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Digitoxin improves cardiovascular autonomic control in rats with heart failure

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6 **Digitoxin improves cardiovascular autonomic control in**
7 **rats with heart failure**
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18 **Running Head:** Digitoxin improves cardiovascular autonomic control

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25 ABSTRACT

26 The effects of chronic treatment with digitoxin on arterial baroreceptor sensitivity
27 for heart rate (HR) and renal sympathetic nerve activity (rSNA) control,
28 cardiopulmonary reflex, and autonomic HR control in an animal model of heart
29 failure were evaluated. Wistar rats were treated with digitoxin, which was
30 administered in their daily feed (1mg/Kg/day), for 60 days. The following three
31 experimental groups were evaluated: Sham, heart failure (HF), and HF treated
32 with digitoxin (HF+DIG). We observed an increase in rSNA in the HF group
33 (190 ± 29 pps, $n=5$) compared with the Sham group (98 ± 14 pps, $n=5$). Digitoxin
34 treatment prevented an increase in rSNA (98 ± 14 pps, $n=7$). Therefore, arterial
35 baroreceptor sensitivity was decreased in the HF group ($-$
36 1.24 ± 0.07 bpm/mmHg, $n=8$) compared with the Sham group ($-$
37 2.27 ± 0.23 bpm/mmHg, $n=6$). Digitoxin did not alter arterial baroreceptor
38 sensitivity in the HF+DIG group. Finally, the HF group showed an increased low
39 frequency band (LFb: 23 ± 5 ms², $n=8$) and a decreased high frequency band
40 (HFb: 77 ± 5 ms², $n=8$) compared with the Sham group (LFb: 14 ± 3 ms²; HFb:
41 86 ± 3 ms², $n=9$); the HF+DIG group exhibited normalized parameters (LFb:
42 15 ± 3 ms²; HFb: 85 ± 3 ms², $n=9$). In conclusion, the benefits of decreasing rSNA
43 are not directly related to improvements in peripheral cardiovascular reflexes;
44 such occurrences are due in part to changes in the central nuclei of the brain
45 responsible for autonomic cardiovascular control.

46 **Keywords:** Heart failure; digitoxin; baroreflex sensitivity; cardiopulmonary
47 reflex; renal sympathetic nerve activity; spectral analysis; sympathetic
48 vasomotor tone

49 INTRODUCTION

50 Heart failure (HF) is a common, chronic, progressive disease and a major
51 cause of mortality and morbidity (Hamdani and Paulus 2011; Mangini et al.
52 2008). Although the mechanisms underlying the cardiovascular alterations
53 related to HF are well-established, strategies avoiding the progression and
54 outcomes of this disease are limited to classes of drugs used to treat
55 compensatory adjustments following HF, including the activation of the renin-
56 angiotensin-aldosterone system and sympathoexcitation (Coons et al. 2011;
57 Hamdani and Paulus 2011). There is a close relationship between the severity
58 of HF and increased sympathetic vasomotor tone (Carillo et al. 2012).

59 In chronic HF, the sympathetic drive to the blood vessels of the heart,
60 kidney and muscle blood is dramatically increased and contributes to the
61 progression of the disease (Parati and Esler 2012). Studies have demonstrated
62 a new potential treatment for heart failure with catheter-based renal
63 denervation, which reduces blood pressure and sympathetic drive (Booth et al.
64 2015). However, the mechanism triggering the increased sympathetic activity at
65 different targets is not well-understood and is a matter of discussion. Increased
66 central sympathetic drive is a reasonable explanation for this phenomenon. The
67 upregulation of the angiotensin II (Ang II) AT1 receptor in conjunction with the
68 downregulation of the AT2 receptor in regions of the brain related to
69 cardiovascular control is present in HF, which would partially explain the
70 increased sympathetic drive associated with this condition (Gao et al. 2008).
71 Impairment of arterial baroreceptor function in HF could be an additional factor
72 leading to increases in sympathetic drive (Han et al. 2010) and a cardiac

73 autonomic imbalance characterized by sympathetic hyperactivity and reduced
74 vagal tone (Morais et al. 2015).

75 One of the pharmacological choices to treat HF is digitalis, which
76 improves systolic and diastolic function, as well as the autonomic balance to the
77 heart ("The effect of digoxin on mortality and morbidity in patients with heart
78 failure. The Digitalis Investigation Group" 1997). Digitalis decreases plasma
79 renin activity and consequently alters the levels of Ang II and aldosterone and
80 decreases serum levels of norepinephrine (Gheorghiade and Ferguson 1991).
81 Digitalis inhibits the Na⁺/K⁺/ATPase and may promote an increase in arterial
82 baroreceptor sensitivity (Gheorghiade and Ferguson 1991).

83 However, the mechanisms triggering increased sympathetic activity at
84 different targets in HF and how digitalis decreases sympathetic drive in this
85 condition are not well-understood. The present study aimed to investigate
86 changes in renal sympathetic nerve activity and autonomic cardiovascular
87 function, an investigation that included a spectral analysis of blood pressure and
88 arterial baroreceptor and cardiopulmonary reflex sensitivity, in HF rats
89 chronically treated with digitoxin.

90 **METHODS**

91 All experimental procedures were conducted according to the National
92 Institutes of Health Guidelines for the Use and Care of Animals, and the study
93 protocol was approved by the Ethics in Research Committee of the University
94 Federal of São Paulo (process No. 0442/11). Male Wistar rats (initially weighing
95 200 g) were obtained from the animal care facility of our institution. The animals
96 were housed in group cages, given access to rat chow and water *ad libitum* and

97 maintained in a temperature-controlled environment (23°C) on a 12-h light/dark
98 cycle.

99 **EXPERIMENTAL PROTOCOLS**

100 The experiments were performed with the following three experimental
101 groups: Sham, HF and HF+DIG. Seven days after surgery to induce myocardial
102 infarction, the rats were submitted to Doppler echocardiography, and the rats
103 with infarctions involving a minimum of 40% of left ventricular cardiac mass
104 were selected and divided into two groups as follows: without treatment (HF)
105 and submitted to digitoxin treatment for 60 consecutive days (HF+DIG). At the
106 end of treatment, the mean arterial pressure (MAP), heart rate (HR), baroreflex
107 control of HR and cardiopulmonary reflex were assessed in conscious rats. The
108 spectral analysis of blood pressure was analysed offline (sample rate 2 KHz).
109 The same group of rats was slowly anesthetized with urethane (1.4 g/kg, i.v.)
110 (Sigma-Aldrich Co, St Louis, MO, USA) to avoid any changes in MAP, and
111 basal renal sympathetic nervous activity (rSNA) and arterial baroreceptor
112 control of rSNA were assessed.

113 **SURGICAL PROCEDURE**

114 After isoflurine-induced anaesthesia (2%), the animals were immobilized
115 in the supine decubitus position. A left thoracotomy was performed in the fourth
116 intercostal space, and two perpendicularly positioned murine retractors
117 separated the ribs. Radiofrequency current (RF) lesions were created with a
118 commercially available, conventional RF generator (model TEB RF10;
119 Tecnologia Eletrônica Brasileira Ltda, São Paulo, Brazil). RF current (1000
120 KHz) was delivered at constant power (12 watts) for 12 seconds. The power
121 output was automatically shut down if the impedance exceeded 200 ohms. The

122 damaged area was immediately confirmed by the presence of a clear white,
123 disk-shaped region of coagulation necrosis. Thereafter, the heart was quickly
124 returned to the thorax; pulmonary hyperinsufflation was performed, and a
125 previously made purse string suture was used to close the chest. The above
126 procedures were performed according to a previous report (Antonio et al. 2009).

127 **DOPPLER ECHOCARDIOGRAPHY AND PULMONARY WATER CONTENT**

128 Doppler echocardiographic examinations were performed using an HP
129 SONOS 5500 (Philips Medical System, Andover, MA) with a 12-MHz transducer
130 at a depth of 2 cm according to a previous report (Antonio et al. 2009; Cury et
131 al. 2005; Kanashiro et al. 2006). Briefly, seven days after surgery, the rats were
132 anesthetized with ketamine + xylazine, and 2-dimensional and M-mode images
133 from the parasternal longitudinal, transverse, and apical views were obtained
134 and recorded on a 0.5-inch videotape. The image analysis and measurements
135 were performed offline. ECHO detected MI based on subjective identification of
136 akinesis or dyskinesis. The MIS was determined posteriorly in the stained
137 myocardium at the completion of the protocol as described below. The end-
138 diastolic (LVAd) and end-systolic (LVAs) LV transverse areas were measured in
139 the 3 transverse planes (basal, medium, and apical), and LV systolic function
140 was assessed by the change in fractional area (FAC: $\text{LVAd} - \text{LVAs} / \text{LVAd} \times$
141 100). Diastolic function was assessed by calculating the peak E and A blood
142 flow mitral velocities and the E/A ratio. For this purpose, the sample volume of
143 the pulsed wave Doppler was positioned at the tips of the mitral valve leaflets in
144 an apical 4-chamber view.

145 Pulmonary water content (%) was obtained based on wet and dry
146 weights. After measuring the right lung wet weight, the tissue was placed in an

oven and maintained at 80°C for 72 hours. After measuring the dry weight of each rat lung, the water content was determined using the following equation: (wet weight and dry weight/wet weight) X 100.

COMPOSITION AND DIET SUPPLEMENTED WITH DIGITOXIN

According to a previous report (Helber et al. 2004), the concentration of digitoxin was determined by taking into account the following: (i) orally administered digitoxin is completely absorbed, (ii) a daily dose similar to the one used in the present study was previously shown to be efficient in promoting cardiac benefits in the rat (100 µg/kg per day) when injected subcutaneously, and (iii) young and adult healthy rats in our laboratory eat approximately 30 g of food daily. Digitoxin (1 mg) was diluted in absolute ethyl alcohol (3 mL sprayed in and mixed with standard diet (100 g)). The resultant chow, with digitoxin added and mixed with water, resulted in dough. Chow pellets were made and left to dry for 24 h at 50°C to allow for the complete evaporation of the alcohol.

ANALYSIS OF CARDIOVASCULAR FUNCTION IN CONSCIOUS RATS

For the intravenous injection of drugs and direct arterial pressure recording, the rats were anesthetized with ketamine and xylazine (40 and 20 mg/kg, i.p., respectively) (Vetbrands, Jacareí, Brazil) and fitted with femoral venous and arterial catheters. After ≥ 24 h of surgical recovery, MAP and HR were recorded in conscious rats using an analog-digital board PowerLab (ADInstruments, Australia).

For the analysis of the arterial baroreceptor function in conscious rats, bolus injections (0.1 ml) of phenylephrine (3, 5 and 7 µg/Kg, i.v.) or sodium nitroprusside (1, 2 and 3 µg/kg, i.v.) (Sigma-Aldrich Co, St Louis, MO, USA) were administered with ≥ 5-min intervals between doses until blood pressure

172 returned to baseline. Values of matching MAP variations (Δ MAP from 20 to 55
173 mmHg) with reflex heart rate (Δ HR) responses were separately plotted for each
174 vasoactive drug to create linear regression curves of baroreceptor function for
175 each group, and their slopes (spikes per second per millimetre of Hg) were
176 compared to analyse the changes in baroreflex sensitivity.

177 **SPECTRAL ANALYSIS OF HEART RATE AND SYSTOLIC BLOOD** 178 **PRESSURE**

179 For the spectral analysis of heart rate and systolic pressure variability,
180 beat-by-beat HR and systolic arterial pressure data were obtained in conscious
181 rats for the first 10 min of the recording period. The spectral power of the
182 various frequency components of HR and systolic pressure were calculated with
183 fast Fourier transform (FFT) algorithm. This analysis requires data collection at
184 equal time intervals. Therefore, heart rate and systolic pressure were calculated
185 every 100 ms with a cubic spline interpolation (10 Hz). The interpolated series
186 were divided into half-overlapping sequential sets of 512 data points (51.2 s).
187 The segments were inspected visually; non-stationary data were not analysed.
188 A Hanning window was used to attenuate the side effects. The power intensity
189 was computed with a direct FFT algorithm for discrete time series. The total
190 power in the low frequency band (LFb: 0.2-0.75 Hz) and high frequency band
191 (HFb: 0.75-3 Hz) was calculated. The LFb/HFb power ratio was calculated and
192 used as an indicator of cardiac sympathovagal balance (Montano et al. 1994).

193 **CARDIAC AUTONOMIC TONE AND INTRINSIC HEART RATE**

194 Tonic sympathetic and vagal influences to the heart were determined by
195 measuring HR changes after the selective pharmacological blockade of cardiac
196 autonomic receptors. The bradycardic response obtained after a β -adrenergic

receptor blockade with atenolol (1 mg/kg, i.v. - Sigma-Aldrich Co, St Louis, MO, USA) was used to estimate sympathetic tone. The tachycardia response after muscarinic cholinergic receptor blockade with methyl atropine (3 mg/kg, i.v. - Sigma-Aldrich Co, St Louis, MO, USA) was used to estimate vagal tone. The HR in the presence of both autonomic blockers was considered the intrinsic HR.

ANALYSIS OF THE BEZOLD-JARISCH REFLEX SENSITIVITY

In conscious rats, bolus injections (0.1 ml) of phenylbiguanide (PBN) (12.5, 25, 50 µg/Kg) were administered with $\geq$ 15-min intervals between doses until blood pressure returned to baseline. The percentages of MAP variation (% Δ MAP) and HR variation (% Δ HR) were compared among the groups.

ANALYSIS OF RENAL SYMPATHETIC NERVE ACTIVITY IN URETHANE ANESTHETIZED RATS

Rats were slowly anesthetized with urethane, and the left renal nerve was retroperitoneally exposed and placed on bipolar silver electrodes. When the conditions for nerve recording were established, the nerve and electrode were covered with paraffin oil. The signal from the renal nerve was displayed on an oscilloscope (Tektronix, TDS 220), and nerve activity was amplified (gain 20000, Neurolog, Digitimer, UK), filtered by a band-pass filter (100–1000 Hz), and collected for display and subsequent analysis using a PowerLab data acquisition system (ADInstruments, Australia). At the end of the experiments, the background noise level was determined following hexamethonium bromide (30 mg/kg, i.v.) (Sigma-Aldrich Co, St Louis, MO, USA) administration. The rSNA was rectified online, integrated from the raw data obtained for each heart period, and expressed as volts-seconds (V.s). Additionally, neural activity was analysed offline using the appropriate software (Spike Histogram,

ADInstruments - Australia). The responses of rSNA to the various stimuli are expressed as the percentage of change compared with the basal value obtained immediately before each test. For this purpose, the raw nerve signal was passed through a spike discriminator (PowerLab) to remove background noise, and the total nerve activity, which is expressed in spikes per second (spikes/s), was computed from the time at which the value changed from the basal value to when it returned to the basal value. The basal rSNA is expressed as spikes/s over a period of 60 s. The mean value obtained was compared with the mean value determined before each test as reported previously. Only experiments in which the level of background noise was confirmed at the end of the experiments following hexamethonium and terminal anaesthesia are included in this report.

For the analysis of arterial baroreceptor function sensitivity for rSNA control in anesthetized rats, continuous infusions of phenylephrine (10 µg/Kg, 0.2 mL, 12 mL/h, i.v.) or sodium nitroprusside (20 µg/kg, 0.1 mL, 6 mL/h, i.v.) (Sigma-Aldrich Co, St Louis, MO, USA) were administered with a 1 ml syringe mounted on a syringe pump (model KDS 100, KD Scientific). Values of matching MAP variations (Δ MAP from 20 to 55 mmHg) with reflex renal sympathetic nerve activity (Δ rSNA) responses were separately plotted for each vasoactive drug to create linear regression curves of baroreceptor function for each group, and their slopes (spikes per second per millimetre of Hg) were compared to analyse the changes in baroreflex sensitivity.

STATISTICAL ANALYSES

The results are shown as the mean $\pm$ standard error of the mean for functional experiments, and the data of the spectral analyses are shown as the

mean $\pm$ standard deviation of the mean. Data were evaluated by one-way ANOVA, followed by the Tukey test. The level of statistical significance was defined as $p<0.05$.

RESULTS

DOPPLER ECHOCARDIOGRAPHY AND PULMONARY WATER CONTENT

Table 1 shows the echocardiogram data, the other parameters of cardiac remodelling and the pulmonary water content. All rats used in this study had an area of infarction greater than 40%. The HF group showed an increase in systolic area (0.30 cm^2 , $n=9$) and systolic circumference (1.81 cm , $n=9$) compared with the Sham group (systolic area: 0.07 cm^2 ; systolic circumference: 0.86 cm , $n=5$). In this case, treatment with digitoxin did not improve these parameters (systolic area: 0.41 cm^2 ; systolic circumference: 2.18 cm , $n=5$). HF ($80.8 \pm 0.2\%$, $n= 9$) and the HF+DIG group ($80.6 \pm 0.1\%$, $n= 5$) presented with an increase in pulmonary water content compared with the Sham group ($78.1 \pm 0.3\%$, $n= 5$).

CARDIOVASCULAR AND AUTONOMIC BASAL PARAMETERS

The HF rats showed decreased MAP ($91 \pm 5.0\text{ mmHg}$, $n=5$) and HR ($314 \pm 9.8\text{ bpm}$, $n=8$) compared with the Sham group (MAP: $109 \pm 3\text{ mmHg}$, $n=5$; HR: $350 \pm 12.7\text{ bpm}$, $n=6$). Digitoxin treatment did not change these values in the HF+DIG group (MAP: $92 \pm 2.1\text{ mmHg}$, $n=7$; HR: $296 \pm 7\text{ bpm}$, $n=9$), as shown in **Figure 1A** and **B**. The HF rats presented with increased rSNA ($190 \pm 30\text{ pps}$, $n=5$) compared with the Sham rats ($98 \pm 14.7\text{ pps}$, $n=5$). Digitoxin treatment significantly decreased and normalized rSNA in the HF+DIG group ($98 \pm 14.3\text{ pps}$, $n=7$), as shown in **Figure 1C**. **Figure 1D** shows a representative

tracing of the cardiovascular and autonomic parameters in the Sham, HF and HF+DIG groups.

EFFECT OF TREATMENT HEART RATE AND SYSTOLIC PRESSURE VARIABILITY

A spectral analysis of HR variability (HRV) showed an increase in LFb in the HF group ($23 \pm 5 \text{ ms}^2$, $n=8$) compared with the Sham group ($14 \pm 3 \text{ ms}^2$, $n=9$); treatment with digitoxin normalized this parameter in the HF+DIG group ($15 \pm 3 \text{ ms}^2$, $n=9$). The HF group also showed decreased HFb ($77 \pm 5 \text{ ms}^2$, $n=8$) compared with the Sham group ($86 \pm 3 \text{ ms}^2$, $n=9$); digitoxin normalized this parameter in the HF+DIG group ($85 \pm 3 \text{ ms}^2$, $n=9$). The results are summarized in **Table 2**.

A spectral analysis of systolic blood pressure variability showed an increase in LFb in the HF group ($6 \pm 1 \text{ mmHg}^2$, $n=8$) compared with the Sham group ($5 \pm 2 \text{ mmHg}^2$, $n=9$); digitoxin treatment decreased this parameter in the HF+DIG group ($2 \pm 0.3 \text{ mmHg}^2$, $n=9$). The HF+DIG group also showed a decrease in HFb ($1 \pm 0.3 \text{ mmHg}^2$, $n=9$) compared to HF group ($3 \pm 0.4 \text{ mmHg}^2$, $n=8$) and Sham group ($2 \pm 0.4 \text{ mmHg}^2$, $n=9$). The results are summarized in **Table 2**.

ARTERIAL BAROREFLEX SENSITIVITY:

A preferential decrease in baroreflex sensitivity for HR control induced by phenylephrine (baroreceptor loading) was observed in the HF and HF+DIG groups compared with the Sham group, as shown in **Figure 2 A**, and the individual slope values are shown in **Table 3**. However, no difference among the groups was found in relation to baroreceptor sensitivity for rSNA control, as shown in **Figure 2 B**, and the individual slope values are shown in **Table 3**.

296

297 **BEZOLD-JARISCH REFLEX:**

298 The HF and HF+DIG groups showed a decrease in $\% \Delta \text{MAP}$ responses in
299 all doses of PBN (HF: 12.5 μg : -17.2 ± 3.6 mmHg; 25 μg : -27.6 ± 3.8 mmHg; 50
300 μg : -30.3 ± 3.4 mmHg, $n=11$; HF+DIG: 25 μg : -26.4 ± 4.8 mmHg, $n=12$)
301 compared with the Sham group (12.5 μg : -34.5 ± 3 mmHg; 25 μg : -47.4 ± 5.6
302 mmHg; 50 μg : -53.3 ± 4 mmHg, $n=7$), as shown in **Figure 3 A**. The HF group
303 also showed a decrease in $\% \Delta \text{HR}$ at intermediate and highest doses of PBN
304 (25 μg : -52.2 ± 3 bpm, $n=8$ and 50 μg : -56.3 ± 2.5 bpm, $n=8$) compared with the
305 Sham group (25 μg : -71 ± 2.5 bpm, $n=4$ and 50 μg : -72.5 ± 2.2 bpm, $n=4$), and
306 digitoxin treatment (HF+DIG) promoted a decrease in $\% \Delta \text{HR}$ for the same
307 doses of PBN (25 μg : -40.4 ± 2.8 bpm, $n=9$ and 50 μg : -40 ± 3.7 bpm, $n=9$)
308 compared with the HF group, as shown in **Figure 3 B**.

309

310 **DISCUSSION**

311 The major new findings of the present study were as follows: 1) digitoxin
312 treatment prevented increases in rSNA and alterations of the autonomic
313 balance to the heart and blood vessels in the HF-treated group (HF+DIG) and
314 2) these improvements in cardiovascular function were independent of cardiac
315 remodelling and peripheral cardiovascular reflexes, including the arterial
316 baroreceptor and cardiopulmonary reflexes.

317 The mechanisms underlying sympathoexcitation in HF are not well-
318 understood. Some hypotheses suggest that reflex sympathoexcitation is a
319 consequence of decreased blood pressure and increased circulating and tissue

320 Ang II (DiBona et al. 1995) or that sympathoexcitation occurs even in response
321 to a decrease in arterial baroreflex sensitivity. These processes may result in a
322 reduction in the inhibitory influence on afferent vasomotor centres in the brain,
323 resulting in neuro-humoral efferent sympathetic outflow (Ferguson et al. 1984*b*).

324 Considering that increased sympathetic activity to the kidneys leads to
325 important changes in kidney function, including renin secretion, vasoconstriction
326 and sodium reabsorption (DiBona et al. 1995) and that all of these changes are
327 present in HF, new strategies to control autonomic dysfunction in HF are
328 important for the outcome of the disease.

329 In the present study, we observed a preferential decrease in
330 baroreceptor reflex sensitivity for HR but not for rSNA in the HF rats compared
331 with the Sham group. These data suggest that the increased rSNA in the HF
332 group was not directly related to changes in baroreceptor reflex sensitivity, as
333 previously reported (Ferguson et al. 1984*a*).

334 The decrease in MAP in the HF rats could trigger other compensatory
335 mechanisms, such as renin-angiotensin-aldosterone system activation, which
336 could contribute to increases in rSNA, as proposed previously (DiBona et al.
337 1995). However, in the present study, a significant decrease in rSNA was found
338 in the HF+DIG group, suggesting that sympathoexcitation is not related to low
339 blood pressure because the HF+DIG and HF groups had similar blood pressure
340 levels. Changes in the central nuclei in the brain related to cardiovascular
341 control could be involved in the increases in rSNA; we also noted an imbalance
342 in the autonomic control of the heart with an increase in LFb and a decrease in
343 HFb in the HF rats. Wang et al. (Wang et al. 2009) found that microinjections of
344 a glutamate receptor antagonist into the rostralventrolateral medulla (RVLM) of

345 HF rats significantly decreased MAP and rSNA, suggesting that increased
346 glutamate receptor expression in the RVLM contributes to increased
347 sympathetic vasomotor drive in HF.

348 However, there is controversy regarding baroreceptor dysfunction in HF.

349 The divergence is related to the animal model used for HF induction, the
350 extension of myocardial infarction and the time after myocardial infarction at
351 which the experiments were performed. According to Ramchandra et al.
352 (Ramchandra et al. 2014), baroreflex control for HR and rSNA, but not for
353 cardiac sympathetic nerve activity (cSNA), was impaired in sheep with HF.
354 However, Zucker et al. (Zucker et al. 1985) reported that arterial baroreceptor
355 sensitivity for rSNA in dogs with HF was normal compared with control animals.
356 This controversy is not limited only to HF animal models. Floras et al. (Floras,
357 2001) found that humans with advanced HF had decreased baroreceptor reflex
358 sensitivity relative to HR control but not to muscle ANS compared with healthy
359 subjects. Others (Dibnerdunlap and Thames 1989; Grassi et al. 1995) noted
360 decreased sensitivity of baroreceptor reflex responses to muscle sympathetic
361 nerve activity in humans with HF.

362 Together, these studies suggest a territory-dependent difference in
363 baroreceptor reflex sensitivity in HF in different species. The same was found in
364 the present study: a decrease was noted in the sensitivity of the baroreceptor
365 reflex for HR control but not for of rSNA control.

366 However, this was the first study to investigate baroreceptor function in a
367 model of HF induced by ablation. The ablation model of HF was chosen due to
368 its low mortality rates and its low variability in the size of the infarct area among
369 animals. In addition, this model does not cause papillary muscle injury, as noted

370 in the occlusion model, and thus does not lead to changes in cardiac valves that
371 could hamper the interpretation of the results. In addition, as presented in Table
372 1 (echocardiography parameters), this model has specific characteristics of HF,
373 as described in other models of HF animals (dos Santos et al. 2008; Moisés et
374 al. 2000).

375 We noted significant impairment of the cardiopulmonary reflex in HF
376 animals; a similar finding was previously reported (Modesti et al. 2004).
377 Ferguson et al. (Ferguson et al. 1984b) found that the decreased sensitivity of
378 the cardiopulmonary and arterial baroreceptor reflexes may result in decreased
379 afferent inhibitory influence on the vasomotor centres of the brain, resulting in
380 increased sympathetic efferent and neurohumoral excitation. Thus, we can infer
381 that the reduction in the sensitivity of the cardiopulmonary reflex is one of the
382 factors that may trigger increased rSNA. However, digitoxin treatment
383 significantly reduced rSNA and caused further impairment of the
384 cardiopulmonary reflex, suggesting that rSNA reduction induced by digitoxin in
385 HF rats is not directly related to the cardiopulmonary reflex.

386 Digitoxin sensitizes the vagal afferents mediating the cardiopulmonary
387 reflex, leading to a reduction in rSNA in dogs (Thames et al. 1982). These
388 findings could have implications concerning the potential mechanism of action
389 of digitalis in heart failure. However, in the present study, there was no
390 correlation between the sympathoinhibition induced by digitoxin and the
391 cardiopulmonary reflex. Therefore, the improvement in survival induced by
392 digitoxin in HF rats could be related to rSNA reduction (Helber and Tucci 2010).

393 Another interesting result was that the animals treated with digitoxin
394 showed no improvement in cardiac remodelling, as shown by the

395 echocardiographic data in Table 1. However, there was improvement and
396 normalization of autonomic balance to the heart and blood vessels, as shown
397 by the HRV results in Table 2. These results demonstrate that digitoxin has
398 cardioprotective effects and promotes the reduction of sympathetic vasomotor
399 drive. Moreover, we believe that digitalis influences autonomic modulation, most
400 likely through central actions in the brain. In addition, digitoxin may act directly
401 on the sinus node, improving the autonomic balance to the heart because
402 digitoxin acts on the $\text{Na}^+/\text{K}^+/\text{ATPase}$, which is present in all cells. In excitable
403 tissues, its activity is essential for regulating membrane potential and is
404 responsible for cellular excitability (Teruya et al. 1997). However, the relative
405 importance of the central versus peripheral actions of digitoxin is still a matter of
406 discussion.

407 Although digitoxin has a very low therapeutic range and can be extremely
408 toxic, we used a study previously conducted in our laboratory as a reference
409 (Helber, et al. 2004) as this study described the importance of serum digitoxin
410 and the mortality rates of infarcted animals. Furthermore, according
411 Wasserstrom and Aistrup (2005), rats are less sensitive to cardiac glycoside
412 due to resistance in alpha isoform 1; therefore, the dose used in this study was
413 higher than that used in humans but was not toxic to the animals (Helber, et al.
414 2004).

415 Conclusion:

416 In the present study, digitoxin reduced and normalized rSNA and
417 improved the autonomic balance to the heart in HF rats; similar data have been
418 previously described (Gheorghiade and Ferguson 1991; Mason and Braunwald
419 1964). However, the mechanism underlying the sympathoinhibition induced by

420 digitoxin remains unknown. Because the HF animals treated with digitoxin
421 showed no improvement in the sensitivities of their arterial and cardiopulmonary
422 baroreceptor reflexes, it is possible that digitoxin acts in the brain to reduce
423 sympathetic vasomotor drive to the kidneys and heart.

424

425

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430

431 **DISCLOSURE**

432 The authors declare no conflicts of interest.

433

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- 540

FIGURE LEGENDS

542

543 **Table 1.** Doppler echocardiographic parameters for the Sham, Heart
544 Failure (HF) and HF treated with digitoxin (HF+DIG) groups. Values are the
545 mean $\pm$ standard error of the mean. * $p < 0.05$ compared with the Sham group.
546 One-way ANOVA followed by Tukey post-test.

547

548 **Figure 1. (A)** Mean Arterial Pressure (MAP). **(B)** Heart Rate (HR). **(C)**
549 renal Sympathetic Nerve Activity (rSNA). **(D)** Typical tracings of MAP, HR and
550 rSNA. Sham and Heart Failure (HF) untreated and treated (HF+DIG). Values
551 are the mean $\pm$ standard error of the mean. * $p < 0.05$ compared with the Sham
552 group; # $p < 0.05$ compared with the Heart Failure (HF) group. One-way ANOVA
553 followed by Tukey post-test.

554

555 **Table 2.** Blood pressure and pulse interval variability for the Sham, Heart
556 Failure (HF) and HF treated with digitoxin (HF+DIG) groups. Values were
557 compared and are presented as the mean $\pm$ standard deviation. ANOVA test
558 followed by Tukey post-test. * $p < 0.05$ vs Sham and # $p < 0.05$ vs HF.
559 Abbreviations: LFb = Low frequency band, HFb = High frequency band.

560

561 **Figure 2. (A)** Baroreceptor reflex function for heart rate (HR) control.
562 Linear regression graph containing **(1)** reflex increases in HR in response to
563 decreases in mean arterial pressure (MAP) produced by bolus injections of (0.1
564 ml) sodium nitroprusside (1, 2 and 3 $\mu\text{g/kg}$, i.v.) and **(2)** reflex decreases in HR
565 in response to increased MAP produced by bolus injections of (0.1 ml)

phenylephrine (3, 5 and 7 µg/Kg, i.v.). **(B)** Baroreceptor reflex function for renal sympathetic nerve activity (rSNA) control. Nonlinear regression graph containing **(1)** reflex increases in rSNA in response to decreases in mean arterial pressure (MAP) produced by continuous infusions of sodium nitroprusside (20 µg/kg, 0.1 mL, 6 mL/h, i.v.) and **(2)** reflex decreases in rSNA in response to increased MAP produced by continuous infusions of phenylephrine (10 µg/Kg, 0.2 mL, 12 mL/h, i.v.). Values are the mean ± standard error of the mean. **p*<0.05 compared with the Sham group before digitoxin; and #*p*<0.05 compared with the Heart Failure (HF) group before digitoxin. **p*<0.05 compared with the Sham group. One-way ANOVA followed by Tukey post-test.

Table 3. Slope values obtained from the linear and nonlinear regression lines of the baroreceptor reflex function control for heart rate (HR) and of the renal sympathetic nerve activity (rSNA), respectively, for the Sham, Heart Failure (HF) and HF treated with digitoxin (HF+DIG) groups. Values are the mean ± standard error of the mean. **p*<0.05 compared with the Sham group. One-way ANOVA followed by Tukey post-test.

Figure 3. Bezold-Jarisch reflex. **(A)** Percentage of mean arterial pressure variation (%ΔMAP) induced by bolus injection (0.1 ml) of phenylbiguanide (PBN) (12.5, 25, 50 µg/Kg). **(B)** Percentage of heart rate variation (%ΔHR) induced by bolus injection (0.1 ml) of phenylbiguanide (PBN) (12.5, 25, 50 µg/Kg). Values are the mean ± standard error of the mean. **p*<0.05 compared with the same dose of phenylbiguanide (PBN) in the Sham group; #*p*<0.05 compared with the

590 same dose of PBN in the Heart Failure (HF) group. One-way ANOVA followed
591 by Tukey post-test.
592

Draft

183x213mm (300 x 300 DPI)

161x250mm (300 x 300 DPI)

222x70mm (300 x 300 DPI)

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Parameters	Groups		
	Sham (N= 5)	HF (N= 9)	HF+DIG (N= 5)
Myocardial Infarction Size	0%	47%	47%
Pulmonary water content	78,1 ± 0,3%	80,8 ± 0,2%*	80,6 ± 0,1%*
Fractional Shortening of the Transverse Area	0,73 ± 0,008%	0,44 ± 0,02%*	0,37 ± 0,06%*
Systolic area	0,07 ± 0,006 cm ²	0,30 ± 0,03 cm ² *	0,41 ± 0,06 cm ² *
Systolic circumference	0,86 ± 0,1 cm	1,81 ± 0,1 cm*	2,18 ± 0,1 cm*

Table 1. Doppler echocardiographic parameters for the Sham, Heart Failure (HF) and HF treated with digitoxin (HF+DIG) groups. Values are the mean ± standard error of the mean. *p<0.05 compared with the Sham group. One-way ANOVA followed by Tukey post-test.

	Sham (N= 9)	HF (N= 8)	HF+DIG (N= 9)
Pulse Interval			
LFb (ms ²)	14 ± 3	23 ± 5*	15 ± 3 [#]
HFb (ms ²)	86 ± 3	77 ± 5*	85 ± 3 [#]
LFb/HFb	0.2 ± 0.05	0,4 ± 0.2*	0.2 ± 0.04 [#]
Systolic Blood Pressure			
LFb (mmHg ²)	5 ± 2	6 ± 1	2 ± 0,3* [#]
HFb (mmHg ²)	2 ± 0.4	3 ± 0.4*	1 ± 0.3* [#]

Table 2. Blood pressure and pulse interval variability for the Sham, Heart Failure (HF) and HF treated with digitoxin (HF+DIG) groups. Values were compared and are presented as the mean ± standard deviation. ANOVA test followed by Tukey post-test. *p<0.05 vs Sham and [#]p<0.05 vs HF. LFb = Low frequency band, HFb = High frequency band.

Reflex responses for heart rate		
Groups	Pressor response Slope (pps/mmHg)	Depressor response Slope(pps/mmHg)
Sham	-2.27 ± 0.23, N= 6	-3.17 ± 0.32, N= 6
HF	-1.24 ± 0.07, N= 8*	-3.54 ± 0.27, N= 8
HF+DIG	-1.18 ± 0.09, N= 9*	-2.86 ± 0.25, N= 9

Reflex responses of renal sympathetic nerve activity		
Grupos	Pressor response Slope (pps/mmHg)	Depressor response Slope(pps/mmHg)
Sham	-1.94 ± 0.45, N= 5	-0.82 ± 0.15, N= 5
HF	-2.62 ± 0.30, N= 7	-0.81 ± 0.35, N= 5
HF+DIG	-1.63 ± 0.49, N= 7	-0.80 ± 0.08, N= 7

Table 3. Slope values obtained from the linear regression line of the baroreceptor reflex function control for heart rate (HR) and renal sympathetic nerve activity (rSNA) for the Sham, Heart Failure (HF) and HF treated with digitoxin (HF+DIG) groups. Values are the mean ± standard error of the mean. *p<0.05 compared with the Sham group. One-way ANOVA followed by Tukey post-test.