Original Paper

N-Acetylcysteine Influence on Oxidative Stress and Cardiac Remodeling in Rats During Transition from Compensated Left Ventricular Hypertrophy to Heart Failure

David R.A. Reyes^a Mariana J. Gomes^a Camila M. Rosa^a Luana U. Pagan^a
Felipe C. Damatto^a Ricardo L. Damatto^a Igor Depra^a Dijon H.S. Campos^a
Ana A.H. Fernandez^b Paula F. Martinez^c Katashi Okoshi^a Marina P. Okoshi^a

^aDepartment of Internal Medicine, Botucatu Medical School, Sao Paulo State University, UNESP, Botucatu, SP, ^bBotucatu Institute of Biosciences, Sao Paulo State University, UNESP, Botucatu, SP,

^cFederal University of Mato Grosso do Sul, Campo Grande, MS, Brasil

Key Words

Aortic stenosis • Cardiac failure • MAPK • Echocardiogram • Ventricular remodeling • Tissue Doppler imaging • Rat

Abstract

Background/Aims: To evaluate the effects of the antioxidant *N*-acetylcysteine (NAC) on cardiac structure and function in rats with long-term ascending aortic stenosis (AS). **Methods:** Four months after inducing AS, Wistar rats were assigned into the groups Sham, AS, and AS treated with NAC (AS-NAC) and followed for eight weeks. Cardiac structure and function were evaluated by echocardiogram. Myocardial antioxidant enzymes activity was measured by spectrophotometry and malondialdehyde serum concentration by HPLC. Gene expression of NADPH oxidase subunits NOX2, NOX4, p22 phox, and p47 phox was assessed by real time RT-PCR and protein expression of MAPK proteins by Western blot. Statistical analyzes were performed with Goodman and ANOVA or Mann-Whitney. **Results:** NAC restored myocardial total glutathione (Sham 20.8±3.00; AS 12.6±2.92; AS-NAC 17.6±2.45 nmol/g tissue; $p < 0.05$ AS vs Sham and AS-NAC). Malondialdehyde serum concentration was lower in AS-NAC and myocardial lipid hydroperoxide was higher in AS (Sham 199±48.1; AS 301±36.0; AS-NAC 181±41.3 nmol/g tissue). Glutathione peroxidase activity was lower in AS than Sham. Echocardiogram showed LV concentric hypertrophy with systolic and diastolic dysfunction before and after treatment; no differences were observed between AS-NAC and AS groups. NAC reduced p-ERK and p-JNK protein expression, attenuated myocardial fibrosis, and decreased the frequency of right ventricular hypertrophy. **Conclusion:** *N*-acetylcysteine restores myocardial total glutathione, reduces systemic and myocardial oxidative stress, improves MAPK signaling, and attenuates myocardial fibrosis in aortic stenosis rats.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Marina P. Okoshi

Address: Faculdade de Medicina de Botucatu, Departamento de Clinica Medica,
Rubiao Junior, S/N CEP 18.618-687 Botucatu, SP (Brazil)
E-Mail mpoliti@fmb.unesp.br

Introduction

The transition from compensated left ventricular (LV) hypertrophy to heart failure is a critical event in patients with long-term pressure overload conditions such as systemic arterial hypertension and aortic stenosis [1]. Although several mechanisms may be involved in the development of heart failure, its pathophysiology is not completely understood [2-4].

Oxidative stress is often observed and considered to play an important role on pathological cardiac remodeling and the transition to cardiac failure [5, 6]. Despite the importance of oxidative stress in inducing myocardial damage, antioxidant therapy is still a matter of controversy in heart failure treatment [7, 8]. Glutathione (L-γ glutamyl-cysteinyl-glycine) is an endogenous tripeptide that plays a central role in cellular defense against oxidative stress [9]. It is synthesized and maintained at high concentrations in cells [10]. In heart failure, glutathione redox status changes and its total concentration decreases in myocardium [11, 12]. *N*-acetylcysteine (NAC) is a molecule with antioxidant properties; it possesses a sulfhydryl group which acts as a source of cysteine to glutathione synthesis. NAC administration has been shown to restore total glutathione levels and reduce oxidative stress in infarcted rat hearts [11]. Furthermore, NAC attenuates cardiac and myocyte hypertrophy, interstitial fibrosis, LV dysfunction, and arrhythmogenic propensity in different experimental cardiac injury models [12-15]. However, the effects of NAC during the transition from compensated LV hypertrophy to clinical heart failure have not been established.

Ascending aortic stenosis in rats is a useful model to study chronic pressure overload-induced cardiac remodeling. In this model, three-four week-old rats are subjected to a clip being placed around the ascending aorta. After clip placement, aorta diameter is preserved; as rats grow, stenosis and LV hypertrophy progressively develop. In this model, ventricular dysfunction and clinical heart failure occur slowly, similarly to what is seen in human chronic pressure overload [16, 17]. In this study, we evaluated the effects of NAC administration on systemic and myocardial oxidative stress and cardiac remodeling in rats during transition from compensated LV hypertrophy to heart failure.

Materials and Methods

Experimental groups

Male Wistar rats weighing 90–100 g were purchased from the Central Animal House, Botucatu Medical School, Sao Paulo State University, UNESP, Brazil. All experiments and procedures were approved by the Animal Experimentation Ethics Committee of Botucatu Medical School, which follows the guidelines established by the Brazilian College for Animal Experimentation (protocol number 1071/2014).

Rats were anesthetized with a mixture of ketamine hydrochloride (50 mg/kg, i.m.) and xylazine hydrochloride (10 mg/kg, i.m.) and aortic stenosis (AS) was induced by placing a 0.6 mm stainless-steel clip on the ascending aorta via a median thoracic incision according to a previously described method [16]. During surgery, the rats were manually ventilated using positive pressure and given 1 mL of warm saline solution intraperitoneally. Sham operated rats were used as controls. All animals were housed in a temperature controlled room at 23 °C and kept on a 12-hour light/dark cycle. Food and water were supplied *ad libitum*.

Four months after surgery, rats were subjected to transthoracic echocardiogram to evaluate the degree of cardiac injury, and assigned to three groups: control (Sham, n=16), AS (n=22), and AS treated with *N*-acetylcysteine (AS-NAC, n=15). *N*-acetylcysteine (Sigma, St. Louis, MO, USA) was added to chow at a dosage of 120 mg/kg/day for eight weeks. At the end of the experimental period, rats were subjected to transthoracic echocardiogram and euthanized the next day. During euthanasia, we determined the presence or absence of clinical and pathological heart failure features. The clinical finding suggestive of heart failure was tachypnea/labored respiration. Pathologic assessment of heart failure included pleuropericardial effusion, atrial thrombi, ascites, hepatic congestion, pulmonary congestion (lung weight/body weight ratio higher than 2 standard deviations above Sham group mean), and right ventricular hypertrophy (right ventricle weight/body weight ratio higher than 0.8 mg/g) [18, 19].

Echocardiography

Cardiac structures and left ventricular (LV) function were evaluated by transthoracic echocardiogram and tissue Doppler imaging (TDI) using a commercially available echocardiograph (General Electric Medical Systems, Vivid S6 model, Tirat Carmel, Israel) equipped with a 5–11.5 MHz multifrequency transducer as previously described [20–22]. After anesthesia with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (1 mg/kg) intramuscularly, the rats were placed in left lateral decubitus. A two dimensional parasternal short-axis view of the LV was obtained at the level of the papillary muscles. M-mode tracings were obtained from short-axis views of the LV at or just below the tip of the mitral-valve leaflets and at the level of the aortic valve and left atrium. M-mode images of the LV were printed on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 200 mm/s. All structures were manually measured by the same observer (KO). Values obtained were the mean of at least five cardiac cycles on M-mode tracings. The following structural variables were measured: left atrium diameter (LA), LV diastolic and systolic diameters (LVDD and LVSD, respectively), LV diastolic (D) and systolic (S) posterior wall thickness (PWT) and septal wall thickness (SWT), and aortic diameter. Left ventricular mass (LVM) was calculated using the formula $[(LVDD + DPWT + DSWT)^3 - LVDD^3] \times 1.04$. LV relative wall thickness (RWT) was calculated by the formula $2 \times DPWT / LVDD$. LV function was assessed by the following parameters: endocardial fractional shortening (EFS), midwall fractional shortening (MFS), ejection fraction (EF), posterior wall shortening velocity (PWSV), early and late diastolic mitral inflow velocities (E and A waves), E/A ratio, E-wave deceleration time (EDT), and isovolumetric relaxation time (IVRT). A joint assessment of diastolic and systolic LV function was performed using the myocardial performance index (Tei index). The study was complemented with evaluation by TDI of systolic (S'), early diastolic (E'), and late diastolic (A') velocity of the mitral annulus (arithmetic average of the lateral and septal walls) and E/E' ratio [23].

Collection of left ventricle and other tissues

One day after final echocardiogram, the rats were weighed and anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and euthanized. After blood collection, hearts (not arrested in diastole) were removed by thoracotomy. Atria and ventricles were dissected, weighed separately, frozen in liquid nitrogen, and stored at -80°C . Lung weight was used to assess the degree of pulmonary congestion [24].

Morphologic study

Frozen LV samples were transferred to a cryostat and cooled to -20°C . Serial transverse 8 μm thick sections were stained with hematoxylin and eosin. At least 50 cardiomyocyte diameters were measured from each LV as the shortest distance between borders drawn across the nucleus [25]. Other slides were stained with Sirius red F3BA and used to quantify interstitial collagen fraction [26]. On average, 20 microscopic fields were analyzed with a 40X lens. Perivascular collagen was excluded from this analysis. Measurements were taking using a microscope (Leica DM LS; Nussloch, Germany) attached to a computerized imaging analysis system (Media Cybernetics, Silver Spring, MD, USA) [27].

Oxidative stress evaluation

Myocardial glutathione concentration

Reduced glutathione was determined using a kinetic method in media consisting of 100 mM phosphate buffer pH 7.4 containing 5 mM EDTA, 2 mM 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB), 0.2 mM NADPH₂ and 2 U of glutathione reductase according to a previously describe method [28]. Total glutathione was measured in the presence of 0.1 M Tris-HCl buffer, pH 8.0 with 0.5 mM EDTA, 0.6 mM DTNB and 0.1 U glutathione reductase [28].

Antioxidant enzymes activity and lipid hydroperoxide concentration

Left ventricular samples (~200 mg) were homogenized in 5 mL of cold 0.1 M phosphate buffer, pH 7.0. Tissue homogenates were prepared in a motor-driven Teflon glass Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 10,000 g, for 15 min at 4°C , and the supernatant was assayed for total protein, lipid hydroperoxide [29], and glutathione peroxidase (GSH-Px, E.C.1.11.1.9), catalase (E.C.1.11.1.6.), and superoxide dismutase (SOD, E.C.1.15.1.1.) activities by spectrophotometry [30]. Enzyme activities were analyzed at 25°C using a microplate reader (Quant-MQX 200) with KCjunior software for computer

system control (Bio-Tech Instruments, Winooski, Vermont, USA). Spectrophotometric determinations were performed in a Pharmacia Biotech spectrophotometer with temperature controlled cuvette chamber (UV/visible Ultrospec 5000 with Swift II applications software for computer system control, Cambridge, UK). All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Malondialdehyde serum concentration

Systemic lipid peroxidation was assessed by measuring malondialdehyde (MDA) serum concentration by high performance liquid chromatography (HPLC), as previously reported [31]. Briefly, 100 μ L of serum were treated with 700 μ L of 1% orthophosphoric acid and vortex-mixed for 10 s for protein precipitation. Then, 200 μ L of thiobarbituric acid (TBA) were added. The mixture was heated to 100 $^{\circ}$ C for 60 min and cooled to -20 $^{\circ}$ C for 10 min. Then, 200 μ L of this reaction were added to a solution containing 200 μ L of NaOH:methanol (1:12). The tubes were centrifuged for 3 min at 13,000 g; 200 μ L of the supernatant were taken for injection in the equipment. Analysis was performed on a Shimadzu HPLC using a C18 5 μ m Gemini Phenomenex column, and an Rf-535 fluorescence detector, which was set to Ex 525 nm and EM 551 nm. The mobile phase consisted of a 60:40 (v/v) mixture of 10 mmol potassium dihydrogen phosphate (pH 6.8): methanol. MDA was quantified by a calibration curve, which was constructed every day for analysis [31].

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Gene expression of NADPH oxidase subunits (NOX2, NOX4, p22 phox, and p47 phox) and reference genes cyclophilin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed by RT-PCR according to a previously described method [32]. Total RNA was extracted from LV myocardium with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and treated with DNase I (Invitrogen Life Technologies). One microgram of RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit, according to standard methods (Applied Biosystems, Foster City, CA, USA). Aliquots of cDNA were then submitted to real-time PCR reaction using a customized assay containing sense and antisense primers and Taqman (Applied Biosystems, Foster City, CA, USA) probes specific to each gene: NOX2 (Rn00576710 m1), NOX4 (Rn00585380 m1), p22 phox (Rn00577357 m1), and p47 phox (Rn00586945 m1). Amplification and analysis were performed using Step One Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression data were normalized to reference gene expressions: cyclophilin (Rn00690933 m1) and GAPDH (Rn01775763 g1). Reactions were performed in triplicate and expression levels calculated using the CT comparative method ($2^{-\Delta\Delta CT}$).

Western blotting

Protein levels were analyzed by Western blotting as previously described [33, 34] using specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA): total JNK1/2 (sc-137019), p-JNK (sc-6254), total p38-MAPK (sc-7972), p-p38-MAPK (sc-17852), total ERK 1 (sc-93), and p-ERK1/2 (sc-16982). Protein levels were normalized to GAPDH (6C5 sc-32233). Myocardial protein was extracted using RIPA buffer (containing proteases and phosphatases inhibitors); supernatant protein content was quantified by the Bradford method. Samples were separated on a polyacrylamide gel and then transferred to a nitrocellulose membrane. After blockade, membrane was incubated with the primary antibodies. The membrane was then washed with TBS and Tween 20 and incubated with secondary peroxidase-conjugated antibodies. Super Signal® West Pico Chemiluminescent Substrate (Pierce Protein Research Products, Rockford, USA) was used to detect bound antibodies. The membrane was then stripped (Restore Western Blot Stripping Buffer, Pierce Protein Research Products, Rockford, USA) to remove previous antibody. After blockade, the membrane was incubated with anti-GAPDH antibody.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation or median and percentiles. Comparisons between groups were performed by one-way ANOVA and Tukey test or Kruskal-Wallis and Dunn test. Frequency of heart failure features was assessed by the Goodman test. Significance level was set at 5%.

Results

At the end of the experimental period, Sham group had 16 animals, AS 22, and AS-NAC had 15 rats. Before treatment, both aortic stenosis groups presented dilated left atrium and concentric LV hypertrophy with diastolic dysfunction and mild systolic dysfunction (Tables 1 and 2). Except for a lower systolic posterior wall thickness, AS-NAC did not differ from AS. At the end of the experiment, AS-NAC and AS preserved the same pattern of LV concentric hypertrophy and dysfunction. No differences were observed between AS-NAC and AS (Tables 3 and 4).

Heart failure feature frequencies are presented in Table 5. One rat from Sham had ascites. AS-NAC had a lower frequency of right ventricular hypertrophy than AS. Anatomical variables are shown in Table 6. Final body weight did not differ between groups. Absolute and normalized LV, right ventricle, and atria weights were higher in AS and AS-NAC than Sham. Lung weight and lung weight-to-body weight ratio were higher in AS than Sham.

Total glutathione was lower in AS than Sham and AS-NAC and reduced glutathione was lower in both AS and AS-NAC (Fig. 1). Systemic and myocardial markers of oxidative stress were reduced in AS-NAC. Malondialdehyde serum concentration was lower in AS-NAC than Sham and AS. Lipid hydroperoxide myocardial concentration was lower in AS-NAC and Sham than AS (Fig. 2). Antioxidant enzyme activities are shown in Fig. 3. Superoxide dismutase was lower in AS and AS-NAC than Sham and glutathione peroxidase was lower in AS than Sham. Catalase activity did not differ between groups. NADPH oxidase subunit gene expression did not differ between groups (Table 7).

Myocyte diameter was higher in AS than Sham and did not differ between AS-NAC and both AS and Sham groups (Table 8). Interstitial collagen fraction was higher in AS than Sham and AS-NAC (Table 8). Myocardial MAPK proteins expression is shown in Table 9. Phosphorylated-ERK/total ERK ratio was higher in AS than Sham and AS-NAC and p-JNK/GAPDH ratio was lower in AS-NAC than AS.

Table 1. Echocardiographic structural data before treatment. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; BW: body weight; LVDD and LVSD: left ventricular (LV) diastolic and systolic diameters, respectively; DPWT and SPWT: LV diastolic and systolic posterior wall thickness, respectively; DSWT and SSWT: LV diastolic and systolic septal wall thickness, respectively; RWT: relative wall thickness; AO: aorta diameter; LA: left atrial diameter; LVM: LV mass; LVMI: LVM index. One-way ANOVA and Tukey or Kruskal-Wallis and Dunn test. Data are mean $\pm$ SD or median and percentiles; * $p < 0.05$ vs Sham; # $p < 0.05$ vs AS

	Sham (n=16)	AS (n=22)	AS-NAC (n=15)
BW (g)	472 $\pm$ 64.0	445 $\pm$ 33.2	488 $\pm$ 64.1
LVDD (mm)	8.03 $\pm$ 0.58	8.80 $\pm$ 0.74*	9.06 $\pm$ 0.84*
LVDD/BW (mm/kg)	17.2 $\pm$ 2.15	19.8 $\pm$ 1.68*	18.8 $\pm$ 2.36
LVSD (mm)	3.92 $\pm$ 0.57	4.10 $\pm$ 1.08	4.62 $\pm$ 1.12
DPWT (mm)	1.41 (1.37-1.44)	2.07 (1.90-2.22)*	2.07 (1.90-3.35)*
SPWT (mm)	2.91 $\pm$ 0.25	3.79 $\pm$ 0.53*	3.35 $\pm$ 0.34**
DSWT (mm)	1.45 (1.40-1.49)	2.14 (1.82-2.18)*	1.94 (1.57-2.07)*
SSWT (mm)	2.49 (2.31-2.61)	3.11 (2.85-3.37)*	3.01 (2.81-3.14)*
RWT	0.34 (0.33-0.38)	0.46 (0.41-0.52)*	0.45 (0.41-0.47)*
AO (mm)	3.98 $\pm$ 0.28	3.88 $\pm$ 0.24	3.92 $\pm$ 0.20
LA (mm)	5.29 (5.11-6.08)	7.87 (6.31-8.58)*	8.54 (7.30-8.94)*
LA/AO	1.39 (1.32-1.43)	1.99 (1.56-2.31)*	1.84 (1.50-2.29)*
LA/BW (mm/kg)	11.4 (10.7-12.7)	18.4 (13.6-19.4)*	17.7 (13.8-19.1)*
LVM (g)	0.77 (0.72-0.88)	1.51 (1.25-1.78)*	1.65 (1.05-2.01)*
LVMI (g/kg)	1.71 (1.56-1.80)	3.45 (2.82-4.01)*	3.46 (2.54-4.28)*

Table 2. Echocardiographic data of left ventricular function before treatment. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; HR: heart rate; EFS: endocardial fractional shortening; MFS: midwall fractional shortening; PWSV: posterior wall shortening velocity; Tei index: myocardial performance index; EF: ejection fraction; TDI S': tissue Doppler imaging (TDI) of systolic velocity of the mitral annulus; E/A: ratio between early (E)-to-late (A) diastolic mitral inflow; IVRT: isovolumetric relaxation time; IVRTn: IVRT normalized to heart rate; EDT: E wave deceleration time; TDI E' and A': TDI of early (E') and late (A') diastolic velocity of mitral annulus. One-way ANOVA and Tukey or Kruskal-Wallis and Dunn test. Data are mean $\pm$ SD or median and percentiles; * $p < 0.05$ vs Sham

	Sham (n=16)	AS (n=22)	AS-NAC (n=15)
HR (bpm)	261 $\pm$ 22.1	278 $\pm$ 37.6	295 $\pm$ 20.2*
EFS (%)	53.0 (47.7-56.3)	54.6 (45.3-60.4)	54.0 (44.0-58.6)
MFS (%)	31.0 $\pm$ 3.77	29.7 $\pm$ 6.43	30.2 $\pm$ 6.37
PWSV (mm/s)	39.3 (36.9-41.0)	30.3 (26.6-35.8)*	30.4 (25.9-36.0)*
Tei index	0.46 $\pm$ 0.07	0.45 $\pm$ 0.09	0.45 $\pm$ 0.08
EF	0.89 (0.84-0.91)	0.91 (0.86-0.94)	0.87 (0.80-0.91)
TDI S' (average, cm/s)	3.47 $\pm$ 0.43	2.83 $\pm$ 0.49*	3.17 $\pm$ 0.62
Mitral E (cm/s)	78.0 (70.3-81.5)	127 (93.5-149)*	140 (100-157)*
Mitral A (cm/s)	50.5 (46.3-55.5)	29.0 (18.5-57.0)	53.0 (25.0-64.0)
E/A	1.52 $\pm$ 0.19	4.92 $\pm$ 3.70*	3.58 $\pm$ 3.14
IVRT (ms)	26.0 (23.0-27.5)	18.0 (15.0-26.0)*	18.0 (15.0-22.0)*
IVRTn (ms)	53.1 (48.8-57.5)	41.5 (32.3-53.7)*	41.9 (33.0-47.1)*
EDT (ms)	47.5 (41.7-56.0)	30.0 (26.0-48.0)*	30.0 (27.5-36.0)*
TDI E' (average, cm/s)	4.16 $\pm$ 0.68	3.82 $\pm$ 0.93	4.09 $\pm$ 0.67
TDI A' (average, cm/s)	3.20 (2.86-3.56)	3.23 (2.73-3.81)	3.35 (3.15-4.45)
E'/TDI E' (average)	19.0 (16.5-22.2)	36.2 (27.6-39.7)*	25.4 (19.4-37.5)*

Discussion

In this study, the effects of NAC were evaluated in aortic stenosis rats during the transition from LV compensated hypertrophy to overt heart failure.

Aortic stenosis in young rats has been often used to evaluate persistent pressure overload from early LV concentric hypertrophy through gradual LV dysfunction and decompensated heart failure [16]. After aortic stenosis induction, rats remain compensated for approximately 20 to 28 weeks [35]. They then begin to present clinical and pathological heart failure features and evolve to death within two to four weeks. In this study, after a chronic period of LV hypertrophy, rats were randomly distributed into different treatment groups. Cardiac structures and LV function were evaluated by transthoracic echocardiogram before and after NAC treatment. The presence of cardiac failure was demonstrated by the high frequency of heart failure features observed at euthanize in the AS group. Lung congestion and right ventricular hypertrophy have been often used to diagnose heart failure in post-mortem rats [36, 37].

Before treatment, aortic stenosis rats had concentric LV hypertrophy characterized by an increase in LV mass, LV wall thicknesses, and relative wall thickness. Mild systolic dysfunction was characterized by reduced posterior wall shortening velocity, and diastolic dysfunction by increased E wave and E/TDI E' ratio, and reduced isovolumetric relaxation time (IVRT) and E wave deceleration time. At the end of the

Table 3. Echocardiographic structural data after treatment. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; BW: body weight; LVDD and LVSD: left ventricular (LV) diastolic and systolic diameters, respectively; DPWT and SPWT: LV diastolic and systolic posterior wall thickness, respectively; DSWT and SSWT: LV diastolic and systolic septal wall thickness, respectively; RWT: relative wall thickness; AO: aorta diameter; LA: left atrial diameter; LVM: LV mass; LVMI: LVM index. One-way ANOVA and Tukey or Kruskal-Wallis and Dunn test. Data are mean $\pm$ SD or median and percentiles; * $p < 0.05$ vs Sham

	Sham (n=16)	AS (n=22)	AS-NAC (n=15)
BW (g)	510 $\pm$ 50.7	482 $\pm$ 41.6	490 $\pm$ 69.9
LVDD (mm)	8.31 $\pm$ 0.57	9.02 $\pm$ 0.90*	9.07 $\pm$ 0.95*
LVDD/BW (mm/kg)	16.8 $\pm$ 2.20	18.9 $\pm$ 2.65	18.5 $\pm$ 3.24
LVSD (mm)	4.02 (3.58-4.58)	4.80 (3.66-5.29)	4.67 (3.82-5.29)
DPWT (mm)	1.42 (1.38-1.45)	2.06 (1.87-2.12)*	1.94 (1.57-2.07)*
SPWT (mm)	2.99 $\pm$ 0.31	3.47 $\pm$ 0.51*	3.48 $\pm$ 0.42*
DSWT (mm)	1.42 (1.38-1.46)	2.11 (1.87-2.16)*	1.94 (1.57-2.11)*
SSWT (mm)	2.53 (2.42-2.68)	2.90 (2.64-3.21)*	3.01 (2.68-3.26)*
RWT	0.34 (0.33-0.36)	0.43 (0.39-0.51)*	0.44 (0.36-0.47)*
AO (mm)	3.98 $\pm$ 0.20	4.00 $\pm$ 0.25	4.08 $\pm$ 0.23
LA (mm)	5.29 (4.93-5.69)	8.36 (7.55-8.71)*	7.48 (6.15-9.26)*
LA/AO	1.36 (1.26-1.44)	2.17 (1.74-2.29)*	1.84 (1.54-2.49)*
LA/BW (mm/kg)	10.5 (9.71-12.4)	17.3 (14.7-19.5)*	17.5 (13.7-19.0)*
LVM (g)	0.81 (0.76-0.88)	1.47 (1.34-1.94)*	1.13 (0.97-2.06)*
LVMI (g/kg)	1.65 (1.40-1.77)	3.01 (2.69-3.99)*	2.72 (2.06-4.01)*

Table 4. Echocardiographic data of left ventricular function after treatment. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; HR: heart rate; EFS: endocardial fractional shortening; MFS: midwall fractional shortening; PWSV: posterior wall shortening velocity; Tei index: myocardial performance index; EF: ejection fraction; TDI S': tissue Doppler imaging (TDI) of systolic velocity of the mitral annulus; E/A: ratio between early (E)-to-late (A) diastolic mitral inflow; IVRT: isovolumetric relaxation time; IVRTn: IVRT normalized to heart rate; EDT: E wave deceleration time; TDI E' and A': TDI of early (E') and late (A') diastolic velocity of mitral annulus. One-way ANOVA and Tukey or Kruskal-Wallis and Dunn test. Data are mean $\pm$ SD or median and percentiles; * $p < 0.05$ vs Sham

	Sham (n=16)	AS (n=22)	AS-NAC (n=15)
HR (bpm)	295 $\pm$ 44.4	292 $\pm$ 40.1	294 $\pm$ 19.2
EFS (%)	51.6 (46.4-55.2)	49.0 (41.8-55.3)	53.2 (44.0-61.8)
MFS (%)	28.7 (26.5-32.4)	30.2 (21.2-35.6)	30.4 (24.6-36.5)
PWSV (mm/s)	39.3 (36.8-41.0)	30.3 (26.6-35.8)*	30.2 (24.5-40.4)*
Tei index	0.39 (0.37-0.45)	0.41 (0.38-0.50)	0.45 (0.42-0.52)
EF	0.89 (0.85-0.91)	0.91 (0.86-0.94)	0.85 (0.82-0.92)
TDI S' (average, cm/s)	4.03 $\pm$ 0.64	2.89 $\pm$ 0.64*	3.15 $\pm$ 0.60
Mitral E (cm/s)	77.5 (70.5-86.5)	141 (89.7-161)*	122 (85.0-142)*
Mitral A (cm/s)	59.5 (50.0-69.3)	27.0 (22.0-53.5)*	36.0 (22.0-57.0)*
E/A	1.35 (1.15-1.58)	5.38 (1.73-7.37)*	3.88 (1.70-8.47)*
IVRT (ms)	26.0 (23.0-27.5)	20.0 (18.0-23.0)*	22.0 (18.0-26.0)*
IVRTn	55.2 (49.0-60.4)	39.2 (33.6-45.9)*	40.7 (32.4-47.1)*
EDT (ms)	44.0 (41.0-51.0)	30.0 (24.0-40.0)*	33.0 (30.0-41.0)*
TDI E' (average, cm/s)	4.70 (4.26-5.21)	3.80 (3.30-4.90)*	4.00 (3.74-4.53)
TDI A' (average, cm/s)	4.38 (3.76-6.04)	3.55 (2.90-4.75)	3.05 (2.40-3.45)*
E/TDI E' (average)	17.5 (13.6-18.8)	35.0 (27.4-41.9)*	30.2 (18.9-37.9)*

experiment, both AS and AS-NAC preserved the same pattern of cardiac remodeling. No differences between AS-NAC and AS groups were observed. Therefore, despite of the fact that AS-NAC had a lower frequency of right ventricular hypertrophy than AS, we failed to show beneficial effects of NAC on cardiac structure and LV function.

NAC is a precursor to an important intracellular defense mechanism against oxidative damage. By providing cysteine to glutathione synthesis, NAC replenishes glutathione, which is usually depleted in several disease states [12]. The replenishment of glutathione in deficient cells plays a central role in the effects of NAC as an antioxidant agent [10]. In fact, NAC is probably ineffective in cells replete in glutathione [10]. This property is important for antioxidant agents because at appropriate concentrations, reactive oxygen species also have fundamental roles in cellular function [8, 38].

In this study, we first analyzed myocardial glutathione status. Total glutathione was decreased in AS and normalized by NAC. However, reduced glutathione was decreased in AS and AS-NAC groups. Depleted cardiac total glutathione has been previously observed in both congestive heart failure patients and post-myocardial infarction rats, as has total glutathione repletion after NAC treatment in infarcted rats [11]. We next evaluated systemic and myocardial markers of oxidative stress and myocardial antioxidant enzyme activity. Myocardial oxidative stress, analyzed by lipid hydroperoxide concentration, was higher in AS and normalized by NAC. Interestingly, malondialdehyde serum concentration was decreased in AS-NAC compared to both Sham and AS groups. As total glutathione was replenished in AS-NAC, glutathione peroxidase activity was normalized and its ability to neutralize free radicals increased, resulting in reduced lipoperoxidation. Therefore, NAC administration restored myocardial total glutathione and glutathione peroxidase activity, and reduced myocardial and systemic oxidative stress markers.

The NADPH oxidase family, composed of enzymes whose main function is producing reactive oxygen species, is involved in the pathophysiology of several cardiovascular diseases [5, 6, 39]. To evaluate the potential role of NADPH oxidase on increased oxidative stress, we assessed gene expression of NOX2 and NOX4 - the main cardiac isoforms, p22 phox - the transmembrane protein, and p47 phox - the NOX2 cytosolic regulatory subunit, all which did not differ between groups. However, as we did not evaluate NADPH oxidase activity, we cannot discard the possible influence of NADPH oxidase on increased oxidative stress in the AS group. We have previously observed that NAC administration decreases NADPH oxidase activity and NOX4 and p22 phox gene expression in the soleus muscle of heart failure rats [40].

Table 5. Frequency of heart failure features (%). AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; n: number of animals. Goodman test; * p < 0.05 vs AS

	AS (n=22)	AS-NAC (n=15)
Ascites	59.1	46.7
Pleural effusion	27.3	13.3
Tachypnea	27.3	6.6
Atrial thrombi	36.4	26.6
Liver congestion	18.2	13.3
Lung congestion	59.1	40.0
Right ventricular hypertrophy	77.3	33.3 *

Table 6. Anatomical data. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; BW: body weight; LVW: left ventricle weight; RVW: right ventricle weight. One-way ANOVA and Tukey or Kruskal-Wallis and Dunn test. Data are mean ± SD or median and percentiles; * p < 0.05 versus Sham

	Sham (n=16)	AS (n=22)	AS-NAC (n=15)
BW (g)	510 ± 50.7	482 ± 41.6	490 ± 69.9
LVW (g)	0.94 (0.88-0.98)	1.84 (1.56-2.09)*	1.41 (1.09-1.56)*
LVW/BW (mg/g)	1.86 (1.69-2.05)	3.80 (2.98-4.33)*	2.82 (2.33-3.67)*
RVW (g)	0.24 (0.21-0.25)	0.46 (0.40-0.55)*	0.38 (0.25-0.49)*
RVW/BW (mg/g)	0.48 (0.45-0.50)	0.99 (0.83-1.11)*	0.76 (0.49-1.01)*
Atria weight (g)	0.10 (0.09-0.11)	0.36 (0.21-0.41)*	0.29 (0.17-0.40)*
Atria/BW (mg/g)	0.20 (0.19-0.23)	0.75 (0.66-0.82)*	0.61 (0.35-0.88)*
Lung weight (g)	1.80 (1.66-1.99)	2.89 (2.11-3.29)*	2.11 (1.57-3.12)
Lung/BW (mg/g)	3.80 (3.12-4.11)	6.30 (4.29-7.05)*	3.94 (3.34-6.12)

Fig. 1. Myocardial concentration of total (A) and reduced glutathione (B). AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; n: number of animals. Data are mean $\pm$ SD; ANOVA and Tukey; * $p < 0.05$ vs Sham; # $p < 0.05$ vs AS.

Fig. 2. Malondialdehyde serum concentration (A) and lipid hydroperoxide myocardial concentration (B). AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; n: number of animals. Data are mean $\pm$ SD; ANOVA and Tukey; * $p < 0.05$ vs Sham; # $p < 0.05$ vs AS.

Experimental studies have shown that the mitogen-activated protein kinase (MAPK) signaling pathway is involved in myocardial response to oxidative stress in several conditions [41-43]. MAPK includes four subfamilies, three of which have been well characterized: extracellular regulated kinase (ERK 1/2), c-jun N-terminal kinase (JNK), and p38-MAPK. After oxidative stress increase, MAPK downstream signaling may lead to myocardial hypertrophy and fibrosis [6, 41, 42, 44]. We therefore evaluated MAPK protein expression and observed that p-ERK and p-JNK were lower in AS-NAC than AS. Probably as a consequence of the reduced oxidative stress and p-ERK and p-JNK signaling, myocardial fibrosis was attenuated by NAC treatment. As myocardial fibrosis is strongly related to poor outcome in cardiovascular diseases [2, 45], this result is important for future clinical studies.

Table 7. Myocardial gene expression of NADPH oxidase subunits. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine. One-way ANOVA and Tukey test. Data are mean $\pm$ SD

	Sham (n=8)	AS (n=6)	AS-NAC (n=8)
p22 phox	1.00 $\pm$ 0.51	1.26 $\pm$ 0.98	0.92 $\pm$ 0.14
p47 phox	1.04 $\pm$ 0.50	0.89 $\pm$ 0.61	0.83 $\pm$ 0.71
NOX 2	0.74 $\pm$ 0.55	1.64 $\pm$ 0.29	0.66 $\pm$ 0.43
NOX 4	1.00 $\pm$ 0.85	3.47 $\pm$ 0.19	1.16 $\pm$ 0.66

Table 8. Myocardial morphometric parameters. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; ICF: interstitial collagen fraction. One-way ANOVA and Tukey test. Data are mean $\pm$ SD; * $p < 0.05$ vs Sham; # $p < 0.05$ vs AS

	Sham (n=10)	AS (n=12)	AS-NAC (n=8)
Myocyte diameter (μ m)	13.4 $\pm$ 1.17	15.3 $\pm$ 0.97*	14.6 $\pm$ 1.19
ICF (%)	4.30 $\pm$ 1.20	9.86 $\pm$ 1.69*	4.77 $\pm$ 1.66#

More recently, NAC has been evaluated in experimental models of cardiac injury, particularly hypertrophic cardiomyopathy in which NAC reduced oxidative stress, reversed established cardiac hypertrophy and fibrosis, prevented cardiac systolic and diastolic dysfunction, and improved arrhythmogenic propensity [12, 14, 46]. Similarly, to our findings, NAC also reduced ERK and JNK phosphorylation [14, 46]. The antifibrotic effect of NAC was also observed in other models and organs [46-48]. Despite the well characterized actions of NAC on hypertrophic cardiomyopathy, its effects on aortic stenosis-induced cardiac remodeling have been poorly addressed. When administered before and after aortic stenosis in rats, NAC attenuated increase in LV weight and electrical remodeling [15]. On the other hand, NAC did not improve cardiac function in aortic stenosis mice [49].

We have therefore shown for the first time that the antioxidant NAC restores myocardial total glutathione and reduces systemic and myocardial oxidative stress in aortic stenosis rats. Probably, as a consequence of this decreased oxidative stress, myocardial MAPK signaling was improved and interstitial fibrosis was attenuated. However, cardiac echocardiographic parameters were not changed and heart failure features were only marginally improved. NAC administration was probably initiated too late when rats had already presented advanced degrees of structural cardiac alterations, thus preventing a reverse remodeling process. Our results therefore allow us to propose the hypothesis that early administration of NAC may be a useful treatment for preventing pathological cardiac remodeling and LV dysfunction during persistent LV pressure overload. A limitation of this study is that as we have not evaluated aorta blood pressure, differences in blood pressure levels derived from aortic stenosis may have influenced cardiac variables.

Table 9. Myocardial expression of MAPK proteins. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine. One-way ANOVA and Tukey or Kruskal-Wallis and Dunn test. Data are mean $\pm$ SD or median and percentiles; * $p < 0.05$ vs Sham; # $p < 0.05$ vs AS

		Sham (n=7)	AS (n=7)	AS-NAC (n=7)
ERK	p-ERK/ERK	1.00 $\pm$ 0.22	1.46 $\pm$ 0.34*	0.97 $\pm$ 0.31#
	p-ERK/GAPDH	1.00 $\pm$ 0.16	1.30 $\pm$ 0.85	1.22 $\pm$ 0.50
	ERK/GAPDH	1.00 $\pm$ 0.21	0.98 $\pm$ 0.41	1.13 $\pm$ 0.22
JNK	p-JNK/JNK	1.01 (0.86-1.07)	0.78 (0.68-1.27)	0.78 (0.55-1.27)
	p-JNK/GAPDH	1.07 (0.95-1.18)	1.15 (0.92-1.26)	0.83 (0.62-1.45)#
	JNK/GAPDH	1.00 $\pm$ 0.23	1.11 $\pm$ 0.19	0.95 $\pm$ 0.40
P38	p-P38/P38	0.74 (0.50-1.68)	1.29 (1.23-1.81)	0.70 (0.57-1.04)
	p-P38/GAPDH	1.00 $\pm$ 0.54	2.01 $\pm$ 1.30	1.47 $\pm$ 0.69
	P38/GAPDH	1.05 (0.81-1.13)	1.00 (0.70-2.58)	1.49 (0.99-2.86)

Fig. 3. Antioxidant enzyme activity in myocardium. (A): superoxide dismutase; (B): catalase; (C): glutathione peroxidase. AS: aortic stenosis; AS-NAC: aortic stenosis treated with N-acetylcysteine; n: number of animals. Data are mean $\pm$ SD; ANOVA and Tukey; * $p < 0.05$ vs Sham.

Conclusion

N-acetylcysteine treatment restores myocardial total glutathione, reduces systemic and myocardial oxidative stress, improves MAPK signaling, and attenuates myocardial fibrosis in aortic stenosis rats during transition from compensated left ventricular hypertrophy to heart failure.

Acknowledgements

The authors are grateful to Jose Carlos Georgette for their technical assistance and Colin Edward Knaggs for English editing. Financial support was provided by CNPq (Proc. n. 306770/2015-6 and 308674/2015-4), FAPESP (Proc. n. 2014/21972-3), CAPES, PROPe, UNESP, and PADEX/AUIP Program.

Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this article.

References

- 1 Drazner MH: The progression of hypertensive heart disease. *Circulation* 2011;123:327-334.
- 2 Crozatier B, Ventura-Clapier R: Inhibition of hypertrophy, per se, may not be a good therapeutic strategy in ventricular pressure overload: other approaches could be more beneficial. *Circulation* 2015;131:1448-1457.
- 3 Schiattarella GG, Hill JA: Inhibition of hypertrophy is a good therapeutic strategy in ventricular pressure overload. *Circulation* 2015;131:1435-1447.
- 4 Zwadlo C, Schmidtman E, Szaroszyk M, Kattih B, Froese N, Hinz H, Schmitto JD, Widder J, Batkai S, Bahre H, Kaefer V, Thum T, Bauersachs J, Heineke J: Antiandrogenic therapy with finasteride attenuates cardiac hypertrophy and left ventricular dysfunction. *Circulation* 2015;131:1071-1081.
- 5 Sag CM, Santos CX, Shah AM: Redox regulation of cardiac hypertrophy. *J Mol Cell Cardiol* 2014;73:103-111.
- 6 Madamanchi NR, Runge MS: Redox signaling in cardiovascular health and disease. *Free Rad Biol Med* 2013;61:473-501.
- 7 Munzel T, Gori T, Keaney Jr JF, Maack C, Daiber A: Pathophysiological role of oxidative stress in systolic and diastolic heart failure and its therapeutic implications. *Eur Heart J* 2015;36:2555-2564.
- 8 Altenhofer S, Radermacher KA, Kleikers PW, Wingler K, Schmidt HH: Evolution of NADPH oxidase inhibitors: selectivity and mechanisms for target engagement. *Antioxid Redox Signal* 2015;23:406-427.
- 9 Fratelli M, Goodwin LO, Orom UA, Lombardi S, Tonelli R, Mengozzi M, Ghezzi P: Gene expression profiling reveals a signaling role of glutathione in redox regulation. *Proc Natl Acad Sci U S A* 2005;102:13998-14003.
- 10 Rushworth GF, Megson IL: Existing and potential therapeutic uses for N-acetylcysteine: the need for conversion to intracellular glutathione for antioxidant benefits. *Pharmacol Ther* 2014;141:150-159.
- 11 Adamy C, Mulder P, Khouzami L, Andrieu-Abadie N, Defer N, Candiani G, Pavoine C, Caramelle P, Souktani R, Le Corvoisier P, Perier M, Kirsch M, Damy T, Berdeaux A, Levade T, Thuillez C, Hittinger L, Pecker F: Neutral sphingomyelinase inhibition participates to the benefits of N-acetylcysteine treatment in post-myocardial infarction failing heart rats. *J Mol Cell Cardiol* 2007;43:344-353.
- 12 Lombardi R, Rodriguez G, Chen SN, Ripplinger CM, Li W, Chen J, Willerson JT, Betocchi S, Wickline SA, Efimov IR, Marian AJ: Resolution of established cardiac hypertrophy and fibrosis and prevention of systolic dysfunction in a transgenic rabbit model of human cardiomyopathy through thiol-sensitive mechanisms. *Circulation* 2009;119:1398-1407.
- 13 Giam B, Chu PY, Kuruppu S, Smith AI, Horlock D, Kiriazis H, Du XJ, Kaye DM, Rajapakse NW: N-acetylcysteine attenuates the development of cardiac fibrosis and remodeling in a mouse model of heart failure. *Physiol Rep* 2016;4:e12757.
- 14 Wilder T, Ryba DM, Wieczorek DF, Wolska BM, Solaro RJ: N-acetylcysteine reverses diastolic dysfunction and hypertrophy in familial hypertrophic cardiomyopathy. *Am J Physiol Heart Circ Physiol* 2015;309:H1720-H1730.

- 15 Foltz WU, Wagner M, Rudakova E, Volk T: N-acetylcysteine prevents electrical remodeling and attenuates cellular hypertrophy in epicardial myocytes of rats with ascending aortic stenosis. *Basic Res Cardiol* 2012;107:290.
- 16 Gomes MJ, Martinez PF, Campos DHS, Pagan LU, Bonomo C, Lima AR, Damatto RL, Cezar MD, Damatto FC, Rosa CM, Garcia CM, Reyes DA, Fernandes AA, Fernandes DC, Laurindo FR, Okoshi K, Okoshi MP: Beneficial effects of physical exercise on functional capacity and skeletal muscle oxidative stress in rats with aortic stenosis-induced heart failure. *Oxid Med Cell Longev* 2016;2016:8695716.
- 17 Moreira VO, Pereira CA, Silva MO, Felisbino SL, Cicogna AC, Okoshi K, Aragon FF, Padovani CR, Okoshi MP, Castro AV: Growth hormone attenuates myocardial fibrosis in rats with chronic pressure overload-induced left ventricular hypertrophy. *Clin Exp Pharmacol Physiol* 2009;36:325-330.
- 18 Damatto RL, Martinez PF, Lima AR, Cezar MD, Campos DH, Oliveira SAJ, Guizoni DM, Bonomo C, Nakatani BT, Dal Pai Silva M, Carvalho RF, Okoshi K, Okoshi MP: Heart failure-induced skeletal myopathy in spontaneously hypertensive rats. *Int J Cardiol* 2013;167:698-703.
- 19 Martinez PF, Okoshi K, Zornoff LA, Carvalho RF, Oliveira Junior SA, Lima AR, Campos DH, Damatto RL, Nogueira CR, Dal Pai-Silva M, Okoshi MP: Chronic heart failure-induced skeletal muscle atrophy, necrosis, and myogenic regulatory factors changes. *Med Sci Monit* 2010;16:374-383.
- 20 Okoshi K, Fioretto JR, Okoshi MP, Cicogna AC, Aragon FF, Matsubara LS, Matsubara BB: Food restriction induces *in vivo* ventricular dysfunction in spontaneously hypertensive rats without impairment of *in vitro* myocardial contractility. *Braz J Med Biol Res* 2004;37:607-613.
- 21 Oliveira Junior SA, Dal Pai-Silva M, Martinez PF, Lima-Leopoldo AP, Campos DH, Leopoldo AS, Okoshi MP, Okoshi K, Padovani CR, Cicogna AC: Diet-induced obesity causes metabolic, endocrine and cardiac alterations in spontaneously hypertensive rats. *Med Sci Monit* 2010;16:BR367-BR373.
- 22 Oliveira-Junior SA, Martinez PF, Fan WYC, Nakatani BT, Pagan LU, Padovani CR, Cicogna AC, Okoshi MP, Okoshi K: Association between echocardiographic structural parameters and body weight in Wistar rats. *Oncotarget* 2017;8:26100-26105.
- 23 Pagan LU, Damatto RL, Cezar MD, Lima AR, Bonomo C, Campos DH, Gomes MJ, Martinez PF, Oliveira SAJ, Gimenes R, Rosa CM, Guizoni DM, Moukbel YC, Cicogna AC, Okoshi MP, Okoshi K: Long-term low intensity physical exercise attenuates heart failure development in aging spontaneously hypertensive rats. *Cell Physiol Biochem* 2015;36:61-74.
- 24 Lima AR, Martinez PF, Damatto RL, Cezar MD, Guizoni DM, Bonomo C, Oliveira SAJ, Dal-Pai Silva M, Zornoff LA, Okoshi K, Okoshi MP: Heart failure-induced diaphragm myopathy. *Cell Physiol Biochem* 2014;34:333-345.
- 25 Guizoni DM, Oliveira-Junior SA, Noor SL, Pagan LU, Martinez PF, Lima AR, Gomes MJ, Damatto RL, Cezar MD, Bonomo C, Zornoff LA, Okoshi K, Okoshi MP: Effects of late exercise on cardiac remodeling and myocardial calcium handling proteins in rats with moderate and large size myocardial infarction. *Int J Cardiol* 2016;221:406-412.
- 26 Matsubara LS, Matsubara BB, Okoshi MP, Franco M, Cicogna AC: Myocardial fibrosis rather than hypertrophy induces diastolic dysfunction in renovascular hypertensive rats. *Can J Physiol Pharmacol* 1997;75:1328-1334.
- 27 Wang K, Lu D, Zhang B, Wang S, Liu Q, Zhang Q, Geng J, Shan Q: Renal denervation attenuates multi-organ fibrosis and improves vascular remodeling in rats with transverse aortic constriction induced cardiomyopathy. *Cell Physiol Biochem* 2016;40:465-476.
- 28 Tietze F: Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969;27:502-522.
- 29 Rosa CM, Gimenes R, Campos DH, Guirado GN, Gimenes C, Fernandes AA, Cicogna AC, Queiroz RM, Falcão-Pires I, Miranda-Silva D, Rodrigues P, Laurindo FR, Fernandes DC, Correa CR, Okoshi MP, Okoshi K: Apocynin influence on oxidative stress and cardiac remodeling of spontaneously hypertensive rats with diabetes mellitus. *Cardiovasc Diabetol* 2016;15:126.
- 30 Gimenes C, Gimenes R, Rosa CM, Xavier NP, Campos DHS, Fernandes AAH, Cezar MDM, Guirado GN, Cicogna AC, Takamoto AHR, Okoshi MP, Okoshi K: Low intensity physical exercise attenuates cardiac remodeling and myocardial oxidative stress and dysfunction in diabetic rats. *J Diabetes Res* 2015:ID457848.
- 31 Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjean P: Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. *Clin Chem* 1997;43:1209-1214.

- 32 Cezar MD, Damatto RL, Martinez PF, Lima AR, Campos DH, Rosa CM, Guizoni DM, Bonomo C, Cicogna AC, Gimenes R, Pagan LU, Okoshi MP, Okoshi K: Aldosterone blockade reduces mortality without changing cardiac remodeling in spontaneously hypertensive rats. *Cell Physiol Biochem* 2013;32:1275-1287.
- 33 Cezar MD, Damatto RL, Pagan LU, Lima AR, Martinez PF, Bonomo C, Rosa CM, Campos DH, Cicogna AC, Gomes MJ, Oliveira-Jr SA, Blotta DA, Okoshi MP, Okoshi K: Early spironolactone treatment attenuates heart failure development by improving myocardial function and reducing fibrosis in spontaneously hypertensive rats. *Cell Physiol Biochem* 2015;36:1453-1466.
- 34 Oliveira-Junior SA, Martinez PF, Guizoni DM, Campos DH, Fernandes T, Oliveira EM, Okoshi MP, Okoshi K, Padovani CR, Cicogna AC: AT1 receptor blockade attenuates insulin resistance and myocardial remodeling in rats with diet-induced obesity. *PLoS One* 2014;9:e86447.
- 35 Okoshi MP, Cezar MD, Iyomasa RM, Silva MB, Costa LC, Martinez PF, Campos DH, Damatto RL, Minicucci MF, Cicogna AC, Okoshi K: Effects of early aldosterone antagonism on cardiac remodeling in rats with aortic stenosis-induced pressure overload. *Int J Cardiol* 2016;222:569-575.
- 36 Lima ARR, Pagan LU, Damatto RL, Cezar MDM, Bonomo C, Gomes MJ, Martinez PF, Guizoni DM, Campos DHS, Damatto FC, Okoshi K, Okoshi MP: Effects of growth hormone on cardiac remodeling and soleus muscle in rats with aortic stenosis-induced heart failure. *Oncotarget* 2017;8:83009-83921.
- 37 Damatto RL, Lima AR, Martinez PF, Cezar MD, Okoshi K, Okoshi MP: Myocardial myostatin in spontaneously hypertensive rats with heart failure. *Int J Cardiol* 2016;215:384-387.
- 38 Arcaro A, Pirozzi F, Angelini A, Chimenti C, Crotti L, Giordano C, Mancardi D, Torella D, Tocchetti CG: Novel perspectives in redox biology and pathophysiology of failing myocytes: modulation of the intramyocardial redox milieu for therapeutic interventions - a review article from the Working Group of Cardiac Cell Biology, Italian Society of Cardiology. *Oxid Med Cell Longev* 2016;2016:6353469.
- 39 Cao TT, Chen HH, Dong Z, Xu YW, Zhao P, Guo W, Wei HC, Zhang C, Lu R: Stachydrine protects against pressure overload-induced cardiac hypertrophy by suppressing autophagy. *Cell Physiol Biochem* 2017;42:103-114.
- 40 Martinez PF, Bonomo C, Guizoni DM, Junior SA, Damatto RL, Cezar MD, Lima AR, Pagan LU, Seiva FR, Fernandes DC, Laurindo FRM, Novelli EL, Matsubara LS, Zornoff LA, Okoshi K, Okoshi MP: Influence of N-acetylcysteine on oxidative stress in slow-twitch soleus muscle of heart failure rats. *Cell Physiol Biochem* 2015;35:148-159.
- 41 Suchal K, Malik S, Gamad N, Malhotra RK, Goyal SN, Chaudhary U, Bhatia J, Ojha S, Arya DS: Kaempferol attenuates myocardial ischemic injury via inhibition of MAPK signaling pathway in experimental model of myocardial ischemia-reperfusion injury. *Oxid Med Cell Longev* 2016;2016:7580731.
- 42 Xu Z, Sun J, Tong Q, Lin Q, Qian L, Park Y, Zheng Y: The role of ERK1/2 in the development of diabetic cardiomyopathy. *Int J Mol Sci* 2016;17:E2001.
- 43 Martinez PF, Bonomo C, Guizoni DM, Junior SA, Damatto RL, Cezar MD, Lima AR, Pagan LU, Seiva FR, Bueno RT, Fernandes DC, Laurindo FR, Zornoff LA, Okoshi K, Okoshi MP: Modulation of MAPK and NF-kappaB signaling pathways by antioxidant therapy in skeletal muscle of heart failure rats. *Cell Physiol Biochem* 2016;39:371-384.
- 44 Ren J, Zhang N, Liao H, Chen S, Xu L, Li J, Yang Z, Deng W, Tang Q: Caffeic acid phenethyl ester attenuates pathological cardiac hypertrophy by regulation of MEK/ERK signaling pathway *in vivo* and *in vitro*. *Life Sci* 2017;181:53-61.
- 45 Roe AT, Aronsen JM, Skardal K, Hamdani N, Linke WA, Danielsen HE, Sejersted OM, Sjaastad I, Louch WE: Increased passive stiffness promotes diastolic dysfunction despite improved Ca²⁺ handling during left ventricular concentric hypertrophy. *Cardiovasc Res* 2017;113:1161-1172.
- 46 Marian AJ, Senthil V, Chen SN, Lombardi R: Antifibrotic effects of antioxidant N-acetylcysteine in a mouse model of human hypertrophic cardiomyopathy mutation. *J Am Coll Cardiol* 2006;47:827-834.
- 47 Demedts M, Behr J, Buhl R, Costabel U, Dekhuijzen R, Jansen HM, MacNee W, Thomeer M, Wallaert B, Laurent F, Nicholson AG, Verbeken EK, Verschakelen J, Flower CD, Capron F, Petruzzelli S, De Vuyst P, van den Bosch JM, Rodriguez-Becerra E, Corvasce G, Lankhorst I, Sardina M, Montanari M: IFIGENIA Study Group. High-dose acetylcysteine in idiopathic pulmonary fibrosis. *N Engl J Med* 2005;353:2229-2242.
- 48 Liu RM, Liu Y, Forman HJ, Olman M, Tarpey MM: Glutathione regulates transforming growth factor-beta-stimulated collagen production in fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L121-L128.
- 49 van Deel ED, Octavia Y, de Boer M, Juni RP, Tempel D, van Haperen R, de Crom R, Moens AL, Merkus D, Duncker DJ: Normal and high eNOS levels are detrimental in both mild and severe cardiac pressure-overload *J Mol Cell Cardiol* 2015;88:145-154.