential of GRx in RV subjected to oxidative

stress. GRx enzymatically catalyses protein disulphide bonds (oxidised −SH groups) by reduction of S-thiol adducts in the presence of GSH to regenerate the native protein with free −SH groups (Shelton et al. 2005). GRx gene therapy prevented ROS-induced cardiac complications in diabetic rats. These effects were partly mediated by activation of redox-sensitive cellular proteins related to cellular survival, which resulted in improved left ventricular function (Lekli et al. 2010).

In this sense, the normalization of an eustress environment (provided by pterostilbene) can promote a positive modulation of sarcoplasmic reticulum proteins, such SERCA, leading to the improved MPI, a global diastolic and systolic cardiac function parameter. In fact, pterostilbene treatment increased protein expression of SERCA and maintained phospholamban levels in rats with pulmonary arterial hypertension, suggesting, in a general context, increased calcium reuptake and improved cardiac lusitropy and inotropy.

The results of the present study show that the PTS:HPβCD complex can increase SERCA expression, improving the calcium handling mechanism, and also has a dose-dependent antioxidant effect, as evidenced by the activation of antioxidants from nonenzymatic and enzymatic systems (i.e. glutathione and glutaredoxin systems). This modulation resulted in increased systolic and cardiac output in experimental right heart failure. These effects suggest that the PTS:HPβCD complex has potential use in right heart failure and may be used as an adjunctive treatment to conventional therapy to minimise oxidative alterations and right ventricular dysfunction.

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Conflict of Interest

The authors have no Conflict of Interest.

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Table 1 Echocardiographic data of control (CTR0) and heart failure (MCT) rats after 14 days of oral administration with the PTS:HPβCD complex in doses corresponding to 25, 50 or 100 mg kg^{-1} of PTS.

Parameter	CTR0	MCT0	MCT25	MCT50	MCT100	P
RVDD (cm)	0.265 ± 0.017	0.317 ± 0.034 [a]	0.299 ± 0.017	0.277 ± 0.026	0.277 ± 0.026	<0.05
RVSD (cm)	0.199 ± 0.003	0.277 ± 0.040 [a]	0.266 ± 0.035 [a]	0.231 ± 0.028	0.242 ± 0.032	<0.05
HR (bpm)	257 ± 18.89	269 ± 15.03	262 ± 20,39	274 ± 20.10	260 ± 11.99	>0.05
MPI	0.26 ± 0.03	0.45 ± 0.05[a]	0.44 ± 0.09[a]	0.35 ± 0.12	0.36 ± 0.08	<0.05

Values expressed as mean ± standard deviation, n= 8 animals per group. One-way ANOVA and Tukey's post-hoc tests were performed. (a) Significantly different compared to CTR; $P< 0.05$. RVSD: right ventricular systolic diameter; RVDD: right ventricular diastolic diameter; HR: heart rate; MPI: myocardial performance index

Figure captions

Figure 1: Effect of 14-day oral administration (gavage) of the PTS:HPβCD complex at different doses (corresponding to 25, 50 and 100 mg/kg of PTS) on functional echocardiographic measurements, RV end-systolic volume (A) and RV cardiac output (B) in control rats (CTR) or rats with heart failure (MCT). A one-way ANOVA and Tukey's post hoc tests were performed. *different to CTR0 ($P < 0.05$), #different to MCT0 ($P < 0.05$).

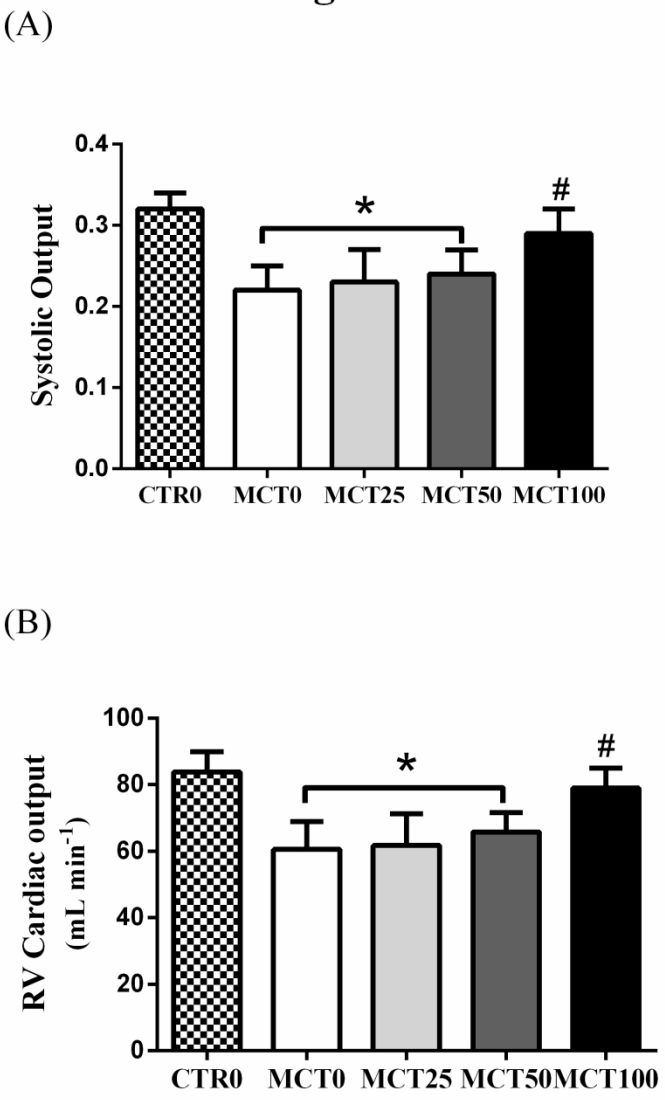
Figure 2: Effect of 14-day oral administration (gavage) of the PTS:HPβCD complex at different doses (corresponding to 25, 50 and 100 mg/kg of PTS) on the content of reduced glutathione (GSH) (A), levels of oxidised glutathione (GSSG) (B), the GSH-to-GSSG ratio (C) and glutathione reductase (GSR) activity (D) in the RV of control rats (CTR) or rats with heart failure (MCT). Values are expressed as mean ± SD with n = 8 per group. A one-way ANOVA and Tukey's post hoc tests were performed. *different to CTR0 ($P < 0.05$), **different to CTR0 ($P < 0.001$), #different to MCT0 ($P < 0.05$), @different to all groups (CTR, MCT0, MCT25 and MCT50) ($P < 0.001$).

Figure 3: Effect of 14-day oral administration (gavage) of the PTS:HPβCD complex at different doses (corresponding to 25, 50 and 100 mg/kg of PTS) on glutathione-S-transferase activity (GST) (A) and glutaredoxin activity (GRx) (B) in the RV of control rats (CTR) or rats with heart failure (MCT). Values are expressed as mean ± SD with n = 8 per group. A one-way ANOVA and Tukey's post hoc tests were performed. *different to CTR0 ($P < 0.05$), #different to MCT0 ($P < 0.05$).

Figure 4: Effect of 14-day oral administration (gavage) of the PTS:HPβCD complex at different doses (corresponding to 25, 50 and 100 mg/kg of PTS) on thiobarbituric acid reactive substances (TBARS) (A) and lipid peroxidation (LPO) (B) in the RV of control rats (CTR) or rats with heart failure (MCT). Values are expressed as mean ± SD with n = 8 per group. A one-way ANOVA and Tukey's post hoc tests were performed. *different to CTR0 ($P < 0.05$), #different to MCT0 ($P < 0.05$), &different to MCT25 and MCT50 ($P < 0.05$).

Figure 5: Effect of 14-day oral administration (gavage) of the PTS:HPβCD complex at different doses (corresponding to 25, 50 and 100 mg/kg of PTS) on the expression of p-phospholamban (A), total phospholamban (B), SERCA (C), SERCA2a/PLB ratio (D) and p-phospholamban/total phospholamban ratio (E) in the RV of control rats (CTR) or rats with heart failure (MCT). Values are expressed as mean ± SD with n = 3-4 per group. A one-way ANOVA and Tukey's post hoc tests were performed. *different to CTR0 ($P < 0.05$), #different to MCT0 ($P < 0.05$)

Figure 1



Effect of 14-day oral administration (gavage) of the PTS:HPβCD complex at different doses (corresponding to 25, 50 and 100 mg/kg of PTS) on functional echocardiographic measurements, RV end-systolic volume (A) and RV cardiac output (B) in control rats (CTR) or rats with heart failure (MCT). A one-way ANOVA and Tukey's post hoc tests were performed. *different to CTR0 ($P < 0.05$), #different to MCT0 ($P < 0.05$).

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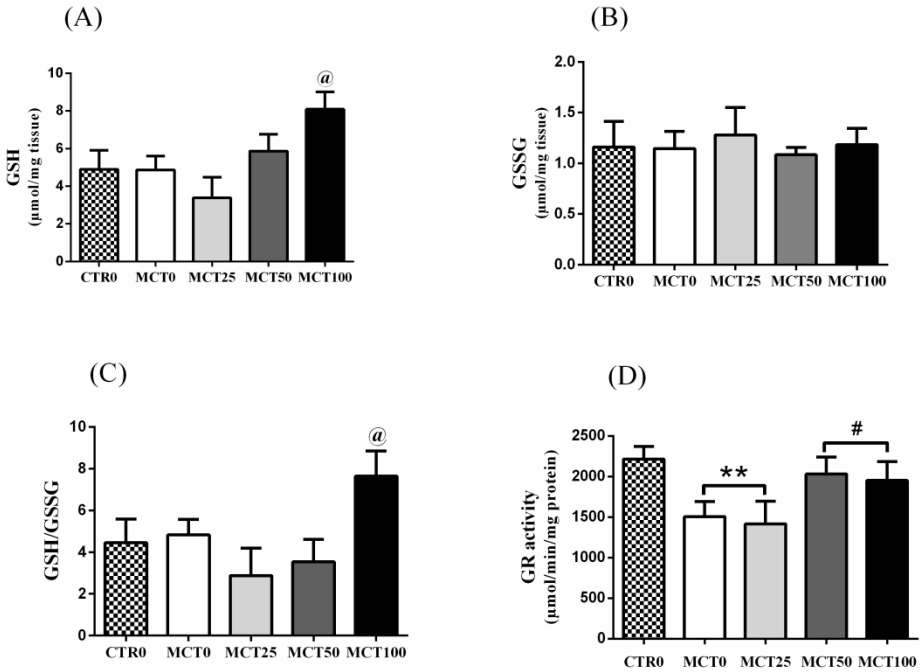
Figure 2



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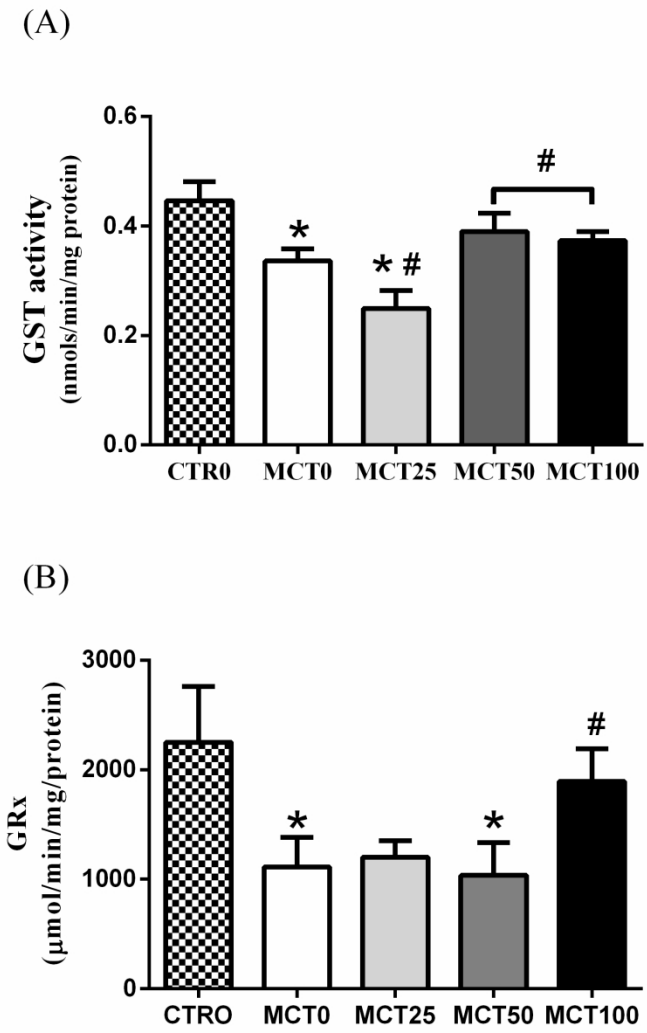
279x216mm (300 x 300 DPI)

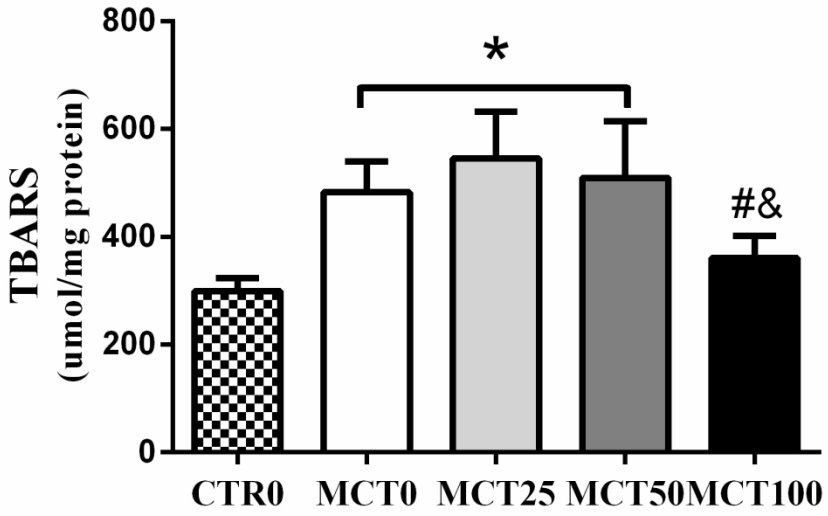
Figure 3



Effect of 14-day oral administration (gavage) of the PTS:HPβCD complex at different doses (corresponding to 25, 50 and 100 mg/kg of PTS) on glutathione-S-transferase activity (GST) (A) and glutaredoxin activity (GRx) (B) in the RV of control rats (CTR) or rats with heart failure (MCT). Values are expressed as mean ± SD with n = 8 per group. A one-way ANOVA and Tukey's post hoc tests were performed. *different to CTR0 ($P < 0.05$), #different to MCT0 ($P < 0.05$).

174x270mm (300 x 300 DPI)

Figure 4



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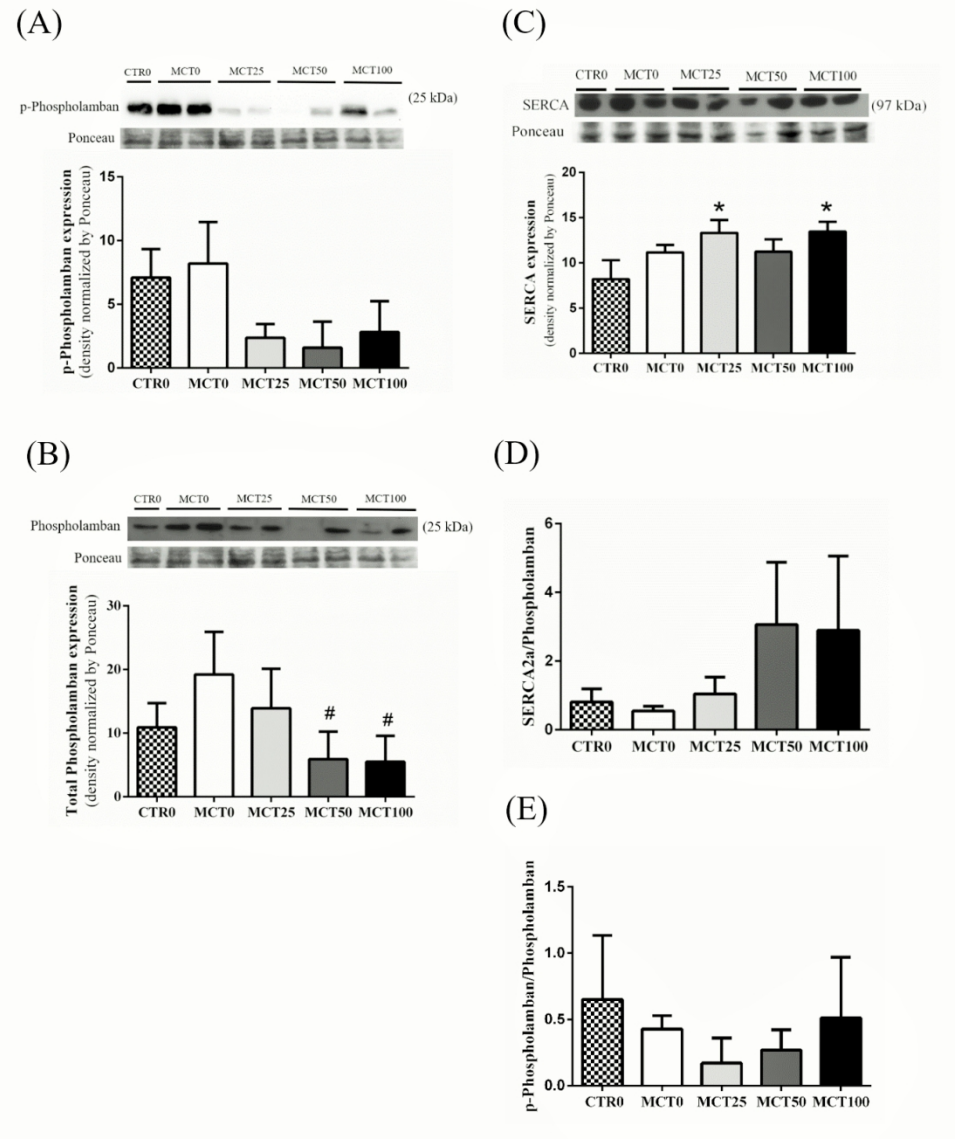
168x166mm (300 x 300 DPI)

Figure 5



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Capítulo IV

4.4 Estilbenoide Pterostilbeno preserva a função ventricular esquerda após infarto do miocárdio em ratos: possível envolvimento de proteínas tiois e modulação de GSK-3β fosforilada

Este capítulo aborda os efeitos farmacológicos do pterostilbeno sobre parâmetros antioxidantes e indução de apoptose no VE pós IAM. Neste estudo mostramos que o pterostilbeno modula a atividade de enzimas tiol-dependentes, aumenta a expressão de antioxidantes e eleva o limiar para a apoptose pós-infarto, resultando em uma redução da peroxidação lipídica e uma melhora da função sistólica.

Artigo Original intitulado:

Stilbenoid Pterostilbene Preserves Left Ventricular Function after Myocardial Infarction in Rats: Possible Involvement of Thiol Proteins and Modulation of Phosphorylated GSK-3β

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Stilbenoid pterostilbene complexed with cyclodextrin preserves left ventricular function after myocardial infarction in rats: possible involvement of thiol proteins and modulation of phosphorylated GSK-3β

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ORIGINAL ARTICLE

Stilbenoid pterostilbene complexed with cyclodextrin preserves left ventricular function after myocardial infarction in rats: possible involvement of thiol proteins and modulation of phosphorylated GSK-3β

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ABSTRACT

Oxidative stress alters signalling pathways for survival and cell death favouring the adverse remodelling of postmyocardial remnant cardiomyocytes, promoting functional impairment. The administration of pterostilbene (PTS), a phytophenol with antioxidant potential, can promote cardioprotection and represents a therapeutic alternative in acute myocardial infarction (AMI). The present study aims to explore the effects of oral administration of PTS complexed with hydroxypropyl-β-cyclodextrin HPβCD (PTS:HPβCD complex) on the glutathione cycle, thiol protein activities and signalling pathways involving the protein kinase B (AKT) and glycogen synthase kinase-3β (GSK-3β) proteins in the left ventricle (LV) of infarcted rats. Animals were submitted to acute myocardial infarction through surgical ligation of the descending anterior branch of the left coronary artery and received over 8 days, by gavage, PTS:HPβCD complex at dose of 100 mg kg^{-1} day^{-1} (AMI + PTS group) or vehicle (aqueous solution with HPβCD) divided into Sham-operated (SHAM) and infarcted (AMI) groups. The results showed that the PBS: HPβCD complex decreased lipid peroxidation, prevented the decrease in thioredoxin reductase (TRxR) activity, and increased the activity of glutathione-S-transferase (GST) and glutaredoxin (GRx). Additionally, the expression of nuclear factor-erythroid two (Nrf2) and p-GSK-3β was increased, whereas the p-GSK-3β/GSK-3β ratio was reduced in the LV of the infarcted animals. Overall, the PTS:HPβCD complex modulates activity of thiol-dependent enzymes and induces to the expression of antioxidant proteins, improving systolic function and mitigating the adverse cardiac remodelling post infarction.

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KEYWORDS
Antioxidant; cardioprotection; cyclodextrin; heart failure; natural products; stilbene

Introduction

Among the cardiovascular diseases, acute myocardial infarction (AMI) is considered the most common, presenting a high rate of morbidity and mortality [1]. AMI can be defined as an ischaemic cardiomyopathy, which generates left ventricular contractile dysfunction, arrhythmias and heart failure, events that arise due to cardiac tissue perfusion deficiency and myocardial necrosis, among other factors [2]. To maintain cardiac function, the remaining cardiomyocytes (area not exposed to ischaemia) are exposed to a higher workload, resulting, at first in, compensatory hypertrophy and a later reduction in wall thickness, adverse remodelling, and cell death through the activation of apoptosis and autophagy [3,4]. The rate of cardiomyocyte death in remnant tissue is a predictor of the severity of adverse cardiac remodelling, which will determine the rate of progression to heart failure [4].

Concomitant to left ventricle (LV) morphological and structural changes, intracellular biochemical changes also contribute to the progression of postinfarction ventricular remodelling. In this sense, we highlight the activation of inflammatory pathways, as well as the increase in reactive oxygen species (ROS) and/or nitrogen reactive species (RNS) associated with a reduction of antioxidant defences and changes in redox balance [3,5]. To maintain a reduced cellular environment, the myocardium has endogenous thiol-dependent

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mechanisms, such as the thioredoxin (Trx), glutaredoxin (GRx), and glutathione (the most abundant intracellular antioxidant) systems, characterised as electron donors that act to eliminate ROS and reduce oxidised proteins through thiol disulphide exchange reactions [6–8]. Particularly, the actions of thioredoxin and glutarredoxin are important in minimising the deleterious effects on cardiovascular diseases [9]. For example, in humans plasma concentration of thioredoxin (TRx) was inversely correlated with the severity of heart failure [10]. Moreover, TRx seems to be pivotal in intracellular redox signalling, and its decreased levels are involved with ventricular dysfunction induced by infarction [11]. Both, redox balance modulation and exogenous administration of thioredoxin are associated with cardioprotection and reduction of cardiomyocyte apoptosis in the AMI model [12,13]. In addition, cardiomyocytes (H9c2) knockdown for GRx are more susceptible to apoptosis and oxidative stress [14].

Also, redox change can modify intracellular signalling pathways that coordinate survival and death responses, since many proteins that participate in these signalling processes undergo redox regulation. For example, an imbalance in the signalling pathway of protein kinase B (Akt) and glycogen synthase kinase-3β (GSK-3-β), redox sensitive proteins that regulate survival and cell death, may result in impaired function and cardiomyocyte damage [15,16]. These effects may directly impact the progress of cardiac remodelling [6]. Considering this, it is of great scientific interest to seek new therapeutic targets and to identify potential pharmacological interventions that are capable of modulating adverse remodelling and improving post-AMI ventricular function.

Studies have shown the biological potential of pterostilbene (PTS), a natural demethylated analogue of resveratrol, found in fruits such as blueberries (Vaccinium spp.) and grapes [17,18]. Several pharmacological actions were highlighted, including antioxidant, anti-inflammatory, and antiapoptotic properties [19–21]. Although structurally similar to resveratrol, the pharmacological effects of pterostilbene have been described as more potent, which is an additional advantage attributed to differences in absorption, bioavailability, distribution, and metabolism [22,23]. Despite its high pharmacological potential, the poor aqueous solubility of PTS can be considered a barrier to its oral bioavailability, which may limit its oral use [24]. Given this, the water solubility of lipophilic substances as PTS can be enhanced by complexing with hydroxypropyl-β-cyclodextrin (HPβCD) [25–27].

Previous studies from our research group found potential beneficial effects of PTS complexed with HPβCD (PTS:HPβCD complex) on systolic function and right ventricular remodelling in a model of heart failure [28]. Moreover, other authors have demonstrated cardioprotective effects of PTS in myocardial ischaemia models, highlighting their antioxidant and anti-inflammatory actions [20,29,30]. However, the effects of PTS on thiol-dependent antioxidant proteins, as well as on cell survival via GSK-3-β in cardiomyocytes remains, unknown. The present study aims to analyse the effects of the oral administration of PTS:HPβCD complex on the glutathione cycle, on the activities of TRxR and GRx, as well as to investigate signalling pathways involving the AKT and GSK-3β proteins in the heart tissue of infarcted rats.

Materials and methods

Reagents

PTS was purchased from Changha Organic Herb (Changha, China). HPβCD was supplied by Roquette Frères (Lestrem, France). All reagents used were of analytical grade or high performance liquid chromatography (HPLC) grade.

Preparation of PTS:HPβCD complex

The complexation method was carried out according to Lacerda and colleagues [28]. Briefly, the resultant dispersion (PTS:HPβCD) was filtered and the supernatant was lyophilised. The content of PTS in the lyophilised powder was quantified by HPLC (Shimadzu LC-20A system; Kyoto, Japan).

Animals

Twenty five male Wistar adult rats (250–300 g) from the Reproduction Centre for Laboratory Animals (CREAL) of the Universidade Federal do Rio Grande do Sul (UFRGS) were housed in polypropylene cages (40 × 33 × 17 cm), four per cage, under standard environmental conditions (room temperature, 22 ± 2 °C; 12-h light-dark cycle, 7 am–7 pm). All rats had free access to food and water. Our experimental protocol was carried out in accordance with the International Guidelines for Use and Care of Laboratory Animals of the National Institutes of Health and with Brazilian Laws for the Scientific Use of Animals. The protocol began after it had been approved by the Ethical Committee for Animal Experimentation at UFRGS (CEUA-UFRGS # 29835). All efforts were made to minimise animal suffering and to

use the minimum number of animals necessary to produce reliable data.

Experimental groups and procedures

Before the surgical procedure, animals were anaesthetised (ketamine 90 mg kg^{-1}; xylazine 20 mg kg^{-1}, i.p.), and myocardial infarctions were produced by a method similar to that previously described (Johns e Olson, 1954). Animals were submitted to a surgical ligation of the descending anterior branch of the left coronary artery (AMI group), or to a sham-operation in which all surgical procedures were performed, except the suture around the coronary artery (SHAM group). All animals were treated for 2 days with the analgesics tramadol (12.5 mg kg^{-1} subcutaneously every 12 h) and dipyrone (50 mg kg^{-1}, by gavage, every 12 h) to minimise pain and discomfort in the postoperative period. Seven days after the surgical procedure, the animals were submitted to an echocardiographic evaluation to confirm the effectiveness of the infarction surgery and were divided in three groups: sham animals (SHAM group ;sevev animals per group), infarcted nontreated animals (IAM group; eight animals per group) and infarcted treated animals (IAM + PTS group; eight animals per group). Subsequently, IAM + PTS rats began to receive PTS:HPβCD complex in dose corresponding to 100 mg kg^{-1} day^{-1} (AMI + PTS) and SHAM and AMI rats received the vehicle (aqueous solution with HPβCD), daily by oral administration, for 8 days. The mortality evaluated 24 h after the surgical procedure was approximately 10% (two animals – one belonged to sham group, and other to IAM group). Mortality rate at the end of the experiment was zero.

Echocardiographic evaluation

Cardiac function was analysed by echocardiography, 14 days after the surgery. Rats were anaesthetised and placed in left lateral decubitus position (45°) to obtain cardiac images. Philips HD7 XE ultrasound system with an L2-13 MHz transducer was used. Left ventricular systolic and diastolic transverse areas (cm^2) were obtained by tracing the endocardial border at three levels: basal, middle, and apical. Left ventricular systolic and diastolic diameters (cm) were measured using the M-Mode, in the three previously described planes [31]. Fractional area change (%), measured as FAC = (diastolic area − systolic area/diastolic area) × 100, was used to determine contractile function and left ventricular function [31]. On each echocardiographic transverse plane, the arch corresponding to the segments with infarction (I) and the total endocardial perimeter (EP) were measured at end-diastole. Infarction size (IS) was estimated as %IS = (I/EP) × 100 [31,32]. The final value for each animal was obtained by taking the average of all three planes [31,32].

Tissue preparation

Samples from LV (it was utilised an area not at risk of infarction, corresponding to remaining myocardium) were homogenised (OMNI Tissue Homogeneizer, Omni International, USA) during 30 seconds in 1.15% KCl buffer containing 1% phenyl methyl sulphonyl fluoride (PMSF). The homogenates were centrifuged (1358 × *g*, 20 min, at 4 °C; ALC Multispeed Refrigerated Centrifuge PK 121R, Thermo Electron Corporation, USA) and the supernatant was collected for subsequent determination of oxidative parameters. The protein concentrations in samples were determined by the Lowry method using bovine serum albumin as standard [33].

Evaluation of protein and lipid damage

Lipid oxidative damage was determined in VE homogenates using chemiluminescence and thiobarbituric acid reactive substances (TBARS) methods.

Lipid peroxidation (LPO) was evaluated using tert-butyl-hydroperoxide-initiated chemiluminescence measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB – Produkter AB, Sweden). Homogenates were added in a medium containing 120 mmol/L KCl, 30-mmol/L phosphate buffer (pH 7.4). Measurements were initiated by the addition of 3 mmol/L tert-butyl hydroperoxide. The results was expressed as cps per mg of protein (cps/mg protein) [34].

Lipid oxidative damage was determined in LV homogenates by the thiobarbituric acid reactive substances (TBARS) method as described by [35]. The absorbance was read at 535 nm (Anthos Zenyth 200 rt, Biochrom, UK). Malondialdehyde was used as standard [35], and the results were expressed as nmol TBARS/mg protein.

Protein oxidative damage in LV homogenates was determined by the carbonyl assay according to [36]. The absorbance (360 nm) was determined (Anthos Zenyth 200 rt, Biochrom, UK). The results were expressed as nmol of DNPH/mg protein.

Determination of oxidised and reduced glutathione concentrations

To measure oxidised (GSSG) and reduced total glutathione (total glutathione (GSH)) concentrations, the heart tissue was deproteinised with 2 M perchloric acid, centrifuged for 10 min at 1000 × *g*, and the supernatant was neutralised with 2-M potassium hydroxide. The determination of total GSH was based on the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and catalysed by glutathione reductase; the absorbance values were measured at 420 nm. To measure GSSG, n-ethylmaleimide was added to the neutralised supernatant to perform the reaction described above. From these data measured (total GSH and GSSG), we calculated GSH, according this stoichiometric formula (total GSH = GSH + 2GSSG). The GSH/GSSG ratio was calculated to estimate cellular redox state [37].

Glutathione reductase activity

Glutathione reductase (GR), a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme, catalyses the oxidative form of glutathione to form GSH. The activity of GR is proportional to the consumption of NADPH and can be spectrophotometrically monitored at 340 nm [38].

Glutathione-S-transferase activity

Glutathione-S-transferase (GST) catalyses the conjugation of GSH with various substrates and plays an important role in the detoxification of alkylating agents. GST activity was evaluated according to Mannervik and Guthenberg [39]. From the reaction medium containing phosphate buffer (0.2 mol L^{-1}), GSH (20 mM), 1-chloro-2-4-dinitrobenzene (CDNB) (20 mM) and homogenate, the formation of dinitrophenyl-S-glutathione (DNP-SG) was quantified spectrophotometrically at 340 nm (Anthos Zenyth 200 rt, Biochrom, UK). Results were expressed as μmol DNP-SG/mg protein [39].

Thioredoxin reductase activity

The antioxidant function of thioredoxin reductase (TRxR) is related to its ability to directly reduce hydrogen peroxide (H_2O_2) and lipid hydroperoxides. The TRxR activity was determined from the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to TNB (a yellow product) detected at 340 nm. In this assay, a reagent mixture containing DTNB (25 mg mL^{-1}), albumin (20 mg mL^{-1}), NADPH (20 mg mL^{-1}) and EDTA (0.2 M) was used [40]. Results were expressed as mmol TNB/mg protein.

Glutaredoxin activity

Glutaredoxin (GRx) uses the GSH reducing energy to reduce mixed disulphides. In this assay, GSH (1 μM) was added in Tris-HCl buffer (100 mM, pH 7.8) containing EDTA (1 mM) and albumin (1 mg mL^{-1}). The amount of GSSG produced was measured by the addition of NADPH (0.2 mM) and glutathione reductase (6 μg mL^{-1}). Absorbance was read at 340 nm [41]. Results were expressed as mmol/mg protein.

Western blot

Tissue homogenisation, electrophoresis, and protein transference were performed as previously described [42]. Fifty micrograms of protein on LV homogenates were submitted to one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a discontinuous system using 8–12% (w/v) separating gel and stacking gel [42]. The immunodetection was processed using the following primary antibodies: anti-Nrf2 (57 kDa), antitotal AKT (60 kDa), anti-p-AKT (60 kDa), anti-GSK-3β (47 kDa), anti-p-GSK-3β (47 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA or Cell Signalling Technology, Beverly, MA). The primary antibodies were detected using antirabbit or antimouse horseradish peroxidase-conjugate secondary antibodies and the membranes were developed using chemiluminescence detection reagents. The autoradiographs generated were quantitatively measured with an image densitometer (Imagemaster VDS CI, Amersham Biosciences Europe, IT). The molecular weights of the bands were determined by reference to a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad, CA, USA). Both total and phosphorylated (GSK-3 beta e AKT) were normalised by Ponceau method [43]. Ponceau staining of proteins has long been applied to quality control of membrane transfer in Western blotting; it is equally useful for this purpose, has similar or improved dynamic range and was previously validated to assess equal loading gel [44]. The membrane was transferred to the appropriate container and the Ponceau solution was added, being stirred in on a rocking platform for 2 min. Finally, successive washes with distilled water were made to remove the excess colouration [43]. After this procedure, we made the relation phosphorylated protein/total protein previously normalised by Ponceau S stain.

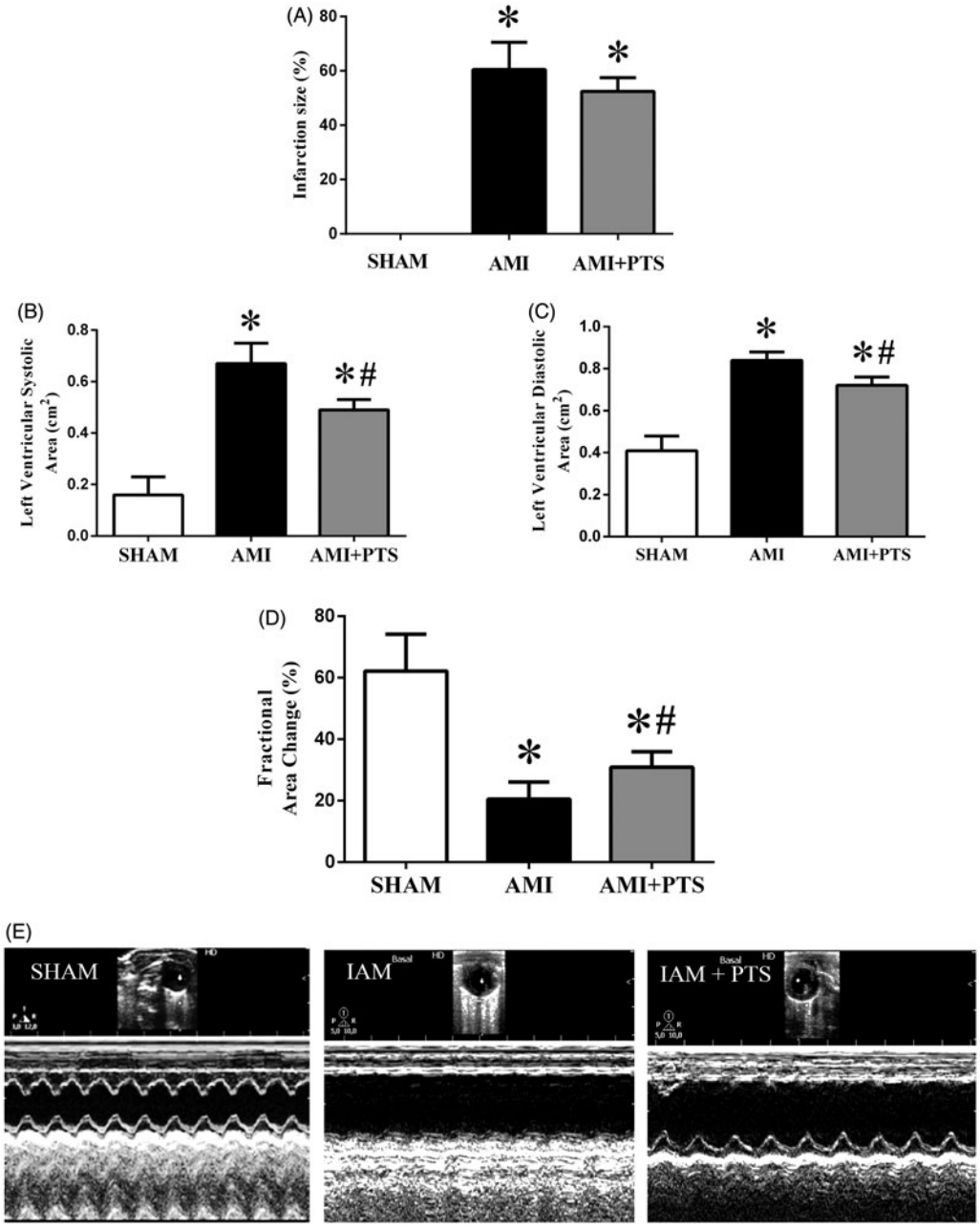

Figure 1. Effect of oral administration of PTS:HPβCD complex in the dose of 100 mg/kg on infarction size (A), left ventricular systolic area (B), left ventricular diastolic area (C) and FAC (D) of rats infarcted treated via gavage for 8 days. Values are expressed as mean ± standard deviation; $n = 6–7$ per group; One-way ANOVA with Tukey's post-hoc was performed. *Different compared to SHAM ($p < 0.05$); #Different compared to AMI ($p < 0.05$).

Statistical analysis

The normal distribution of data was evaluated by Shapiro–Wilk test. Data were expressed as mean ± standard derivation (SD). Parametric data were analysed using one-way ANOVA with Tukey's post-hoc test to detect differences between groups. Differences were considered significant when $p < 0.05$. The data were analysed using the Sigma Stat Program (Jandel, Scientific Co, v. 11.0, San Jose, CA, USA).

Results

Echocardiography functional data

Echocardiographic data were measured after 14 days of AMI induction. When evaluating the infarct area, we observed that there was no difference between AMI and AMI + PTS groups (Figure 1(A)). Infarcted animals present LV dilatation compared to SHAM group, as observed by the increased LV diameter in systole and

diastole (AMI and AMI + PTS) ($p < 0.05$) (Figure 1(B) and 1(C)). Regarding FAC, which is a parameter that evaluates cardiac contractility, we observed that the infarction induction reduced this parameter when compared to control (SHAM) ($p < 0.05$). Remarkably, administration of PTS:HPβCD complex increased FAC in infarcted animals ($p < 0.05$) (Figure 1(D)).

Oxidative parameters

Lipid peroxidation levels evaluated by the chemiluminescence method in LV homogenate, revealed increase in lipid oxidation in AMI group compared to SHAM and AMI + PTS groups ($p < 0.05$) (Figure 2(A)). However, we observed that PTS:HPβCD complex reduced the lipid oxidation in infarcted animals (AMI + PTS group) ($p < 0.05$) (Figure 2(A)).

Increased lipid damage was also observed in AMI group by the evaluation of the TBARS levels in the LV ($p < 0.001$), which was reduced by administration of PTS:HPβCD complex ($p < 0.05$) (Figure 2(B)). However, about protein oxidation (carbonyls content) there was no change in any of the groups tested ($p = 0.99$) (Figure 2(C)).

When evaluating the redox state, it was observed no difference regarding GSH, GSSG, GSH/GSSG ratio and GR activity among the groups ($p > 0.05$) (Figure 3(A), 3(B), 3(C), and 3(D)). On the other hand, GST activity was increased by the administration of PTS:HPβCD complex in infarcted animals in comparison to the other groups ($p < 0.05$) (Figure 3(E)).

The analysis of the activity of antioxidant enzymes showed a reduction in TRxR activity in infarcted animals ($p < 0.05$) (Figure 4(A)), while GRxR showed increased activity ($p < 0.05$) (Figure 4(B)). The PTS:HPβCD complex prevented the decrease in TRxR activity ($p < 0.05$) (Figure 4(A)) and the increase in GRx in infarcted rats. In fact, these animals presented a similar enzymatic activity profile that was observed in SHAM animals ($p < 0.05$) (Figure 4(B)).

Protein content evaluation

Molecular analyses of proteins related to cellular survival pathways were performed in LV cardiac tissue. The data revealed that administration of PTS:HPβCD complex increased the expression of nuclear factor-erythroid two (Nrf2) ($p < 0.05$) (Figure 4(C)) and p-GSK-3β ($p < 0.05$) (Figure 5(B)), whereas the p-GSK-3β/GSK-3β ratio was reduced in the LV of the infarcted animals ($p < 0.05$) (Figure 5(C)). In contrast, expression of the total AKT, p-AKT, as well as the p-AKT/total AKT ration and total GSK-3β were not altered (Figure 6(A), (B), (C), and 5(A)).

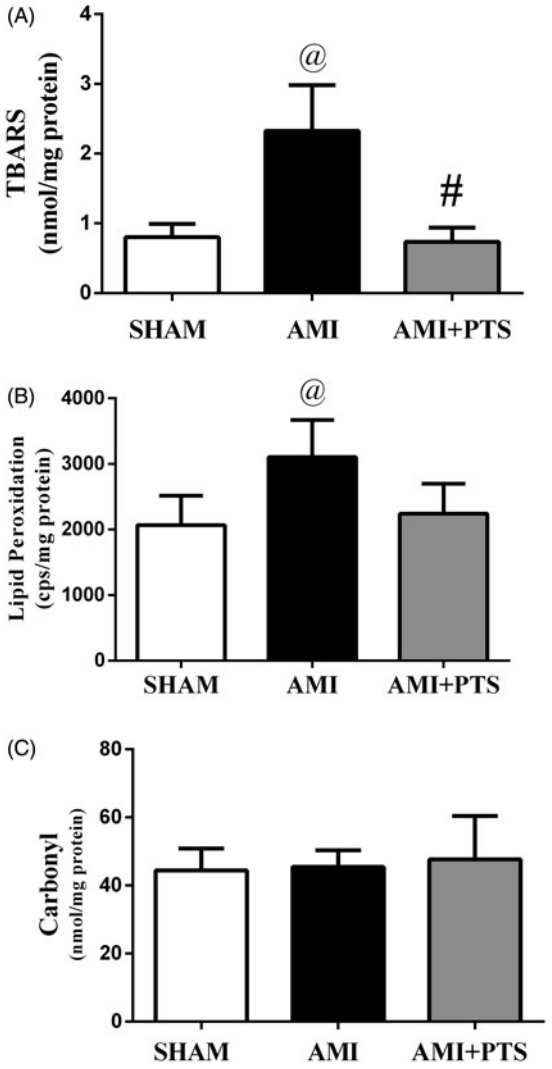

Figure 2. Effect of oral administration of PTS:HPβCD complex in the dose of 100 mg/kg on thiobarbituric acid reactive substances (TBARS) levels (A) lipid peroxidation (B) and protein carbonyl content (C) in the left ventricle of rats infarcted treated via gavage for 8 days. Values are expressed as mean ± standard deviation; $n = 6$–7 per group; One-way ANOVA with Tukey's post-hoc was performed.@Different compared to SHAM and AMI + PTS ($p < 0.05$); #Different compared to AMI ($p < 0.001$).

Discussion

Oxidative stress alters signalling pathways for survival and cell death, favouring the adverse remodelling and apoptosis of postmyocardial infarction remnant cardiomyocytes [4,6]. Recent studies have shown that the administration of phytophenol PTS has cardioprotective effects in ischaemic myocardium, reported that PTS reduced myocardial ischaemia/reperfusion injury and improved oxidative stress through activation of the

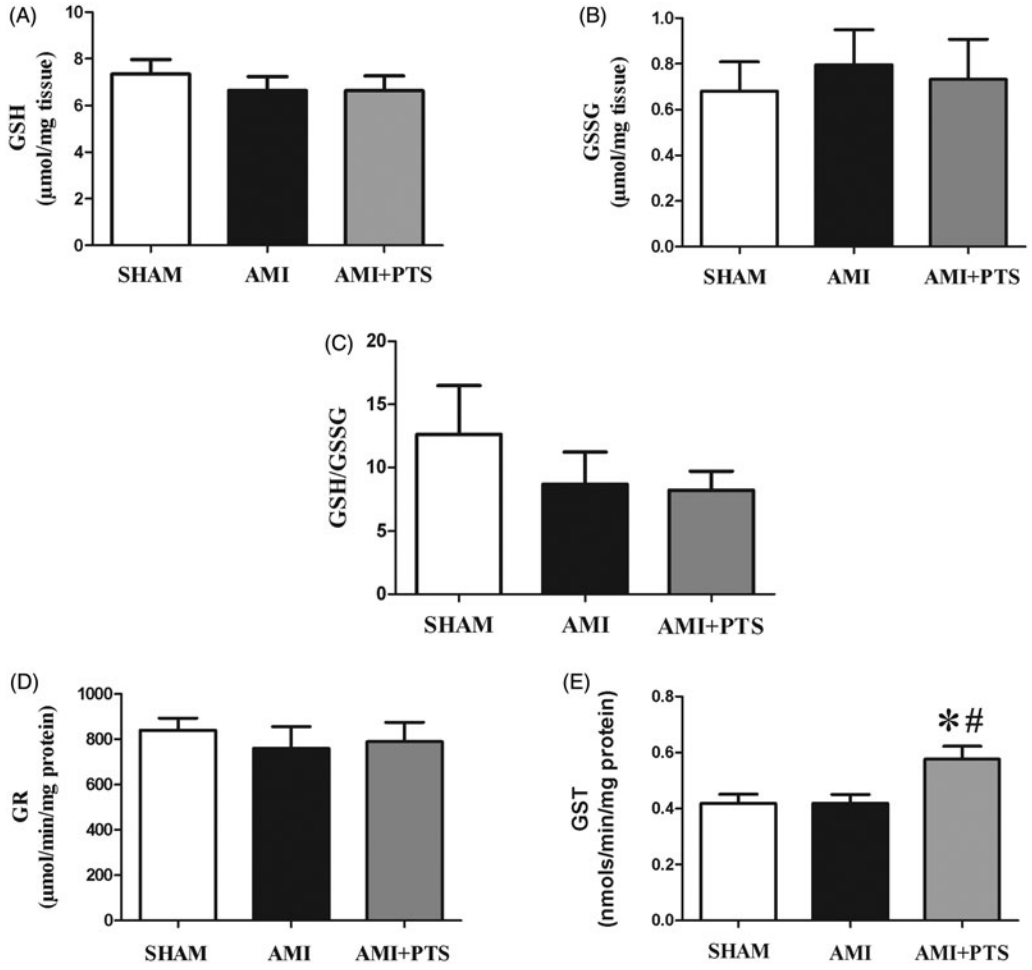

Figure 3. Effect of oral administration of PTS:HPβCD complex in the dose of 100 mg/kg on reduced GSH content (A), oxidised glutathione (GSSG) content (B), GSH/GSSG ration (C), GR activity (D) and GST activity in the left ventricle of rats infarcted treated via gavage for 8 days. Values are expressed as mean ± standard deviation; $n = 6–7$ per group; One-way ANOVA with Tukey's post-hoc was performed. *Different compared to SHAM ($p < 0.05$); #Different compared to AMI ($p < 0.05$).

inducible nitric oxide synthase enzyme in diabetic rats [29]. PTS prevented apoptosis *in vitro* by stimulating the function of sirtuin 1 in cardiomyocyte cells (H9c2) submitted to ischaemia/reperfusion [20]. Moreover, PTS improved cardiac function, reduced peroxynitrite content, and lipid oxidation, and decreased the levels of TNF-α, interleukin-1β, and myeloperoxidase activity, demonstrating its anti-inflammatory effects in ischaemia/reperfusion [45]. Our study differs from the others because we show, for the first time, that the PTS:HPβCD complex increased expression of phosphorylated GSK-3β and this was associated to the increase in Nrf2 expression, in the activity of GST and TRxR enzymes, as well as to the restoration of GRx activity and reduction of lipid oxidation. In this context, the PTS:HPβCD complex plays a key role in providing a redox environment favourable to cell survival and keeping the cardiac function postinfarction.

In the present study, ligature of the left coronary artery caused ischaemia and tissue injury, resulting in a scar. The similarity of the infarct area found in our animals denotes the reproducibility of surgical procedure [46]. The echocardiographic findings indicate dilation of the LV chamber, as demonstrated by an increase in systolic and diastolic diameters in AMI animals; similar results were also described by other authors [46,47].

The variation in the LV area between diastole and final systole is described by FAC and indicates the effectiveness of ventricular systole and cardiac contractility [48]. Administration of the PTS:HPβCD complex prevented AMI-induced reduction in FAC, demonstrating its beneficial effect on LV systolic function. Our research group has been exploring the effects of PTS:HPβCD complex on cardiac function in other models of heart disease and systolic function was also preserved by the administration of PTS in the right ventricle of rats affected by pulmonary hypertension and this cardiac function ameliorate was associate with maintenance of status redox [28].

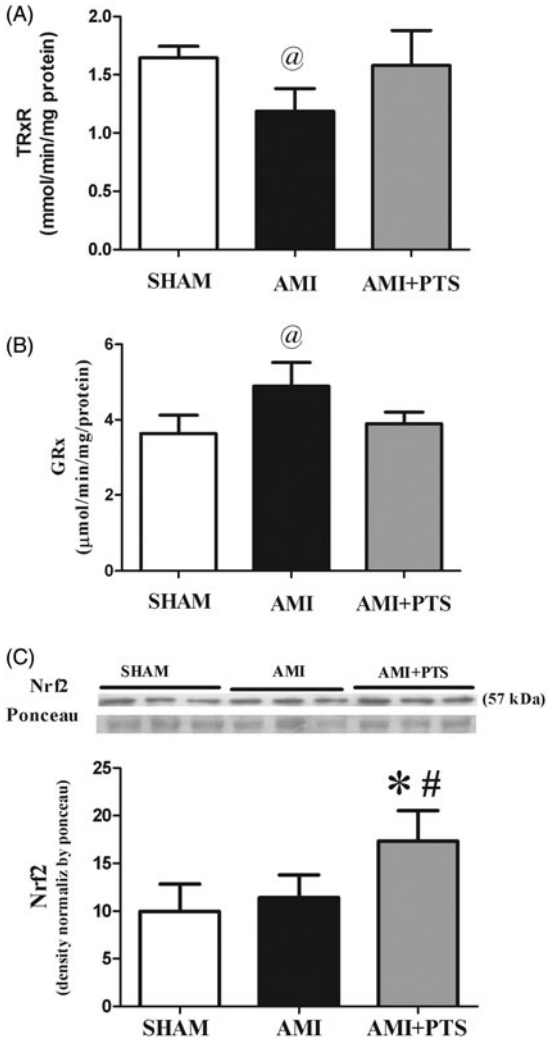

Figure 4. Effect of oral administration of PTS:HPβCD complex in the dose of 100 mg/kg on thioredoxin reductase (TRxR) activity (A), glutaredoxin reductase (GRx) activity (B) and Nuclear factor-erythroid 2 (Nrf2) expression (C) in the left ventricle of rats infarcted treated via gavage for 8 days. Values are expressed as mean ± standard deviation; $n = 6–7$ per group; One-way ANOVA with Tukey's post-hoc was performed. @Different compared to SHAM and AMI + PTS ($p < 0.05$); *Different compared to SHAM ($p < 0.001$); # Different compared to AMI ($p < 0.001$).

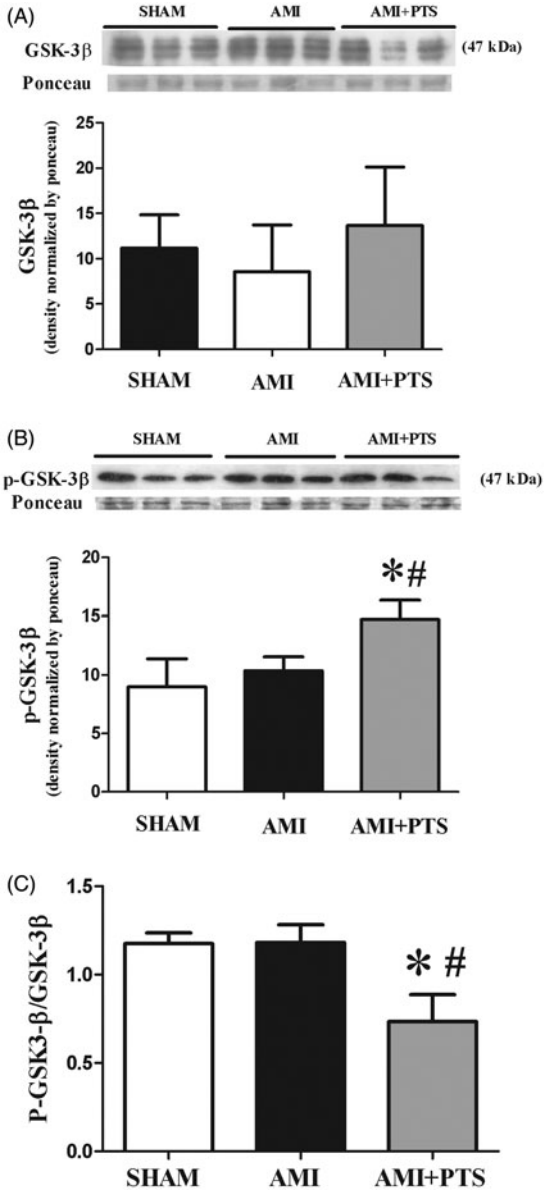

Figure 5. Effect of oral administration of PTS:HPβCD complex in the dose of 100 mg/kg on the expression of the total GSK-3β (A), p- GSK-3β expression (B) and p-GSK-3β/GSK-3β ratio (C) in the left ventricle of rats infarcted treated via gavage for 8 days. Values are expressed as mean ± standard deviation; $n = 6–7$ per group; One-way ANOVA with Tukey's post-hoc was performed. *Different compared to SHAM ($p < 0.05$); #Different compared to AMI ($p < 0.05$).

Alterations in redox homeostasis and sustained oxidative stress can trigger apoptotic stimuli, contributing to the transition from post-AMI cardiac remodelling to heart failure [49]. In this context, GSK-3β, a protein activated by cellular damage, stimulates the intrinsic apoptotic signalling pathway mediated by mitochondria, culminating in activation of the proapoptotic caspase protein [50]. GSK-3β has its activity inhibited by serine-9 phosphorylation promoted by different signalling pathways, including the AKT protein, protein kinase A (PKA), protein kinase C, mitogen activated protein kinases p-38 (p-38-MAPK), among others, resulting in inhibition of the mitochondrial permeability transition pore opening and, consequently reducing the threshold for apoptosis [50–52]. In our study, we observed that the increase of the phosphorylated GSK-3β seems not to have been induced by AKT pathway, since the expression of this protein remained unchanged. On the other hand, Paul et al. [53] reported that mechanism by which PTS stimulated GSK-3β phosphorylation occurs via p38-MAPK signalling. The inhibition of GSK-3β has been reported as a promising therapy in myocardial

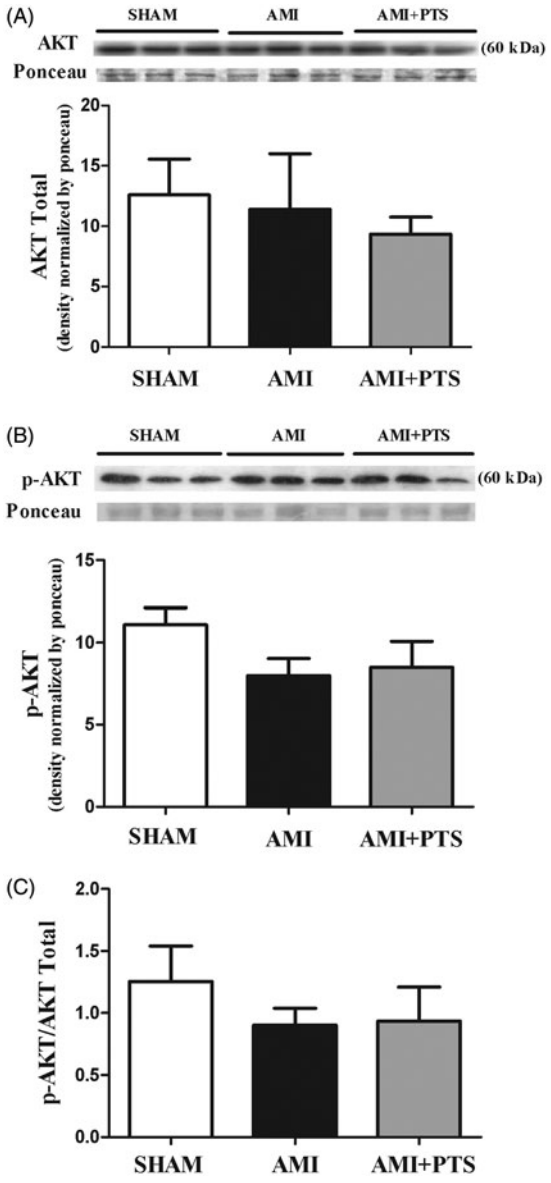

Figure 6. Effect of oral administration of PTS:HPβCD complex in the dose of 100 mg/kg on the expression of the total AKT (A), p-AKT expression (B) and p-AKT/total AKT ratio (C) in the left ventricle of rats infarcted treated via gavage for 8 days. Values are expressed as mean ± standard deviation; $n = 6–7$ per group; One-way ANOVA was performed .

ischaemia/reperfusion injury, as this intervention reduced the infarct area and promoted cardioprotection. Moreover, Yadav and colleagues [54] suggested that the administration of GSK-3β inhibitors potentiates the cardioprotective effects of ischaemic preconditioning in diabetic rats. In the present study, we detected that PTS:HPβCD complex increased the expression of postinfarction phosphorylated GSK-3β, demonstrating its possible inhibitory effect on cell death in remaining cardiomyocytes (area not exposed to ischaemia). This effect is very important because the reduction of apoptosis, in the remote myocardium, it has been inversely related to to the remodelling post-AMI, although infarct sizes were identical [55,56]. In this context, the deletion of GSK-3β in mouse protects against post-AMI remodelling and could be a strategy to both prevent heart failure [56]. Additionally, Jarr and colleagues [55] showed that decreased pGSK3β/GSK3β was observed in conjunction with increased left ventricle end diastolic diameter (LVEDD), which elicit a possibly action of pGSK3β in restraining left ventricle dilation. These data agree with findings of Woulfe and colleagues [56], which showed that mice GSK-3β knockout model presented decreased LVEDD and increased LV ejection fraction 2 weeks post-AMI (same period analysed by our study). Furthermore, another possible mechanism through which increased pGSK3β improves cardiac cells function in remote myocardium is via promoting enhancements in cellular redox balance. In fact, Schenkel and colleagues [57], previously demonstrated decreased pGSK3β was associated with decreased GSH/GSSG ratio and increased H2O2 content in the myocardium of infarcted rats.

Despite all the mechanisms exposed so far for increased pGSK3β in remote myocardium, a positive effect in cell death in the area not at risk cannot be excluded and it can be considered as a protective effect, since it may be important to prevent the development of heart failure in a latter stage. Also, GSK-3β inactivation avoids to degradation, by proteosomal system, of the nuclear factor (erythroid-derived two)-like two (Nrf2) a key regulator of the antioxidant response, establishing a redox balance favourable to cellular survival.

In this context, the cytoprotective mechanism of PTS could be related to the activation of the Nrf2 nuclear factor. This mechanism is associated not only to inhibition of the interaction of kelch-like ECH-associated protein-1 (Keap1) with Nrf2 (Keap1-Nrf2), but also with GSK-3β inactivation. This process culminate in Nrf2 activation and its translocation to the nucleus, leading to the transcription of several genes that encode antioxidant enzymes [19]. Consistent studies have reported that PTS activates the Nrf2 signalling pathway [30,58]. However, to the best of our knowledge, the present study is the first to describe how PTS increases the expression of Nrf2 in cardiac tissue after AMI, contributing to the antioxidant and cardioprotective effect of this phytochemical. Others reports have shown that PTS activates Nrf2 and inhibits the production of ROS and inflammation in an osteoarthritis model, as well as reducing oxidative damage in the pancreas by regulating altered glucose metabolism present in

diabetes [30,58]. Nrf2 promotes expression wide number of antioxidant proteins, through gene regulation of antioxidant response element (ARE), provoking the synthesis of enzymes and peptides involved with control redox homeostasis, such as thioredoxin, glutaredoxin, and glutathione system.

The efficiency of thiol-dependent systems (glutathione, thioredoxin, and glutaredoxin) in the myocardium is an important mechanism of cellular homeostasis due to its redox buffering capacity and neutralisation of oxidative damage [6–8]. Our results show that the PTS:HPβCD complex modulates the activity of some antioxidant enzymes, contributing to the reduction of lipoperoxidation in remnant cardiomyocytes of LV in AMI. In this way, we observed that the PTS:HPβCD complex increased the activity of the GST enzyme, re-established the TRxR activity, and restored GRx activity to baseline levels. This result can be considered beneficial since increased oxidative stress plays an important role in post-AMI injury and dysfunction [4,6].

In cardiac tissue, GST is responsible for the regulation of glutathione homeostasis through the inactivation of electrophilic and xenobiotic compounds and the inhibition of apoptosis, contributing to the maintenance of redox balance, metabolism and cell survival [59]. As reported by Pari and Satheesh (2006), the upregulation of GST activity, induced by PTS, contributed to the reduction of oxidative stress in different tissues [60].

Also, the participation of TRxR contributes to the reduction of oxidative stress, since this enzyme catalyses the NADPH-dependent reduction of TRx – a key protein that regulates redox stress in the heart [8]. The increased availability of TRx favours the conversion of hydrogen peroxide into water, reducing the formation of hydroxyl radical, a potent oxidant of unsaturated bonds of membrane lipids [61]. With antioxidant function similar to TRx, GRx catalyses the reduction of protein disulphides, transferring NADPH electrons via GR and GSH [7].

Interestingly, we found an increase in GRx activity in infarcted animals, whereas TRxR was decreased in this same group, suggesting that the reduction of TrxR was offset by the increase in GRx activity, reflecting an adaptive response induced by stress conditions [62]. Given this, we assume that PTS:HPβCD complex stimulated the activity of TRxR increasing the availability of TRx, not requiring the glutaredoxin system to exert the antioxidant function. Similarly, modulation of the thioredoxin system by phenolic compounds has also been reported by other authors. Kaga et al. reported that resveratrol increased the expression of TRx in the ischaemic myocardium of mice promoting cardioprotection [63]. Moreover, trans-resveratrol promoted an increase in TRx redox activity, and it was related to the reduced expression of the TRx inhibitory protein (Txnip) [64]. Furthermore, the administration of grape juice, which presents high amounts of phenolic compounds, increased the expression of TRx, leading to a reduction of lipid peroxidation and an improvement of right ventricular function [65]. Corroborating our results, Liu et al. and Lv et al. reported that PTS reduced lipid oxidation in the myocardium submitted to ischaemia/reperfusion [29,66].

Overall, we suggest that the PTS:HPβCD complex modulates the activity of thiol enzymes, enhances the expression of antioxidants (via Nrf2 activation), as well as the expression of phosphorylated GSK-3β postinfarction, thereby increasing the threshold for cell death. These effects may be related to the reduction of lipid peroxidation and an improvement of systolic function, promoted, in part, by the antioxidant potential of PTS.

Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5. DISCUSSÃO GERAL

Diversos estudos evidenciam propriedades terapêuticas do PTS, tanto em abordagens pré-clínica quanto clínica (RICHE et al., 2014; WU et al., 2017). Tais efeitos estão relacionados a ações antioxidante, anti-inflamatória, anticarcinogênica, anti-hipertensiva, antidiabética dentre outras (BHAKKIYALAKSHMI et al., 2016; GUO et al., 2016; PARI; SATHEESH, 2006; YANG et al., 2013; Wang et al., 2016). Estes estudos, em sua maioria, utilizaram o PTS em suspensão, devido à lipofilia inerente ao composto, característica química que pode limitar a sua absorção nos fluidos gastrintestinais bem como sua biodisponibilidade oral (BETHUNE et al., 2011; HURST et al., 2007, JAMBHEKAR; BREEN, 2013). As substâncias administradas em solução aquosa são mais rapidamente absorvidas do que aquelas em suspensão, porque se misturam de forma mais efetiva com a fase aquosa do local absortivo, podendo exercer substancial efeito sobre a eficácia e a toxicidade de um composto (KHADKA et al., 2014). Então, a fim de aumentar a hidrossolubilidade e a estabilidade do PTS, além obter um perfil farmacocinético mais favorável para determinar a dose de PTS efetiva, optamos em produzir uma formulação utilizando a HPβCD como veículo.

Neste estudo, o complexo PTS:HPβCD foi preparado por método em solução de acordo com Yeo et al., (2013). Mediante aos ensaios de caracterização do complexo obtido, verificamos a presença de interações entre as moléculas de PTS e HPβCD, sugerindo que a metodologia de complexação foi efetiva. Adicionalmente, mostramos pela primeira vez que a complexação com PTS pode ser confirmada usando a técnica de ressonância magnética nuclear (homonuclear 2D-ROESY ^{1}H), por meio da interação entre o hidrogênio H_a presente no anel aromático (anel A) do PTS com o hidrogênio (H_5) presente no interior da cavidade da HPβCD. Foi possível verificar que a complexação com HPβCD aumentou a solubilidade do PTS cerca de 7000 vezes quando comparado ao PTS não complexado (em suspensão) (BETHUNE et al., 2011). Há relatos de que o aumento da hidrossolubilidade por complexação com ciclodextrina quadriplica a biodisponibilidade oral do PTS, aumentando sua concentração sérica e exposição tecidual, efeitos decorrentes da melhoria da absorção e saturação de mecanismos de eliminação hepática (CHOO et al., 2014; YEO et al., 2013). Embora a farmacocinética não tenha sido foco deste estudo, é importante conhecer o comportamento cinético do PTS no organismo para identificar a dose terapêutica, assim como risco de toxicidade.

Verificamos que a complexação não altera a capacidade antioxidante do PTS in *vitro*, e demonstra potencial antioxidante nos modelos *in vivo* aqui utilizados. Nossos resultados deixam claro que a dose mais alta testada (100 mg/kg) exerceu a maioria dos efeitos terapêuticos no fígado de ratos hígidos e no coração de ratos com insuficiência cardíaca (IC) e, que esta mesma dose parece não induzir efeitos tóxicos. Estas conclusões podem ser suportadas pela capacidade em aumentar antioxidantes não enzimáticos, reduzir a concentração de EROs totais e lipoperoxidação no fígado dos ratos. Corroborando nossos resultados, outros autores também destacam o efeito antioxidante do PTS no fígado de ratos (EL-SAYED et al., 2015; ACHARYA; GHASKADBI, 2013; PARI; SATHEESH, 2006).

Poucos estudos avaliam os efeitos terapêuticos do PTS complexado a ciclodextrinas no coração, o que nos motivou a explorar diferentes vias sensíveis ao estado redox envolvidas com remodelamento mal adaptativo e progressão para IC, tanto no coração direito quanto no esquerdo. Neste contexto, utilizamos os modelos de remodelamento ventricular: na IC direita, causada por sobrecarga de pressão/volume (secundário a HAP); e ventricular na IC esquerda, devido à perda de tecido contrátil, causada por IAM.

O complexo PTS:HPβCD também reduziu a hipertrofia do VD e a dilatação VE, demonstrando efeitos benéficos deste tratamento no remodelamento ventricular. Evidencias mostram que o PTS inibe a proliferação e crescimento de células musculares, eventos que estão relacionados ao desenvolvimento da hipertrofia (MCCORMACK; MCFADDEN, 2013; PARK et al., 2010). Neste estudo, verificamos que o PTS preservou a função sistólica evidenciado mediante avaliação ecocardiográfica. Houve aumento da variação de área percentual entre a diástole e a sístole final pela administração do PTS, tanto no ventrículo direito quanto no esquerdo, sendo esta variação descrita pela FAC, indicando melhora na função sistólica (LANG et al., 2015). Similarmente, o estilbeno resveratrol previne a hipertrofia e a disfunção sistólica esquerda em ratos hipertensos, efeitos atribuídos a redução de estresse oxidativo (THANDAPILLY et al., 2010). Por outro lado, as respostas específicas oriundas do VD ou VE em nosso protocolo experimental, podem ser decorrentes em parte, de características inerentes a cada ventrículo, embora, em nível celular, as respostas moleculares de remodelação (matriz extracelular e do citoesqueleto) de ambos ventrículos sejam em grande parte similares (REDDY; BERNSTEIN, 2015). Por exemplo, o VD e o VE diferem em sua carga de trabalho e, portanto, em suas necessidades energéticas e metabolismo mitocondrial. Com

base apenas na pós-carga ventricular, a carga de trabalho do VE é 5 vezes maior que o VD, devido à maior resistência vascular sistêmica comparada com o leito vascular pulmonar de baixa resistência (FRIEDBERG; REDINGTON, 2014). No estado patológico, embora haja aumento na produção de ROS em ambos ventrículos, o VD é mais suscetível ao estresse oxidativo do que o VE. Isto ocorre porque no VD as defesas antioxidantes fracassam mais precocemente, enquanto no VE permanecem intactas até um estágio mais avançado de falência (TSUTSUI et al., 2001; REDDY; BERNSTEIN, 2015). De forma conjunta, essas diferenças poderiam explicar a especificidade das respostas encontradas em cada um dos ventrículos sob condições de remodelamento patológico.

De fato, ao compararmos o impacto do complexo PTS:HPβCD nos desenhos experimentais aqui utilizados, encontramos alguns efeitos que foram comuns a ambos modelos. Dentre os efeitos comuns, mostramos que o complexo PTS:HPβCD reduziu a lipoperoxidação tanto no fígado quanto nos ventrículos direito e esquerdo. A oxidação de lipídeos ocorre pela perda de elétrons dos ácidos graxos polinsaturados da membrana celular para os radicais livres, formando radical peroxil ($ROO^{\bullet}$) que é posteriormente convertido em malondialdeído, por meio de uma série de reações em cadeia, alterando a arquitetura da membrana celular e mitocondrial (FRANKEL, 1984). Adicionalmente, os produtos de oxidação lipídica formam complexos que inativam proteínas, desencadeiam apoptose induzida pela via mitocondrial intrínseca, assim como medeiam a sinalização inflamatória (MCINTYRE; HAZEN, 2010). É importante notar que a lipoperoxidação contribui para o remodelamento cardíaco e acarreta disfunção ventricular (DUARTE et al., 2009). Similarmente ao nosso achado, recentes pesquisas fornecem evidências da habilidade do PTS para proteger células cardíacas e mitocôndrias hepáticas isoladas contra lipoperoxidação (ACHARYA; GHASKADBI, 2013; LIU et al., 2017; YU et al., 2017). Semelhante ao α-tocoferol, a capacidade antioxidante do PTS frente à lipoperoxidação pode ser facilitada pela sua característica lipossolúvel, que permite facilmente sua difusão pela membrana plasmática celular, local onde as reações de oxidação de lipídeos ocorrem com notável frequência (TAPPEL, 1962).

De forma agregada, outro importante contribuinte para o potencial terapêutico do complexo PTS:HPβCD sobre o remodelamento ventricular, comum tanto ao VD quanto ao VE, está centrado na modulação de enzimas tiois antioxidantes, como GST e GRx. Nossos resultados indicam que o complexo PTS:HPβCD estimulou a atividade da GST, uma transferase que catalisa a conjugação de GSH para uma variedade de compostos

eletrofílicos e substratos tóxicos, a qual promove desintoxicação celular e contribui de forma significativa para a manutenção do equilíbrio redox e sobrevivência celular (RÖTH et al., 2011; WU et al., 2004). Similar ao nosso resultado Ghazali e cols. (2013) e Satheesh, Pari (2006) também observaram aumento na atividade da GST após tratamento com PTS tanto em culturas de células hepáticas, quanto em tecidos renal e hepático, respectivamente. É importante ressaltar que a inibição da GST em cardiomiócitos submetidos à injúria oxidativa, resulta em apoptose por meio da ativação das proteínas da via MAP cinase (RÖTH et al., 2011). Interessantemente, detectamos que o complexo PTS:HPβCD modulou a atividade da GRx nos ventrículos de forma oposta, ou seja, aumentou a atividade da GRx no VD que foi reduzida pela *Cor pulmonale*, enquanto que no VE sua atividade foi reduzida. Esta resposta paradoxal pode estar relacionada à concentração de agentes estressores (EROs), ao estresse submetido e à ativação de sistema antioxidante específico nos diferentes ventrículos (LUSHCHAK, 2014). De fato, a HAP induzida pela MCT é considerada a forma mais agressiva da doença em modelos animais, tendo o estresse oxidativo como um importante mecanismo patológico (DUMITRASCU et al., 2008; SINGAL et al., 2004). De forma agregada, VD é mais sensível ao estresse oxidativo do que o VE, devido à falha precoce do seu sistema antioxidante (TSUTSUI et al., 2001; REDDY; BERNSTEIN, 2015). Dessa forma, o efeito estimulatório do PTS sobre a atividade da GRx, parece ser um importante mecanismo para minimizar os danos do estresse oxidativo neste tecido. Por outro lado, a administração do complexo PTS:HPβCD no VE intensificou a atividade enzimática de outros tiois, (como TRx e GST), a fim de recompor a homeostase do cardiomiócito e, restabeleceu em níveis basais a atividade da GRx. Então, supomos que devido a ação antioxidante do PTS, não foi necessária a ação do sistema da GRx, sendo a sua atividade poupada no VE.

Ao observarmos em conjunto os resultados obtidos no VD, concluímos que complexo PTS:HPβCD apresenta habilidade antioxidante multialvo, dose dependente, evidenciadas pela capacidade de manter o ambiente intracelular mais reduzido, pela redução da lipoperoxidação e do estresse oxidativo. Estes efeitos podem ser decorrentes da baixa produção de ânion superóxido dependente de NADPH oxidase, associada a ativação do sistema da glutationa pelo aumento de disponibilidade de GSH e modulação da sua reciclagem. Concomitantemente, o complexo PTS:HPβCD estimulou o sistema antioxidante enzimático, através das enzimas SOD, GST e GRx. A inibição de NADPH oxidase pela administração do PTS é benéfica porque reduz a concentração de ânion

superóxido, uma espécie altamente produzida durante a hipertrofia do VD, que poderá originar outras EROs e contribuir para o dano tecidual e disfunção ventricular (KURODA; SADOSHIMA, 2010). O aumento intracelular de ânion superóxido origina altas concentrações de peróxido de hidrogênio, uma espécie com capacidade de inativar a SOD (HALLIWELL; GUTTERIDGE, 2007). A melhora na atividade da SOD pode ser decorrente da capacidade redutora do PTS que transfere elétrons da sua hidroxila fenólica aos radicais livres (PERRON; BRUMAGHIM, 2009). Além disso, a disponibilidade de GSH elimina EROs e protege proteínas contra a oxidação e degradação proteasomal (forma dissulfetos com o grupamento tiol das proteínas) e ainda contribui para a eficiência antioxidante das enzimas GPx, GST e GRx (HANSCHMANN et al., 2013; LUSHCHAK, 2012; NAGARAJAN et al., 2017). É importante ressaltar que a integridade deste sistema é fundamental para a sobrevivência de células cardíacas e, a ineficiência destas enzimas resulta em apoptose de cardiomiócitos (RÖTH et al., 2011; GALLOGLY et al., 2010). Assim, a modulação destes tiois pelo ressalta o potencial terapêutico deste fitofenol sobre o remodelamento ventricular direito.Adicionalmente, houve aumento da sinalização para o relaxamento do VD, favorecendo a contratilidade. Ambos mecanismos parecem prevenir a hipertrofia da parede livre do VD, resultando em proteção da função sistólica, melhora do volume sistólico e débito cardíaco.

Ao na alisarmos os efeitos farmacológicos do complexo PTS:HPβCD sobre o VE pós IAM, verificamos que o sistema da glutationa não foi alterado, fato que nos surpreendeu por se tratar do antioxidante intracelular mais abundante, diferentemente do que foi observado no VD. Por outro lado, no VE o complexo PTS:HPβCD reduziu a lipoperoxidação, aumentou a atividade da GST assim como preservou a atividade da TRxR. Estas modulações preveniram a dilatação da câmara esquerda restabelecendo a função sistólica. Corroborando nosso resultado, outros autores também evidenciaram que o PTS exerce efeito positivo sobre atividade da GST, reduz a lipoperoxidação e o estresse oxidativo (PARI; SATHEESH, 2006; LIU et al., 2017). Embora, outros estudos também reportem que a administração do PTS produz efeitos cardioprotetores em miocárdio isquêmico (YU et al., 2017; LIU et al., 2017), este estudo é pioneiro em descrever que o complexo PTS:HPβCD aumenta a expressão de Nrf2 e da GSK-3β fosforilada em cardiomiócitos pós IAM, mecanismo que parece contribuir para o efeito antioxidante e cardioprotetor deste fitoquímico. Neste contexto, o mecanismo citoprotetor do PTS também pode ser relacionada a regulação da resposta antioxidante, através da síntese de antioxidantes por ativação transcricional de Nrf2 (Bhakkiyalakshmi et al., 2014; Elango

et al., 2014). Adicionalmente, a inibição de GSK-3β tem sido reportada como uma promissora terapia na recuperação da lesão miocárdica por isquemia/reperfusão, já que esta intervenção reduz a área de infarto e promove cardioproteção (MIURA et al., 2009). Adicionalmente, Yadav e cols. (2010) sugerem que a administração de inibidores de GSK-3β potencializa os efeitos cardioprotetores do pré-condicionamento isquêmico em ratos diabéticos. Além disso, há relatos de que GSK-3β fosforilada, além de inibir a sinalização apoptótica, pode inativar a degradação de Nrf2, favorecendo seu direcionamento para o núcleo celular, regulando positivamente a resposta antioxidante (ROJO et al., 2008).

Na figura 4 pode-se observar um resumo esquemático do potencial farmacológico do complexo PTS:HPβCD em diferentes alvos.

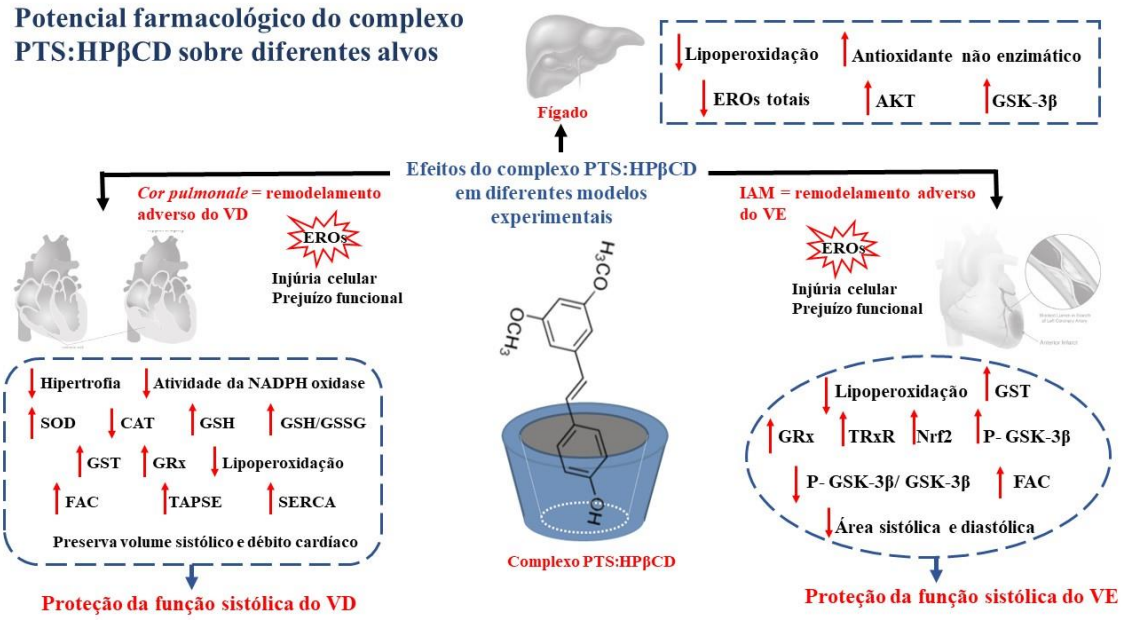

Figura 4: Potencial farmacológico do complexo PTS:HPβCD em diferentes alvos. AKT: serina/treonina quinase; CAT: catalase; EROs: espécies reativas de oxigênio; FAC: variação de área fracional; GSK-3β: glicogênio sintase quinase 3β; GRx: glutaredoxina; GSH: reduzida da glutationa; GSSG: glutationa oxidada; GST: glutationa-S-transferase; HPβCD: hidroxipropil-β-ciclodextrina; IAM: infarto agudo do miocárdio; Nrf2: nuclear factorerythroid 2-related factor 2; SERCA: proteína cálcio ATPase; SOD: superóxido dismutase; TAPSE: excursão sistólica do plano do anel da tricúspide; TRxR: tiorredoxina redutase; VD: ventrículo direito; VE: ventrículo esquerdo. ↑: aumento; ↓: redução.

6. CONSIDERAÇÕES FINAIS

Na presente tese nosso foco inicial foi explorar os efeitos do complexo PTS:HPβCD sobre o remodelamento do VD, desta forma apresentamos mais resultados abordando diferentes mecanismos fisiopatológicos em modelo de *Cor pulmonale* comparado ao modelo de IAM. Uma justificativa plausível seria o fato do mecanismo de remodelamento e IC direita ser pouco estudada, apresentar-se como uma doença muito debilitante, com mau prognóstico e opções terapêuticas limitadas. Assim como no VD, os efeitos do PTS sobre o VE pós IAM são muito promissores, sendo novas investigações necessárias para identificar outros alvos farmacológicos deste fitoquímico durante a progressão e remodelamento pós IAM.

De forma geral, o conjunto de resultados da presente Tese destaca o papel antioxidante do complexo PTS:HPβCD, especialmente na dose de 100 mg/kg, e sua modulação sobre a expressão proteínas de sinalização redox sensíveis tanto no no fígado quanto em ambos os ventrículos sob condições de remodelamento patológico. Adicionalmente, estes achados reforçam outros dados da literatura que apontam a participação de mecanismos de estresse na fisiopatologia desta doença. As ações cardioprotetoras do complexo PTS:HPβCD parecem prevenir o remodelamento ventricular e melhoram a função sistólica direita e esquerda, efeitos que provavelmente foram favorecidos pelo aumento da biodisponibilidade oral promovido pela presença da HPβCD no complexo. Diante disso, o complexo PTS:HPβCD poderia ser usado como um tratamento complementar a terapia farmacológica disponível a fim de minimizar os efeitos deletérios do desequilíbrio oxidativo sobre a homeostase e função cardíaca.

7. PERSPECTIVAS

Embora os resultados apontem para o potencial antioxidantes do PTS, outros alvos farmacológicos carecem ser explorados, tais como:

- ✓ Determinar o efeito do tratamento com do complexo PTS:HPβCD sobre parâmetros inflamatórios, como interleucina 10 (IL-10) e interleucina 6 (IL-6), fator de necrose tumoral alfa (TNF-α), proteína de choque térmico Hsp 70 e fator nuclear kappa B (NF-κB) sobre o remodelamento em *Cor pulmonale* e pós IAM;

- ✓ Explorar o efeito do tratamento com o complexo PTS:HPβCD sobre a sinalização apoptótica, através da expressão das proteínas Bcl2, Bax e caspase em ambos os modelos estudos na presente Tese;

- ✓ Investigar se a administração do complexo PTS:HPβCD terá algum efeito sobre a autofagia tanto no VD quanto no VE, através da expressão das proteínas fosfoinusitol-3-cinase (PI3K), beclina 1, proteína alvo da rapamicina em mamíferos (mTOR);

- ✓ Avaliar a biodisponibilidade oral e tecidual (Fígado, VD e VE) do complexo PTS:HPβCD comparado ao PTS não complexado.

- ✓ Avaliar o efeito do complexo PTS:HPβCD quando co-administrado com fármacos já utilizados na clínica que constem nas diretrizes de tratamento da insuficiência cardíaca, como por exemplo, a associação com inibidor da fosfodiesterase 5 (o sildenafila) durante *a Cor pulmonale* experimental*;* e em modelo de IAM a administração concomitante com um betabloqueador (como metropolol) ou com um bloqueador do receptor de angiotensina (como losartana).

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9. ANEXO

Aprovação da Comissão de Ética no Uso de Animais – CEUA - UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 28218
Título: AVALIACAO DAS PROPRIEDADES ANTIOXIDANTES E ANTI-INFLAMATORIAS DO PTEROSTILBENO NO MODELO EXPERIMENTAL DE INSUFICIENCIA CARDIACA DIREITA

Pesquisadores:

Equipe UFRGS:

ALEX SANDER DA ROSA ARAUJO - coordenador desde 01/02/2015
Denise dos Santos Lacerda - Aluno de Doutorado desde 01/02/2015

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 23/03/2015 - Sala 330 - Prédio do Anexo I da Reitoria - Campus Centro - Porto Alegre - RS, em seus aspectos éticos e metolodológicos, para a utilização de 172 ratos Wistar, machos, pesando 250 g, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quarta-Feira, 1 de Abril de 2015

Cristiane Matte

CRISTIANE MATTE
Vice Coordenador da comissão de ética