

Original Paper

Aldosterone Blockade Reduces Mortality without Changing Cardiac Remodeling in Spontaneously Hypertensive Rats

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Key Words

Heart failure • Myocardial function • Echocardiogram • Spironolactone • Ventricular function • Papillary muscle

Abstract

Background: The role of aldosterone blockers during transition from long-term compensated hypertrophy to dilated failure is not completely understood. In this study we evaluated the effects of early administration of spironolactone on cardiac remodeling, myocardial function, and mortality in spontaneously hypertensive rats (SHR). **Methods:** Sixteen-month-old SHR received no treatment (SHR-C, n=72) or spironolactone (SHR-SPR, 20 mg/kg/day, n=34) for six months. Echocardiogram was performed before and after treatment. Myocardial function was analyzed in left ventricular (LV) papillary muscle preparations. Myocardial collagen and hydroxyproline concentration were evaluated by morphometry and spectrophotometry, respectively. LV gene expression was assessed by real time RT-PCR. Statistics: Student's t test; Log rank test (Kaplan Meyer). **Results:** SHR-C and SHR-SPR presented mortality rates of 71 and 38%, respectively (p=0.004). Systolic arterial pressure did not differ between groups (SHR-C 199±43; SHR-SPR 200±35 mmHg). Initial and final echocardiograms did not show significant differences in cardiac structures or LV function between groups. Myocardial function was similar between groups at basal and after inotropic stimulation. Collagen fractional area, hydroxyproline concentration, gene expression for α - and β -myosin heavy chain, atrial natriuretic peptide, and Serca2a were not different between groups. **Conclusion:** Early spironolactone administration reduces mortality without changing cardiac remodeling in spontaneous hypertensive rats.

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Introduction

Chronic pressure overload is a major cause of heart failure. During sustained pressure overload, stable cardiac hypertrophy develops and may progress to a decompensated state with left ventricular dilation and systolic pump failure [1, 2]. The mineralocorticoid hormone aldosterone has been shown to be involved in the pathophysiology of heart failure [3, 4]. However, the role of aldosterone blockers during transition from long-term compensated hypertrophy to dilated failure is not completely understood.

Experimental models have shown that aldosterone induces deleterious cardiovascular effects such as myocardial fibrosis, myocyte hypertrophy and apoptosis, oxidative stress, vascular injury, endothelial dysfunction, renal retention of sodium and water, loss of potassium and magnesium, electrical remodeling, and sudden death [5-11]. Two aldosterone blockers, spironolactone and eplerenone have been evaluated in clinical and experimental studies [12-24]. In different models of cardiac injury, aldosterone blockers can prevent or attenuate left ventricular structural, functional, and molecular changes [12-20]. Two large clinical trials have shown that aldosterone receptor blockers reduce mortality and hospitalizations in patients with systolic heart failure of any cause and New York Heart Association functional classes III and IV (RALES) [21], and in patients with systolic dysfunction post-myocardial infarction (EPHESUS) [22]. The EMPHASIS-HF study showed that aldosterone blocker eplerenone reduced mortality and hospitalizations in patients with heart failure of any cause in functional class II and left ventricular ejection fraction ≤ 0.35 [23]. Despite being now recommended for symptomatic systolic heart failure patients [25], evidence is less consistent on the benefits of the aldosterone blockers, in milder forms of heart failure.

The spontaneously hypertensive rat (SHR) is a widely used experimental model for studying heart failure [1, 26, 27]. It presents early arterial hypertension and left ventricular hypertrophy which evolves to heart failure during maturity and senescence. As cardiac remodeling and heart failure development is slow, SHRs are considered a useful model to mimic clinical heart failure settings. In this study we evaluated the effects of chronic administration of aldosterone blocker spironolactone, introduced before the appearance of heart failure clinical signs, on cardiac remodeling, myocardial function, and mortality in spontaneously hypertensive rats.

Materials and Methods

Experimental groups

Male spontaneously hypertensive rats (SHR) were purchased from the Central Animal House at Botucatu Medical School, UNESP. All animals were housed in a room under temperature control at 23 °C and kept on a 12-hour light/dark cycle. Food and water were supplied *ad libitum*. All experiments and procedures were approved by the Ethics Committee of Botucatu Medical School, UNESP, Botucatu, SP, Brazil.

Sixteen-month-old SHR were divided into two groups: control (SHR-C, n=72) and spironolactone (SHR-SPR, n=34). Spironolactone (Biolab Pharmaceutical, Sao Paulo, Brazil) was added to rat chow at 20 mg/kg/day for six months. Rats were weighed once weekly to adjust for drug dosage. Systolic arterial pressure was measured by tail-cuff method at 16 and 22 months old. During euthanasia, we assessed pathological features of congestive heart failure. Rats with pleuropericardial effusion, left atrial thrombi, and/or right ventricular hypertrophy (right ventricle weight-to-body weight ratio > 0.8 mg/g) were considered to present heart failure [1, 2, 26, 28].

Echocardiographic study

Echocardiographic evaluation was performed using a commercially available echocardiograph (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel) equipped with a 5 - 11.5 MHz multifrequency probe. Rats were anesthetized by intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (0.5 mg/kg). A two-dimensional parasternal short-axis view of the left ventricle (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 [29-32]. M-mode images of

the LV were printed on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. All LV structures were manually measured by the same observer according to the leading-edge method of the American Society of Echocardiography [33]. The measurements obtained were the mean of at least five cardiac cycles on the M-mode tracings. The following structural variables were measured: left atrium (LA) diameter, LV diastolic and systolic dimensions (LVDD and LVSD, respectively), LV diastolic posterior wall thickness (PWT), LV diastolic septal wall thickness (SWT), and aortic diameter (AO). Left ventricular weight (LVW) was calculated using the formula $[(LVDD + PWT + SWT)^3 - (LVDD)^3] \times 1,04$ [34]. Left ventricular function was assessed by the following parameters: endocardial fractional shortening (EFS), midwall fractional shortening (MWFS), ejection fraction (EF), posterior wall shortening velocity (PWSV), early and late diastolic mitral inflow velocities (E and A waves), E/A ratio, and isovolumetric relaxation time (IVRT).

Myocardial functional study

At the end of the experimental period, two days after the echocardiographic study, myocardial intrinsic contractile performance was evaluated in isolated LV papillary muscle preparation as previously described [35-37]. The rats were anesthetized (pentobarbital sodium, 50 mg/kg, intraperitoneally) and decapitated. Hearts were quickly removed and placed in oxygenated Krebs-Henseleit solution at 28 °C. Left ventricular anterior or posterior papillary muscle was dissected free, mounted between two spring clips, and placed vertically in a chamber containing Krebs-Henseleit solution at 28 °C and oxygenated with a mixture of 95 % O₂ and 5 % CO₂ (pH 7.38). The composition of the Krebs-Henseleit solution in mM was as follows: 118.5 NaCl, 4.69 KCl, 1.25 CaCl₂, 1.16 MgSO₄, 1.18 KH₂PO₄, 5.50 glucose, and 25.88 NaHCO₃. The spring clips were attached to a Kyowa model 120T-20B force transducer and a lever system, which allowed for muscle length adjustment. Preparations were stimulated 12 times/min at a voltage 10 % above threshold.

After a 60-min period, during which the preparations were permitted to shorten while carrying light loads, muscles were loaded to contract isometrically and stretched to the apices of their length-tension curves (L_{max}). After a 5-min period, during which preparations performed isotonic contractions, muscles were again placed under isometric conditions, and the apex of the length-tension curve was determined. A 15-min period of stable isometric contraction was imposed prior to the experimental period. One isometric contraction was then recorded for later analysis.

The following parameters were measured from isometric contraction: peak of developed tension (DT, g/mm²), resting tension (RT, g/mm²), time to peak of tension (TPT, ms), maximum rate of tension development (+dT/dt, g/mm²/s), and maximum rate of tension decline (-dT/dt, g/mm²/s). To evaluate contractile reserve and myocardial response to β-adrenergic stimulation, mechanical performance of papillary muscles were evaluated in basal condition and after the following inotropic stimulation: post-rest contraction, extracellular Ca²⁺ concentration increase, and beta-adrenergic agonist isoproterenol addition to the nutrient solution.

Papillary muscle cross-sectional area was calculated from muscle weight and length by assuming cylindrical uniformity and a specific gravity of 1.0. All force data were normalized for the muscle cross-sectional area.

After dissecting papillary muscle, lungs, atria, and ventricles were separated and weighed. Fragments of ventricles, atria, lung, and liver were weighed before and after drying sessions (65 °C for 72 h) to evaluate the wet-to-dry weight ratio. Tibia was removed and dried and the lengths measured. Atria and left and right ventricular wet weight normalized by body weight and tibia length were used as indices of ventricular hypertrophy.

Morphologic study

Transverse LV sections were fixed in 10 % buffered formalin and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin-eosin and the collagen-specific stain picrosirius red (Sirius red F3BA in aqueous saturated picric acid) [38]. At least 150 fiber cross-sectional areas were measured from each heart. On average, 20 microscopic fields were used to quantify interstitial collagen fractional area. Perivascular collagen was excluded from this analysis. Measurements were performed using a Leica microscope (magnification 40X) attached to a video camera and connected to a computer equipped with image analysis software (Image-Pro Plus 3.0, Media Cybernetics, Silver Spring, MD, USA).

Myocardial hydroxyproline and serum electrolytes concentration

Myocardial hydroxyproline (HOP) concentration was assessed for tissue collagen content estimation. HOP was measured in LV tissue as previously described [39]. Briefly, the tissue was dried using a Speedvac

Concentrator SC 100 attached to a refrigerated condensation trap (TRL 100) and vacuum pump (VP 100, Savant Instruments, Inc., Farmingdale, NY, USA). Tissue dry weight was measured and the samples were hydrolyzed overnight at 100 °C with 6 N HCl (1 ml/10 mg dry tissue). A 50 µl aliquot of the hydrolysate was transferred to an Eppendorf tube and dried in the Speedvac Concentrator. One milliliter of deionized water was added and the sample transferred to a tube with a Teflon screw cap. One milliliter of potassium borate buffer (pH 8.7) was added to maintain constant pH and the sample was oxidized with 0.3 ml of chloramine T solution at room temperature for 20 min. The addition of 1 ml of 3.6 mol/l sodium thiosulfate and thorough mixing for 10 s stopped the oxidative process. The solution was saturated with 1.5 g KCl. The tubes were capped and heated in boiling water for 20 min. After cooling to room temperature, the aqueous layer was extracted with 2.5 ml of toluene. One and a half milliliters of toluene extract were transferred to a 12 X 75 mm test tube. Then 0.6 ml of Ehrlich's reagent was added and the color allowed to develop for 30 min. Absorbances were read at 565 nm against a reagent blank. Deionized water and 20 µg/ml HOP were used as the blank and standard, respectively.

Sodium, potassium, and magnesium serum concentration was analyzed by flame photometry (FC-280, CELM) using a commercial standard solution.

Gene expression

To evaluate expression of the fetal gene program, we assessed α - and β -myosin heavy chain, atrial natriuretic peptide (ANP), and sarcoplasmic reticulum calcium ATPase (Serca 2a) gene expression by real time RT-PCR (transcription polymerase chain reaction after reverse transcription). Total RNA was extracted from LV myocardial sample with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as previously described [40-42]. Frozen muscles were mechanically homogenized on ice in 1 ml of ice-cold TRIzol reagent. Total RNA was solubilized in RNase-free H₂O, incubated in DNase I (Invitrogen Life Technologies) to remove any DNA in the sample, and quantified by measuring optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio of approximately 2.0. One µg of RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit in a total volume of 20 µl, according to standard methods (Applied Biosystems, Foster City, CA, EUA). Aliquots of 2.5 µl (10-100 ng) cDNA were then submitted to real-time PCR reaction using 10 µl 2X TaqMan® Universal PCR Master Mix (Applied Biosystems) and 1 µl of customized assay (20X) containing sense and antisense primers and Taqman (Applied Biosystems, Foster City, CA, EUA) probe specific to each gene, alpha myosin (myosin heavy polypeptide 6, cardiac muscle, alpha; Taqman assay Rn00568304_m1; Ref. seq. Genbank NM_017239.1), beta myosin (myosin heavy polypeptide 7, cardiac muscle, beta; Taqman assay Rn00568328_m1; Ref. seq. Genbank NM_017240.1), natriuretic peptide precursor type A (Taqman assay Rn00561661_m1; Ref. seq. Genbank NM_012612.1), and Serca2a (Taqman assay Rn00568762; Ref. seq. Genbank NM_017290). Amplification and analysis were performed using a StepOnePlus™ Real Time PCR System (Applied Biosystems, Foster City, CA, EUA) according to manufacturer's recommendation. Expression data were normalized to cyclophilin expression (reference gene; Taqman assay Rn00690933_m1; Ref. seq. Genbank NM_017101). Reactions were performed in triplicate and expression levels calculated using the CT comparative method ($2^{-\Delta\Delta CT}$).

Western blotting analysis

To evaluate expression of the proteins involved in intracellular calcium transient, we performed Western blot analyses in LV samples according to a previously described method [43] with specific anti-Serca 2 ATPase (MA3-910, IID8) and anti-phospholamban (MA3-922, 2D12) antibodies (Thermo Fisher Scientific, Rockford, IL). Protein levels were normalized to those of GAPDH (6C5, sc-32233, Santa Cruz Biotechnology). Muscle protein was extracted using Tris-Triton buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate). 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, the membrane was incubated with the primary antibody and then washed with TBS and Tween 20 and incubated with secondary peroxidase-conjugated antibody. Super Signal® West Pico Chemiluminescent Substrate (Pierce Protein Research Products, Rockford, USA) was used to detect bound antibodies.

Statistical analysis

Data are expressed as mean \pm standard deviation or median and 25th and 75th percentiles. Comparisons between the groups were performed by Student's *t* test or Mann-Whitney test. Mortality was assessed by

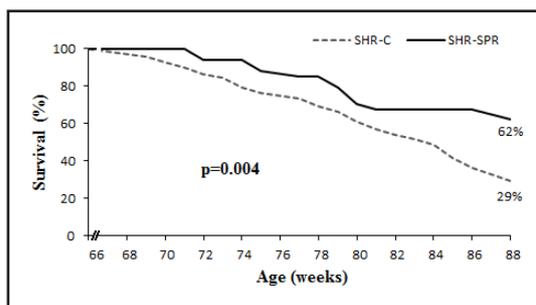


Fig. 1. Probability of survival; Log rank test; Kaplan-Meier. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone.

Variables	SHR-C (n=21)	SHR-SPR (n=21)	P value
Initial BW (g)	393±23	393±27	0.985
Final BW (g)	347±34*	348±33*	0.975
Initial SBP (mmHg)	206±17	215±21	0.259
Final SBP (mmHg)	199±43	200±35	0.932

Table 1. Body weight and systolic blood pressure. Data are expressed as mean ± standard deviation. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone; BW: body weight; SBP: systolic blood pressure. * p<0.05 vs. Initial BW (paired Student's *t* test); P value: SHR-SPR vs. SHR-C (unpaired Student's *t* test)

Variables	SHR-C (n=11)	SHR-SPR (n=19)	P value
HR (bpm)	329±29	334±28	0.665
LVDD (mm)	8.87±1.26	8.66±1.13	0.641
LVSD (mm)	4.85±1.58	4.96±1.35	0.849
LVPWT (mm)	1.93±0.14	1.82±0.15	0.059
LVSWT (mm)	1.94±0.13	1.86±0.15	0.189
AO (mm)	4.97±0.34	4.78±0.40	0.199
LA (mm)	7.52±1.42	7.65±1.30	0.802
LV weight (g)	1.45±0.39	1.31±0.36	0.338
RWT	0.44±0.07	0.42±0.06	0.473

Table 2. Echocardiographic cardiac structural parameters. Data are expressed as mean ± standard deviation. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone. HR: heart rate; LVDD and LVSD: left ventricular (LV) diastolic and systolic diameter, respectively; LVPWT: LV posterior wall thickness; LVSWT: LV septal wall thickness; AO: aorta diameter; LA: left atrial diameter; RWT: relative wall thickness. Unpaired Student's *t* test

Variables	SHR-C (n=11)	SHR-SPR (n=19)	P value
EFS (%)	46.4±10.9	43.7±8.81	0.453
MWFS (%)	26.8±5.65	25.2±4.93	0.426
EF	0.83±0.10	0.81±0.09	0.575
PWSV (mm/s)	33.2±6.73	29.7±4.78	0.115
E-wave (cm/s)	57.0 (47.5–86.8)	70.0 (52.0–125)	0.464
A-wave (cm/s)	82.4±35.7	69.0±34.3	0.320
E/A	0.63 (0.54–0.74)	0.71 (0.59–3.65)	0.606
IVRT (ms)	36.5±7.08	38.7±4.68	0.327

Table 3. Echocardiographic left ventricular functional parameters. Data are expressed as mean ± standard deviation or median and 25th and 75th percentiles. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone. EFS: endocardial fractional shortening; MWFS: midwall fractional shortening; EF: ejection fraction; PWSV: posterior wall shortening velocity; E-wave and A-wave: early and late diastolic mitral inflow, respectively; IVRT: isovolumetric relaxation time. Unpaired Student's *t* test

log-rank test (Kaplan Meier). Analysis of papillary muscle studies using different inotropic stimulation was performed by repeated measures ANOVA. Statistical significance was accepted at the level of p<0.05.

Results

During the experimental period, SHR-C and SHR-SPR presented mortality rates of 71 % and 38 %, respectively (p=0.004). Survival curves according to animal age are shown in Figure 1. In surviving animals (n=21 per group) no difference was seen in the percentage of pathologically diagnosed heart failure (76.2 %) between groups.

Initial and final body weight was not statistically different between groups; both groups presented a significant reduction in final body weight compared to initial weight. Initial and final systolic blood pressure was not different between groups (Table 1). Transthoracic echocardiography was performed at the beginning and end of the experiment. The initial exam aimed to ensure homogeneity between groups (data not shown). In the final exam, no significant differences were observed in cardiac structural and functional parameters (Tables 2 and 3).

Variables	SHR-C (n=18)	SHR-SPR (n=20)	P value
DT (g/mm ²)	4.49±1.79	4.70±1.67	0.706
RT (g/mm ²)	0.88±0.24	0.89±0.28	0.918
TPT (ms)	221±16	218±17	0.611
+dT/dt (g/mm ² /s)	38.0±17.9	39.1±15.7	0.838
-dT/dt (g/mm ² /s)	19.0±6.28	19.5±5.66	0.779
PM CSA (mm ²)	1.19±0.38	1.15±0.27	0.699

Table 4. Basal data of isolated papillary muscle. Data are expressed as mean ± standard deviation. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone. DT: peak of developed tension; RT: resting tension; TPT: time to peak of tension; +dT/dt: maximum rate of tension development; -dT/dt: maximum rate of tension decline; PM CSA: papillary muscle cross sectional area. Unpaired Student's *t* test

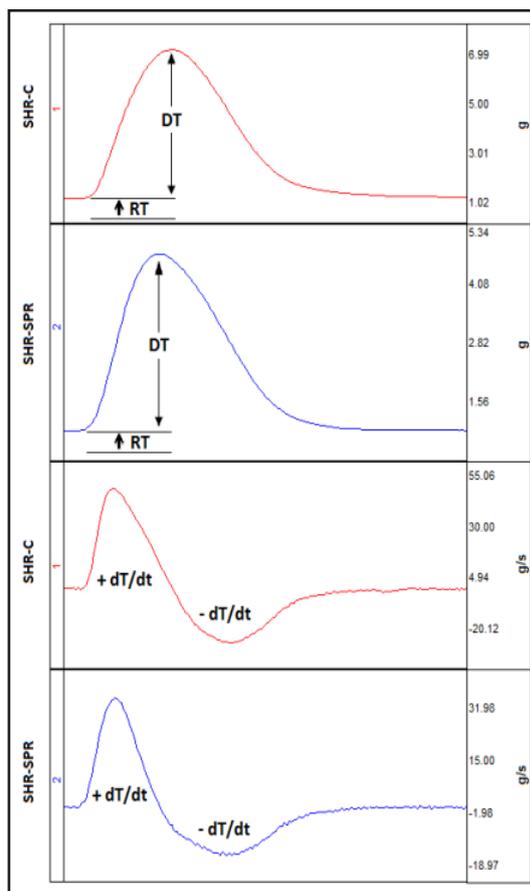


Fig. 2. Illustrative recordings of papillary muscles during isometric contractions at extracellular calcium concentration of 1.25 mM. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone; DT: peak of developed tension; RT: resting tension; +dT/dt: maximum rate of tension development; -dT/dt: maximum rate of tension decline.

Papillary muscle functional data at basal conditions with an extracellular calcium concentration of 1.25 mM were not different between groups (Table 4). Figure 2 shows representative recordings of isometric contractions for both SHR-C and SHR-SPR groups. All parameters obtained at post-rest contractions and after extracellular Ca²⁺ concentration increase were not different between groups (data not shown). Table 5 shows papillary muscle data under isometric contraction after stimulation with different doses of β -adrenergic agonist isoproterenol. There were no differences between groups for all parameters.

Anatomical parameters LV, right ventricle, atria, and lung weights and their wet/dry ratio were not statistically different between groups. Also, LV, right ventricle, and atria weights normalized to body weight or tibia length did not differ between groups (Table 6). Myocyte cross sectional area was lower in the SHR-SPR than SHR-C. Myocardial interstitial collagen fractional area and hydroxyproline concentration were not statistically different between groups (Table 7). Serum electrolytes sodium (SHR-C 145 ± 4.38; SHR-SPR 141 ± 3.62 mEq/L), potassium (SHR-C 4.31 ± 0.36; SHR-SPR 4.46 ± 0.42 mEq/L), and magnesium (SHR-C 2.11 ± 0.21; SHR-SPR

	mM	DT (g/mm ²)	RT (g/mm ²)	TPT (ms)	+dT/dt (g/mm ² /s)	-dT/dt (g/mm ² /s)
SHR-C (n=18)	ISO 10 ⁻⁸	4.30±1.76	0.75±0.24	190±12	40.0±16.9	19.0±6.15
	ISO 10 ⁻⁷	4.09±1.85	0.74±0.24	162±14	43.5±20.8	23.0±6.49
	ISO 10 ⁻⁶	3.92±1.82	0.72±0.24	156±8	44.7±22.9	27.6±10.3
SHR-SPR (n=20)	ISO 10 ⁻⁸	4.32±1.62	0.76±0.27	200±18	41.8±17.5	19.8±6.78
	ISO 10 ⁻⁷	3.91±1.69	0.74±0.26	168±10	43.1±19.1	22.6±9.60
	ISO 10 ⁻⁶	3.75±1.66	0.73±0.28	154±13	44.4±20.3	26.4±11.1

Table 5. Papillary muscle data under isometric contraction after stimulation with the β-adrenergic agonist isoproterenol. Data are expressed as mean ± standard deviation. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone. ISO: isoproterenol; DT: peak of developed tension; RT: resting tension; TPT: time to peak of tension; +dT/dt: maximum rate of tension development; -dT/dt: maximum rate of tension decline. Repeated measures ANOVA

Table 6. Anatomical data. Data are expressed as mean ± standard deviation. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone. LVW: left ventricle weight; BW: body weight; RVW: right ventricle weight; wet/dry: wet weight-to-dry weight ratio. Unpaired Student's *t* test

Variables	SHR-C (n=21)	SHR-SPR (n=21)	P value
Tibia length (cm)	4.28±0.08	4.27±0.09	0.714
LVW (g)	1.19±0.19	1.22±0.20	0.654
LVW/BW (g/kg)	3.41±0.42	3.51±0.57	0.552
LVW/tibia (mg/cm)	278±46	285±47	0.628
RVW (g)	0.36±0.09	0.37±0.10	0.808
RVW/BW (g/kg)	1.06±0.27	1.07±0.28	0.848
RVW/tibia (mg/cm)	85.1±21.1	87.0±23.2	0.780
Atria (g)	0.20±0.06	0.20±0.05	0.966
Atria/BW (g/kg)	0.59±0.18	0.59±0.15	0.972
Atria/tibia (mg/cm)	47.7±15.0	47.6±11.8	0.992
LV wet/dry	4.26±0.28	4.30±0.18	0.601
RW wet/dry	4.24±0.60	4.27±0.54	0.868
Atria wet/dry	4.66±0.26	4.81±0.26	0.069
Lung (g)	3.81±0.70	3.71±0.81	0.666
Lung wet/dry	5.21±0.42	5.32±0.47	0.458
Liver wet/dry	3.33±0.30	3.27±0.28	0.470

Variables	SHR-C (n=8)	SHR-SPR (n=8)	P value
CSA (μm ²)	550±34	440±51	0.001
ICF (%)	8.20±3.00	9.90±6.30	0.535
HOP (mg/g)	7.11±1.65	7.18±1.74	0.928

Table 7. Left ventricular morphometric parameters and hydroxyproline concentration. Data are expressed as mean ± standard deviation. SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone. CSA: myocyte cross-sectional area; ICF: interstitial collagen fractional area; HOP: hydroxyproline. Unpaired Student's *t* test

Variables	SHR-C (n=8)	SHR-SPR (n=7)	P value
α-MHC	2.22±1.85	3.77±1.57	0.108
β-MHC	1.77±1.25	2.34±0.77	0.311
ANP	1.32±0.77	1.15±0.47	0.627
Serca2a	1.52±0.80	1.83±0.52	0.385

Table 8. Gene expression. Data are expressed as mean ± standard deviation (arbitrary units). SHR-C: spontaneously hypertensive rats without treatment; SHR-SPR: spontaneously hypertensive rats treated with spironolactone. MHC: myosin heavy chain; ANP: atrial natriuretic peptide; Serca2a: sarcoplasmic reticulum calcium ATPase. Unpaired Student's *t* test

2.25 ± 0.19) did not differ between groups. Gene expression for α- and β-myosin heavy chain, ANP, and Serca 2a was not different between groups (Table 8). Serca 2 and phospholamban protein levels were not statistically different between groups (Serca 2: SHR-C 1.00 ± 0.36; SHR-SPR 0.93 ± 0.27 arbitrary units; p=0.68; phospholamban: SHR-C 1.00 ± 0.51; SHR-SPR 1.27 ± 0.19 arbitrary units; p=0.21).

Discussion

In this study we showed that chronic administration of aldosterone blocker spironolactone to spontaneously hypertensive rats without the clinical features of heart failure reduces mortality without changing cardiac remodeling.

To the best of our knowledge, this is the first study to evaluate the long-term effects of early spironolactone treatment on SHR mortality, cardiac remodeling, and myocardial function. The spontaneously hypertensive rat is a well-established model of genetic hypertension [1, 2, 26]. At one month old, SHR begin to develop arterial hypertension and left ventricular hypertrophy, which often maintains cardiac performance despite the elevated systemic arterial pressure [44]. However, if pressure overload is sustained, cardiac decompensation may ensue, usually beginning at 18-22 months of age. After developing heart failure, rats evolve to death usually within two to four weeks [1, 2]. In this study we evaluated the effects of aldosterone blocker during the transition from left ventricular hypertrophy to heart failure. Therefore, spironolactone was started when rats were 16 months old with no evidence of heart failure, and evaluated survival and ventricular and myocardial function six months later. Spironolactone has long been used to treat water and sodium retention. However, only after the study by Pitt et al. [21] has this drug attracted substantial attention from cardiologists and cardiovascular physiologists due to its potential to reduce the risk of morbidity and death in heart failure patients.

In this study, spironolactone did not change arterial systolic blood pressure. This result is in agreement with previous studies in SHR using similar spironolactone doses [45, 46]. As arterial pressure was unchanged by the treatment, we were able to exclude the influence of hemodynamic effects on the cardiac remodeling process and mortality. Untreated and treated groups presented a decrease in body weight during the experimental period. Body weight loss accompanying heart failure is an important predictor of mortality [47]. In our study, the body weight loss illustrates the advanced degree of heart failure.

Cardiac structures and left ventricular functional parameters evaluated by transthoracic echocardiogram did not differ between groups. Also, myocardial function, contractile reserve, and responsiveness to β -adrenergic stimulation assessed in papillary muscle preparations were not different between groups. Although not statistically significant, developed tension progressively decreased with increased isoproterenol doses in both groups (Table 5). Blunted responsiveness to β -adrenergic stimulation occurs early during myocardial hypertrophy and failure [48]. In SHR with long-standing hypertrophy, isoproterenol decreased papillary muscle isometric tension at L_{max} [48]. Our study showed, therefore, that spironolactone does not modulate myocardial responsiveness to β -adrenergic stimulation.

Myocardial collagen content assessed by both biochemical and histological analyses was not different between groups, which is in accordance with unchanged myocardial and LV diastolic function. Cardiac remodeling is usually associated with changed gene expression from several myocardial proteins such as α - and β -myosin heavy chain, atrial natriuretic peptide, and Serca 2a. In our study, spironolactone treatment did not change expression of fetal genes program. Also, levels of Serca 2 and phospholamban, the proteins involved in intracellular calcium transient and myocardial contractility did not differ between groups. The results from gene and protein expression are in agreement with the lack of effects seen in cardiac structures and function.

In the clinical multicentric trials Rales [21], Ephesus [22], and Emphasis-HF [23], cardiac structural and functional evaluation was not performed after treatment with aldosterone blockers. Smaller clinical studies have shown conflicting results. In patients with mild-to-moderate systolic heart failure, some authors have observed aldosterone blockers-induced beneficial effects on structural and functional echocardiographic parameters [49-51], whereas others have found unchanged LV remodeling [52].

The effects of aldosterone blockers have been evaluated in different experimental models. In infarcted rodents, they have improved cardiac remodeling [14, 15, 19, 53, 54], whereas in dogs with rapid ventricular pacing-induced heart failure, eplerenone failed to prevent LV

changes [55]. In SHR, the aldosterone blockade was evaluated during the cardiac remodeling process but not during heart failure development. In young [45, 56-59] and mature [20, 46] SHR, treatment with aldosterone blockers have resulted in improved or unchanged LV hypertrophy and myocardial fibrosis. The conflicting results after aldosterone blockers treatment suggest that their role in the remodeling process and heart failure development are not completely understood but are probably influenced by treatment period and experimental cardiac injury model.

Despite unchanged ventricular and myocardial remodeling and heart failure development, mortality rate was decreased in the spironolactone treated SHR. In the RALES [21] and EPHEBUS [22] clinical trials, aldosterone blockers not only decreased cardiovascular and general mortality, but also sudden death. In patients with functional class III heart failure, spironolactone reduced ventricular extrasystole and non-sustained ventricular tachycardia [60]. Decreased urinary magnesium and potassium excretion has been suggested to play a role in lower ventricular arrhythmia rates [61]. However, in our study serum electrolytes concentration remained unchanged after treatment. In heart failure patients, spironolactone increased RR variability and reduced heart rate, QT interval, and QT dispersion, suggesting that it can increase parasympathetic tonus and have anti-arrhythmic properties [62].

During cardiac hypertrophy and heart failure, increased aldosterone production can induce electrical remodeling and cardiac arrhythmia by changing ion channels density or function [4, 63-69]. *In vivo* studies have shown that aldosterone blockers attenuate ventricular electrical remodeling and tachyarrhythmia vulnerability in different models of cardiac injury [19, 55, 70]. Our results suggest that aldosterone blockade reduced mortality by decreasing cardiac arrhythmia and sudden death. We cannot refute, however, that cardiac remodeling was not changed because cardiac and myocardial evaluation was performed late after all non-treated rats with worse mechanical performance had died. Therefore, it is possible that by evaluating cardiac function and structures earlier, improved spironolactone-induced cardiac remodeling can be shown in spontaneous hypertensive rats.

In conclusion, early spironolactone administration reduces mortality without changing cardiac remodeling in spontaneous hypertensive rats.

Conflict of Interest

No conflict of interest declared

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