Effects of Chronic Cholinergic Stimulation Associated With Aerobic Physical Training on Cardiac Morphofunctional and Autonomic Parameters in Spontaneously Hypertensive Rats

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Abstract

We investigated in spontaneously hypertensive rats (SHR) the hemodynamic, cardiac morphofunctional, and cardiovascular autonomic adaptations after a protocol of aerobic physical training associated with chronic cholinergic stimulation. Fifty-four SHR were divided into two groups: trained and untrained. Afterward, each group was subdivided into three smaller groups: vehicle, treated with pyridostigmine bromide at 5mg/kg/day, and at 15mg/kg/day. The following protocols were assessed: echocardiography, autonomic double pharmacological blockade, analysis of heart rate variability (HRV), blood pressure variability (BPV), and baroreflex sensitivity (BRS). Physical training and pyridostigmine bromide reduced blood pressure and heart rate and increased vagal participation in cardiac tonic autonomic balance. Associated the responses were potentialized. Pyridostigmine bromide increased the oscillation of low frequency (LF:0.2-0.75Hz) and high frequency (HF:0.75-3Hz) of HRV. However, the association with physical training attenuated HF oscillations. Pyridostigmine bromide also increased LF oscillations of BPV. Both treatments promoted morphofunctional adaptations and associated increased the ejection volume, ejection fraction, cardiac output, and cardiac index. In conclusion, the association of pyridostigmine bromide and physical training promoted greater benefits in hemodynamic parameters and increase vagal influence on cardiac autonomic tonic balance. Nonetheless, pyridostigmine bromide alone seems to negatively affect BPV, while the association of treatment negatively influences HRV.

Introduction

Systemic arterial hypertension is accompanied by important cardiovascular autonomic impairments, characterized by a decrease in baroreflex sensitivity (BRS) and changes in heart rate (HRV) and blood pressure variability (BPV) ^{1,2}. These autonomic impairments are associated with cardiovascular morphological and functional changes, and when they are not identified and treated promptly, might contribute to the development of heart failure, reducing quality and life expectancy.

Thus, the search for new pharmacological and non-pharmacological therapies with cardioprotective actions is of great relevance to the aim of preventing and/or reversing the development and evolution of hypertension ³⁻⁵. In this case, clinical and experimental studies have demonstrated that pyridostigmine bromide, a drug regularly used to treat myasthenia gravis, promotes cardiac and cardiovascular autonomic benefits by reducing the heart rate (HR) at rest ^{6,7} and enhancing HRV ⁸⁻¹¹. On the other hand, lifestyle modifications, such as regular physical exercise, also promote important benefits in cardiovascular autonomic control, in addition to positive cardiac remodeling ^{4,12}.

In fact, some cholinergic stimulation effects of pyridostigmine bromide on cardiac autonomic control are similar to those observed in aerobic physical training, both in human and experimental models ^{5,10,13,14}. An experimental study in spontaneously hypertensive rats (SHRs) showed that cholinergic stimulation with pyridostigmine bromide for two weeks reduced blood pressure (BP) and HR. However, the ejection fraction, a parameter evaluated by radioisotopic ventriculography, was also reduced. Additionally, autonomic parameters showed an increase in vagal participation in autonomic tonic balance and a reduction in systolic BPV ⁹.

The hemodynamic and autonomic improvements are important and suggest a beneficial effect; however, the reduction of ejection fraction is worrying and might be related to the pyridostigmine bromide dosage used in the study (25 mg/kg/day), or perhaps, to the methodology employed to investigate the ejection fraction. However, with only one functional parameter evaluated, it was not possible to precisely state the real effects of pyridostigmine bromide on cardiac function. In this case, our hypothesis was that treatment with low doses of pyridostigmine bromide at 5 mg/kg/day and 15 mg/kg/day in SHRs would promote positive hemodynamic and cardiovascular autonomic effects, in addition to changes in morphological and functional cardiac parameters but would not affect the ejection fraction. In turn, the association with aerobic physical training will promote a catalytic effect, possibly increasing the gains obtained with pyridostigmine bromide.

Materials And Methods

Animals and procedures

Fifty-four SHR 18-week-old subjects were divided into two treatment groups: untrained (n = 27) and trained (n = 27). Each group was subdivided into three smaller groups (n = 9): vehicle (H₂O), pyridostigmine bromide (Sigma-Aldrich, Saint Louis, MO, USA) diluted in drinking water at a dose of ~5 mg/kg/day, and pyridostigmine bromide diluted in drinking water at a dose of ~15 mg/kg/day for 2

weeks, between the 11th and 12th weeks of aerobic physical training. The dose was determined based on the results of a previous study⁹. During the experiments, the animals were housed in the Animal Facility at the Ribeirão Preto Medical School, which was maintained at 23°C and 60%–70% humidity. The rats were kept on a 12/12-hour light/dark cycle and had free access to food and water. The experimental protocols used in the present study were in accordance with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation and were evaluated and approved by the Animal Experimentation Ethics Committee (CETEA) of the Ribeirão Preto Medical School, University of São Paulo (Protocol 035/2014). The study was carried out in compliance with the ARRIVE guidelines.

On day 15 of treatment (with pyridostigmine bromide 5 or 15 mg/kg or only H₂O), polyethylene catheters (PE-10/PE-50, Intramedic; Becton Dickinson and Company, Sparks, MD, USA) were implanted into the left femoral artery and vein under ketamine (5 mg/kg, intraperitoneal [i.p.]; Sigma-Aldrich, USA) and xylazine (30 mg/kg, intraperitoneal [i.p.]; Sigma-Aldrich, USA) anesthesia to record pulsatile BP and to administer drugs, respectively. The catheters were subcutaneously tunneled and exteriorized in the nape. Twenty-four hours after the surgical procedures, BP was measured in conscious rats kept in a quiet environment. The BP was recorded using a pressure transducer (MLT0380; AD Instruments, Bella Vista, Australia), and the amplified signal (ML110; AD Instruments, Bella Vista, Australia) was fed to a computer acquisition system (PowerLab 8/30; AD Instruments, Bella Vista, Australia). The mean BP (MBP) and HR were calculated from the pulsatile BP.

Physical Training

The rats in the training groups underwent a protocol of aerobic physical training that consisted of swimming sessions in a glass tank (100 cm long × 80 cm wide × 80 cm high), which allowed for the simultaneous training of six animals. The tank was filled with 50 cm of warm water (30 ± 2°C), which was changed after every group training session. The training program was conducted in two different stages over a total of 12 (from 18 to 30 weeks of age). The first stage consisted of a two-week adaptation period, during which the session length was gradually increased from 5 to 30 min per day, five times per week (in increments of 5 min per day). The second stage consisted of 10 weeks, with 30 min of physical training sessions conducted five times per week¹⁵. To evaluate physical training intensity, blood was collected from the tail veins of the animals at the fourth, seventh, and tenth weeks immediately before and after the 30-minute exercise sessions, and the lactate concentration was measured (Accutrend® Plus, Roche Diagnostics, Mannheim, Germany). The expected lactate level ranged from 5.5 to 6 mmol/L, as previously determined. If the animals did not achieve the expected lactate concentration, the level of training exertion was increased by fastening a leaded, impermeable Velcro strap to the chest to increase body weight by 2%–6%¹⁶.

Echocardiography

At 30 weeks of age, all animals underwent an echocardiographic evaluation. We used a Vevo 2100® High-Resolution Imaging System ultrasound (VisualSonics, Toronto, ON, Canada) instrument with a high-resolution transducer (21 MHz). For the procedure, the anterior regions of the thorax were previously trichotomized (Veet®, Reckitt Benckiser, São Paulo, SP, Brazil), and all animals were anesthetized with 1.5% isoflurane supplemented with 1% O₂ and placed on a heated (37°C) platform. Echocardiography and temperature measurements were monitored.

High-resolution B-mode and M-mode images were acquired. Wall thickness and left ventricle dimensions were obtained from a short-axis view at the level of the papillary muscles. Diastolic measurements were performed at the point of maximum cavity dimension, and systolic measurements were performed at the point of minimal cavity dimension. All measurements were performed according to the standards of the American Society of Echocardiography and by an evaluator who was blinded to which group the rats were assigned at the time of measurement¹⁷. The following parameters were obtained from the images: interventricular septum thickness (IVST), posterior wall thickness (PWT), end-diastolic diameter of the left ventricle (LVEDD), and end-systolic diameter of the left ventricle (LVESD). The shortening fraction was calculated as $FS\ Irb\% = [(LVEDD - LVESD) \div LVEDD] \times 100$, and the ejection fraction (EF) was calculated using the Teichholz method: $[(LVEDV - LVESV) \div LVEDV] \times 100$. The left ventricle mass (LV mass/final body weight) was calculated using the formula: $1.047 \times [(LVEDD + PWT + IVST)^3 - LVEDD^3]$, and the relative wall thickness (RWT) was calculated as follows: $[2 \times PWT \div LVEDD]$. Left ventricular volumes were quantified using the following formula: $LVEDV\ (\mu L) = [LVEDD^3 \times (7 \div 2.4 + LVEDD^3)]$ and $LVESV\ (\mu L) = [LVESD^3 \times (7 \div 2.4 + LVESD^3)]$ ^{15,18,19}.

Surgical procedure

Forty-eight hours after echocardiography, the rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and polyethylene catheters made in our laboratory (PE-50 spliced by melting to PE-10; Intramedic, Clay Adams, Parsippany, New Jersey, USA) were implanted into the left femoral artery and vein. Catheters were tunneled subcutaneously and exteriorized at the nape. To prevent blood clotting, the catheters were filled with heparinized saline solution (500 IU/mL). The rats were then allowed to recover for 24 hours before the cardiac sympathovagal assessment protocol, which was performed without anesthesia.

Experimental protocols

Heart rate variability and systolic blood pressure variability

Twenty-four hours after the surgical procedure, arterial pulse (AP) pressure was measured in conscious rats kept in a quiet environment. BP was recorded using a pressure transducer (MLT0380, ADInstruments). Additionally, the amplified signal (ADInstruments ML110) was fed to a computer acquisition system (LabChart 7 Pro). MBP and heart rate (HR) were calculated from the arterial pulse pressure.

HR (pulse interval) and systolic BPV analysis were performed using custom computer software (CardioSeries v2.4, Dias, DPM, University of São Paulo, Brazil <http://sites.google.com/site/cardioseries>)²⁰. The software was designed to perform time-frequency analysis of cardiovascular variability, allowing precise adjustment of parameters related to frequency domain analysis (e.g., interpolation rate, segment length, and boundaries of frequency bands). Because the software does not perform data sampling, a beat-by-beat time series must be generated and loaded into the CardioSeries software. The BP baseline and pulse interval (PI) series, obtained from 60-minute recordings, were processed using computer software (LabChart v7.0, ADInstruments, Bella Vista, Australia) that applies an algorithm to detect cycle-to-cycle inflection points in the pulsatile BP signal, thus determining beat-by-beat values of systolic blood pressure. Beat-by-beat PI series were generated from the pulsatile BP signal by measuring the time interval between adjacent systolic peaks. Next, a beat-by-beat series of PI and SBP were converted to data points every 100 ms using cubic spline interpolation (10 Hz). The interpolated series were divided into half-overlapping sequential sets of 512 data points (51.2 seconds), which were tested for stationarity. It is important to mention that cardiovascular variability analysis requires at least a weakly stationary data series (i.e., mean and covariance stable over time; Berntson *et al.*, 1997). Data series stationarity can be verified by means of stationarity tests (i.e., enhanced reproducibility of the results among users and laboratories²², as well as through visual inspection of the data series^{23,24}.

In our study, a well-experienced researcher visually inspected the segments of interpolated time series (i.e., PI or SBP values) searching for transients that could affect the calculation of the power spectral density (PSD). To confirm that the visual inspection of the time series was properly performed, a Hanning window was used to attenuate side effects, and the spectrums of all segments were calculated using a direct fast Fourier transform algorithm for discrete time series. All segments were visually inspected for abnormal spectra. Finally, considering the results from the time series and spectra inspections, non-stationary data were not taken into consideration for PSD calculation. The spectra were integrated in low-frequency (LF; 0.2–0.75 Hz) and high-frequency (HF; 0.75–3 Hz) bands, and the results were expressed in absolute (ms^2 or mmHg^2) and normalized units (nu). The normalized values were created by calculating the percentage of LF and HF power of the total spectrum power minus the very low frequency band (VLF; < 0.2 Hz) and power^{24,25}. To assess the sympathovagal balance, the LF/HF ratio of PI variability was calculated²⁶.

Spontaneous baroreflex sensitivity

Baroreflex sensitivity (BRS) was assessed in the time domain using the sequence technique, as described by Di Rienzo *et al.* (1985). Custom computer software (CardioSeries v2.4, <http://sites.google.com/site/cardioseries>) scanned the beat-by-beat time series of SBP and PI, searching for sequences of at least four consecutive beats in which increases in SBP were followed by PI lengthening (up sequence) and decreases in SBP followed by PI shortening (down sequence) with a linear correlation higher than 0.8. The slope of the linear regression lines between SBP and PI was used as a measure of spontaneous BRS²⁸.

Assessment of cardiac sympathovagal balance

The influence of sympathetic and parasympathetic autonomic tone on HR was assessed by administering propranolol (5 mg/kg, intravenous [i.v.], Sigma-Aldrich, USA) and methylatropine (4 mg/kg, i.v.; Sigma-Aldrich, USA), respectively. For this purpose, the femoral artery catheter was attached to a pressure transducer (MLT844, AD Instruments, Bella Vista, Australia), which converts the

AP fluctuations into electrical signals. Next, signals were amplified using a bridge amplifier (FE117, AD Instruments, Bella Vista, Australia), and pulsatile AP was continuously sampled (2 kHz) using a computer equipped with an analog-digital interface (ML866, AD Instruments, Bella Vista, Australia). After 60 minutes of basal HR recording, methylatropine was injected into half of the rats in each group, and HR was recorded for the following 15 min to assess the effect of vagal blockade on HR. Propranolol was then injected into the same rats, and HR was recorded for another 15 min to determine the intrinsic HR (IHR). In the other half of the rats, the methylatropine–propranolol sequence was reversed to assess the effect of sympathetic blockade on HR, following the same recording procedure (15 min each) for each drug, as described previously, to determine the IHR. The data from methylatropine–propranolol and propranolol–methylatropine sequences were pooled to provide the basal HR (before any drugs) and the IHR (after drugs).

Statistical analysis

The results are presented as mean $\pm$ standard error of the mean (SEM). The effects of hypertension and pharmacological treatments were assessed using two-way analysis of variance (ANOVA). When appropriate, post-hoc comparisons were performed using the Student-Newman-Keuls test. For comparison between two groups, the Student's t-test for independent measures or the Mann-Whitney Rank Sum test was used as required. Differences were considered significant at $P < 0.05$. All statistical tests were performed using SigmaStat software (version 3.5; Systat Software Inc., San Jose, CA, USA).

Results

Baseline parameters

Treatment with pyridostigmine bromide at a dosage of 15/mg/kg/day, associated with aerobic physical training, promoted an increase in body weight. Both aerobic physical training and pyridostigmine bromide reduced the BP and HR values. When associated, the reduction in SBP and diastolic BP (DBP) was more prominent.

Double pharmacological blockade with atropine and propranolol-sympathovagal balance

Table 1 shows the HR values after administration of atropine and propranolol, as well as the pacemaker intrinsic HR after administration of both drugs. Aerobic physical training and pyridostigmine bromide increased the HR response after atropine administration and reduced the HR response after propranolol administration. This association enhanced the response. Figure 1 shows the cardiac autonomic tonic balance in percentage values for each group.

Aerobic physical training and treatment with pyridostigmine bromide reduced the predominance of sympathetic autonomic components in determining the baseline HR. However, only the association of physical training and pyridostigmine bromide (15 mg/kg/day) promoted an inversion in cardiac autonomic tonic balance, characterized by a vagal predominance in determining the baseline HR.

Heart rate variability (HRV) and systolic blood pressure variability (BPV)

Table 2 presents the results of HRV. The aerobic physical training increased the total variance, oscillation of LF in absolute units, and LF/HF ratio. In turn, the pyridostigmine bromide treatment increased the oscillation of LF and HF in absolute units. On the other hand, the association of both attenuated the oscillation of HF in absolute units and increased the LF/HF ratio. Table 2 also shows the BPV results. The aerobic physical training decreased the LF oscillation of BPV, whereas the pyridostigmine bromide treatment enhanced it. However, the association increased the values of LF oscillation even more than the pyridostigmine bromide isolated treatment.

Baroreflex sensitivity (BRS)

Table 2 shows the BRS values. Both aerobic physical training and pyridostigmine bromide increased the BRS to tachycardic responses induced by a reduction in BP (gain down, ms/mmHg). In turn, only pyridostigmine bromide treatment enhanced the BRS for bradycardic responses induced by an increase in BP (gain up, ms/mmHg).

Cardiac morphological and functional parameters

The cardiac morphological and functional results obtained using two-dimensional echocardiography are shown in Table 3.

Physical training increased LVEDD and LVESD, whereas pyridostigmine bromide treatment reduced RWT and IVST and enhanced LV mass. The association between physical training and pyridostigmine bromide treatment, mainly at a dose of 15 mg/kg/day, increased IVST, LVEDD, and LV mass.

Physical training increased LVEDV, LVESV, and ejection fraction, while pyridostigmine bromide treatment increased LVEDV and cardiac output. The association between the two treatments did not promote any additional effects. The pyridostigmine bromide treatment at a dose of 15 mg/kg/day promoted an increase in cardiac output and cardiac index, compared to the untrained vehicle group. In turn, the aerobic physical training groups had higher values of LVEDV and LVESV than the untrained group. The association of physical training and pyridostigmine bromide, specifically at a dose of 15 mg/kg/day, increased the ejection volume, ejection fraction, cardiac output, and cardiac index compared to the trained vehicle group and its respective untrained group.

Discussion

Aerobic physical training and pyridostigmine bromide treatment had similar effects on cardiac autonomic tonic balance, characterized by an increase in vagal influence and/or a reduction in sympathetic influence, in addition to a reduction in hemodynamic parameters, such as BP, baseline HR, and IHR. In turn, the association between both did not enhance the effects.

In SHRs the accentuated predominance of the sympathetic autonomic drive over the vagal drive is often observed and it contributes to a high baseline HR, accompanied by adverse cardiac morphological and functional adaptations^{16,28,29}. The reduction in baseline HR, induced only by pyridostigmine bromide treatment, results from a greater acetylcholine level. In this case, there is a tendency to reduce the baseline HR-dependent dose; that is, a dose of 15 mg/kg/day promotes greater effects, including in trained animals. In turn, the baseline HR reduction induced by aerobic physical training seems to involve a more complex mechanism, characterized by adaptation in central sites of cardiovascular control^{30–32}, downregulation of β -adrenergic receptors^{33,34}, and intrinsic cardiac adaptations. According to the literature, autonomic adaptations in central sites involve hypothalamic nuclei, such as the paraventricular and supra-optic nuclei, and nuclei located in the brainstem, such as the nucleus of the solitary tract (NTS) and rostral-ventrolateral medulla (RVLM)³⁵. These adaptations seem to involve a series of factors, such as neural remodeling and the influence of endogenous factors, which result in a decrease in sympathetic autonomic drive and/or an increase in the participation of the vagal autonomic drive in cardiac tonic control^{20,36–42}. On the other hand, intrinsic cardiac adaptations also seem to contribute to baseline HR reduction, together with IHR reduction, which might be due to cardiac morphological and functional changes.

In our study, we observed that trained SHR showed an increase in LVEDV and LVESF, resulting in increases in LVEDV and LVESV as well as an expressive increase in ejection volume. These adaptations probably arise from the greater venous return induced by aerobic physical training. However, we did not observe an increase in cardiac output and cardiac index at rest, since the baseline HR reduction offset the greater diastolic filling. In turn, the echocardiography analysis of the animals treated with pyridostigmine bromide did not show changes in left ventricle diameter but showed modifications in IVST and RWT associated with an increase in LV mass, mainly in animals previously submitted to aerobic physical training. The causes of these morphological adaptations are uncertain, but they suggest that changes in autonomic dynamics induced by physical training associated with pyridostigmine bromide treatment result in morphological adaptations that favor better cardiac performance. In addition, animals treated with pyridostigmine bromide also showed an increase in cardiac output. This adaptation is also associated with the increase in LVEDV found in these animals, resulting in a tendency to increase the ejection volume ($P < 0.074$). In addition to the increased cardiac output, mainly in the 15 mg/kg/day treatment group, the cardiac index (mL/g) remained unchanged.

Some studies have related BP reduction to the effects of chronic acetylcholinesterase block on endothelial function and a reduction in oxidative stress, which is also associated with aerobic physical training⁴³. On the other hand, the mechanism responsible for BP reduction is still much discussed. However, other physical training effects can be noted, such as the attenuation of vascular and cardiac sympathetic activity, decreased serum levels of vasoconstrictor factors, and increased endothelial vasodilator factor levels as a result of the enhanced shear stress induced by aerobic physical training, resulting in reduced peripheral vascular resistance^{36,44,45}. The causes of the reduction observed after pyridostigmine bromide treatment were more complex. One hypothesis is that

pyridostigmine bromide might also block the non-neural acetylcholinesterase action that is produced in other locations, such as endothelium and/or lymphocytes, promoting hemodynamic changes, as previously mentioned ⁹.

The results of HRV, BPV, and BRS were interesting. Both aerobic physical training and pyridostigmine bromide treatment increased HRV LF oscillations in absolute units. However, only the treatment with pyridostigmine bromide, more specifically at a dose of 15 mg/kg/day, increased the HRV HF oscillation in absolute units. Until then, we observed that the effects of pyridostigmine bromide treatment on baseline HR, IHR, and cardiac autonomic tonic balance were somewhat similar, and that the association with physical training potentiated some responses such as increased vagal tone and reduced baseline HR, BP, and IHR. In contrast, the HRV results showed that the association of treatments did not potentiate the results obtained with the isolated treatments, but rather attenuated or even reversed the beneficial effects of aerobic physical training and pyridostigmine bromide treatment when applied alone. Regarding HRV, there was a reduction in HF oscillation, which seems to indicate a saturation of vagal stimulation from the association of both treatments ⁴⁶. Nevertheless, the obtained values were still greater than those obtained for the untrained vehicle group. Although, when the autonomic modulation balance represented by the LF/HF ratio was observed, the association treatment effects were even more evident. This occurrence can be explained by the pyridostigmine bromide effect, which increases both oscillation bands, LF and HF, mainly at a dose of 15 mg/kg/day. The causes of this increase are still uncertain, but it might be associated with an increase in sympathetic modulation oscillation, as well as vagal oscillations, since the LF band seems to be mediated by both autonomic components.

In the case of BPV, the findings were also surprising. While only the trained animals showed a reduction in LF oscillation modulation, the animals treated with pyridostigmine bromide, trained or not, showed a significant enhancement of the LF band. In fact, SHR animals are classically known to have a high vascular sympathetic influence, resulting in increased LF oscillations of the BPV ^{47–51}. The cause of this increase is unknown and cannot be attributed to baroreflex malfunction, since the BRS was increased, even in animals treated only with pyridostigmine bromide. This statement is based on the concept that baroreflex is one of the main mechanisms involved in LF oscillations of BPV, together with vascular sympathetic drive ⁵² and relaxing factors derived from the vascular endothelium, such as nitric oxide (NO). The baroreflex and NO would act as a buffer system for BP fluctuations induced by sympathetic activation ⁵³. Therefore, some authors suggest that the increase in LF oscillation of BP is related to reductions in BRS and decrease in NO production and release. However, a study has demonstrated that long-term administration of acetylcholinesterase inhibitors, pyridostigmine or donepezil, attenuates vascular reactivity dysfunction in SHRs by decreasing reactive oxygen species generation and increasing NO bioavailability, possibly via increased endothelial NO synthase activity and inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity ⁴³. In fact, as noted, the explanation of the effects of pyridostigmine bromide on vascular modulation is even more complex than that of the effects on cardiac control, although both require further investigation.

The treatment with pyridostigmine bromide, mainly at a dose of 15 mg/kg/day, appears to be beneficial to cardiac autonomic regulation; it increased vagal autonomic tonic influence in determining baseline HR and increased HRV and BRS. In turn, the enhancement in BPV is worrying. The association with aerobic physical training potentiates the reduction in hemodynamic parameters and results in a greater vagal autonomic tonic influence on the heart. However, this association reduces vagal modulation of the heart. The mechanism involved in these findings is still uncertain and requires further investigation.

Declarations

Authors Contribution

H.C.D.S. and C.B.G. conceived and designed the research. C.B.G, A.C.V.O. and B.A.A. acquired the data. A.C.V.O. and B.A.A. analyzed the data. A.C.V.O., B.A.A., S.V.P. and H.C.D.S. interpreted the data. A.C.V.O., B.A.A. and S.V.P. drafted the work, and along with H.C.D.S., revised it critically for important intellectual content. All authors approved the final version of the manuscript.

Competing of interest

The author(s) declare no competing interests.

Data availability

All data used during the current study are included in this published article or are available from the corresponding author on reasonable request.

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Tables

Table 1. Baseline parameters and values of cardiac autonomic tonus after the pharmacological blockade with atropine and propranolol

	UNTRAINED			TRAINED			Training Factor		Drug Factor		Interaction	
	Vehicle	Pyr-5mg	Pyr-15mg	Vehicle	Pyr-5mg	Pyr-15mg	F _(d.f.)	P	F _(d.f.)	P	F _(d.f.)	P
Basal Parameters												
Body Weight (g)	201 ± 4	195 ± 3	200 ± 3	193 ± 3	201 ± 3	215 ± 2	F _(1,53) : 1.42	0.239	F _(2,53) : 3.37	0.043	F _(2,53) : 3.19	0.050
SBP, mmHg	191 ± 1	178 ± 2	166 ± 4	169 ± 1	158 ± 2	136 ± 2	F _(1,53) : 72.55	<0.001	F _(2,53) : 35.95	<0.001	F _(2,53) : 1.40	<0.001
DBP, mmHg	152 ± 3	140 ± 1	130 ± 4	145 ± 2	130 ± 2	109 ± 3	F _(1,53) : 22.42	<0.001	F _(2,53) : 40.75	<0.001	F _(2,53) : 2.44	<0.001
MBP, mmHg	165 ± 2	152 ± 2	142 ± 3	153 ± 1	139 ± 2	118 ± 2	F _(1,53) : 34.63	<0.001	F _(2,53) : 36.54	<0.001	F _(2,53) : 1.83	0.172
HR, bpm	379 ± 5	344 ± 8	326 ± 4	350 ± 7	335 ± 7	314 ± 7	F _(1,53) : 10.20	0.002	F _(2,53) : 25.22	<0.001	F _(2,53) : 2.2	0.233
Tonic autonomic control												
HR / atropine, bpm	397 ± 4	376 ± 3	357 ± 5	380 ± 5	362 ± 3	360 ± 2	F _(1,53) : 7.75	0.008	F _(2,53) : 26.03	<0.001	F _(2,53) : 3.15	0.052
Δ HR / atropine, bpm	18 ± 2	32 ± 1	31 ± 3	30 ± 1	27 ± 1	46 ± 4	F _(1,53) : 18.21	<0.001	F _(2,53) : 23.39	<0.001	F _(2,53) : 13.99	<0.001
HR / propranolol, bpm	307 ± 7	299 ± 9	285 ± 2	300 ± 8	288 ± 5	278 ± 7	F _(1,53) : 4.23	0.045	F _(2,53) : 8.97	<0.001	F _(2,53) : 0.08	0.925
Δ HR / propranolol, bpm	72 ± 5	45 ± 7	41 ± 5	50 ± 2	47 ± 8	36 ± 5	F _(1,53) : 7.89	0.007	F _(2,53) : 21.17	<0.001	F _(2,53) : 5.61	0.006
IHR, bpm	331 ± 5	305 ± 2	296 ± 6	315 ± 5	295 ± 4	295 ± 2	F _(1,53) : 5.75	0.002	F _(2,53) : 17.49	<0.001	F _(2,53) : 1.29	0.283

All values are presented as the mean ± S.E.M. Pyr-5 mg, pyridostigmine bromide treatment at a dose of 5 mg/kg/day; Pyr-15 mg, pyridostigmine bromide treatment at a dose of 15 mg/kg/day; mmHg, millimeters of mercury; bpm, beats per minute; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate; Δ HR, heart rate variation delta; IHR, intrinsic heart rate; F, factor; df, degrees of freedom.

Table 2. Values for heart rate (pulse interval) variability, systolic arterial pressure variability, and spontaneous baroreflex sensitivity

	UNTRAINED			TRAINED			Training Factor		Drug Factor		Interaction	
	Vehicle	Pyr-5mg	Pyr-15mg	Vehicle	Pyr-5mg	Pyr-15mg	F _(d.f.)	P	F _(d.f.)	P	F _(d.f.)	P
PI Variability												
PI, ms	158 ± 2	174 ± 5	184 ± 2	171 ± 3	179 ± 3	191 ± 5	F _(1,53) : 4.79	0.033	F _(2,53) : 12.06	<0.001	F _(2,53) : 0.36	0.700
Variance, ms ²	30 ± 2	37 ± 2	37 ± 1	40 ± 3	42 ± 3	42 ± 2	F _(1,53) : 6.44	0.014	F _(2,53) : 1.12	0.334	F _(2,53) : 0.38	0.687
LF, ms ²	1,5 ± 0,1	1,4 ± 0,1	2,3 ± 0,2	1,7 ± 0,1	2,1 ± 0,06	2,5 ± 0,1	F _(1,53) : 8.76	0.005	F _(2,53) : 15.8	<0.001	F _(2,53) : 1.82	0.173
LF, nu	21 ± 2	21 ± 3	17 ± 0,6	22 ± 0,7	20 ± 3	25 ± 2	F _(1,53) : 2.36	0.131	F _(2,53) : 0.14	0.874	F _(2,53) : 3.10	0.054
HF, ms ²	4,5 ± 0,3	5,6 ± 0,4	11 ± 0,3	6,6 ± 0,4	5,7 ± 0,5	7,0 ± 0,4	F _(1,53) : 3.74	0.059	F _(2,53) : 52.3	<0.001	F _(2,53) : 32.2	<0.001
HF, nu	79 ± 2	79 ± 3	83 ± 0,6	78 ± 1	80 ± 3	74 ± 2	F _(1,53) : 1.66	0.204	F _(2,53) : 0.1	0.906	F _(2,53) : 1.22	0.304
LF/HF	0,29 ± 0,03	0,24 ± 0,01	0,20 ± 0,01	0,25 ± 0,03	0,31 ± 0,05	0,34 ± 0,02	F _(1,53) : 5.86	0.019	F _(2,53) : 0.06	0.945	F _(2,53) : 5.72	0.006
SBP Variability												
Variance, mmHg ²	19 ± 2	26 ± 1	44 ± 2	27 ± 1	34 ± 1	46 ± 2	F _(1,53) : 5.63	0.022	F _(2,53) : 27.1	<0.001	F _(2,53) : 0.58	0.561
LF, mmHg ²	6 ± 0,2	8,5 ± 0,3	9,0 ± 0,3	4,7 ± 0,3	9,8 ± 1	13,1 ± 0,3	F _(1,53) : 11.5	0.001	F _(2,53) : 68.9	<0.001	F _(2,53) : 16.2	<0.001
Spontaneous BRS												
Gain down, ms/mmHg	1,16 ± 0,09	1,12 ± 0,07	1,63 ± 0,06	1,33 ± 0,10	1,41 ± 0,05	1,73 ± 0,06	F _(1,53) : 12.9	<0.001	F _(2,53) : 35.9	<0.001	F _(2,53) : 1.53	0.226
Gain up, ms/mmHg	1,21 ± 0,07	1,30 ± 0,08	1,90 ± 0,07	1,16 ± 0,09	1,18 ± 0,04	1,78 ± 0,07	F _(1,53) : 0.74	0.395	F _(2,53) : 74.8	<0.001	F _(2,53) : 3.12	0.053

All values are presented as the mean ± S.E.M. Pyr-5 mg, pyridostigmine bromide treatment at a dose of 5 mg/kg/day; Pyr-15 mg, pyridostigmine bromide treatment at a dose of 15 mg/kg/day; PI, pulse interval; LF, low frequency band; ms, milliseconds; nu, normalized units; HF, high frequency band; SBP, systolic arterial pressure; mmHg, millimeters of mercury; BRS, baroreflex sensitivity; gain down, baroreflex sequence with progressive decreases in blood pressure followed by progressive decreases in pulse interval; gain up, baroreflex sequence with progressive increases in blood pressure followed by progressive increases in pulse interval; F, factor; df, degrees of freedom.

Table 3. Values for cardiac function and morphology observed in the untrained and trained groups

	UNTRAINED			TRAINED			Training Factor		Drug Factor		Interaction	
	Vehicle	Pyr-5mg	Pyr-15mg	Vehicle	Pyr-5mg	Pyr-15mg	F _(d.f.)	P	F _(d.f.)	P	F _(d.f.)	P
Cardiac Morphology												
RWT, mm/kg	0.5 ± 0.01	0.49 ± 0.02	0.43 ± 0.01	0.5 ± 0.03	0.44 ± 0.01	0.43 ± 0.01	F _(1,53) : 1.98	0.165	F _(2,53) : 9.87	<0.001	F _(2,53) : 2.6	0.084
IVST, mm/kg	2.16 ± 0.08	1.95 ± 0.1	1.86 ± 0.08	1.93 ± 0.06	1.74 ± 0.08	2.13 ± 0.04	F _(1,53) : 1.17	0.285	F _(2,53) : 5.53	0.007	F _(2,53) : 10.2	<0.001
LVEDD, mm/kg	32 ± 0.8	31 ± 0.9	32 ± 1.2	31 ± 0.8	34 ± 0.9	34 ± 0.5	F _(1,53) : 6.4	0.014	F _(2,53) : 1.28	0.288	F _(2,53) : 3.6	0.036
LVESD, mm/kg	21 ± 0.7	20 ± 0.1	23 ± 1.2	23 ± 0.9	24 ± 0.8	24 ± 0.8	F _(1,53) : 22.6	<0.001	F _(2,53) : 3.1	0.055	F _(2,53) : 2.2	0.126
LV mass, mg/g	3,22 ± 0.1	2,91 ± 0,1	3,35 ± 0,3	3,0 ± 0,05	3,11 ± 0,1	3,76 ± 0,1	F _(1,53) : 2.71	0.106	F _(2,53) : 16.9	<0.001	F _(2,53) : 5.2	0.009
Cardiac Function												
LVEDV (μL)	272 ± 16	264 ± 14	280 ± 17	317 ± 11	312 ± 12	350 ± 22	F _(1,53) : 54.1	<0.001	F _(2,53) : 5.01	0.011	F _(2,53) : 1.23	0.301
LVESV (μL)	65 ± 4	71 ± 5	73 ± 4	85 ± 6	86 ± 4	76 ± 8	F _(1,53) : 10.6	0.002	F _(2,53) : 0.43	0.656	F _(2,53) : 1.79	0.179
Stroke Volume, μL	205 ± 19	193 ± 19	208 ± 14	231 ± 22	226 ± 19	275 ± 14	F _(1,53) : 12.7	<0.001	F _(2,53) : 2.75	0.074	F _(2,53) : 1.21	0.307
Cardiac Output, mL/min	63 ± 3	67 ± 5	75 ± 4	72 ± 8	71 ± 5	90 ± 5	F _(1,53) : 3.5	0.068	F _(2,53) : 3.73	0.031	F _(2,53) : 0.41	0.664
Ejection fraction, %	76 ± 1	73 ± 2	74 ± 1	73 ± 1	72 ± 2	79 ± 2	F _(1,53) : 0.04	0.852	F _(2,53) : 0.8	0.455	F _(2,53) : 1.58	0.216
Cardiac Index, mL/g	0,31 ± 0,02	0,34 ± 0,02	0,37 ± 0,02	0,36 ± 0,03	0,37 ± 0,02	0,44 ± 0,02	F _(1,53) : 3.43	0.070	F _(2,53) : 2.80	0.071	F _(2,53) : 0.24	0.788

All values are presented as the mean ± S.E.M. Pyr-5 mg, pyridostigmine bromide treatment at a dose of 5 mg/kg/day; Pyr-15 mg, pyridostigmine bromide treatment at a dose of 15 mg/kg/day; RWT, relative wall thickness; mm, millimeter; kg, kilogram; IVST, interventricular septum thickness; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LV, left ventricular; mg, milligram; g, gram; LVEDV, left ventricular end-diastolic volume; μL, microliter; LVESV, left ventricular end-systolic volume; mL, milliliter; min, minute; %, percentage; F, factor; df, degrees of freedom.

Figures

Figure 1

Evaluation of cardiac autonomic control through double pharmacological blockade. The bars show the percentage fluctuation of heart rate (HR) and intrinsic heart rate (IHR) after the administration of atropine (solid box) and propranolol (cross-hatched box) in vehicle and pyridostigmine bromide (Pyr-B) treatment, both trained and untrained. a versus untrained SHR vehicle; b versus untrained SHR Pyr-B 5mg; c versus untrained SHR Pyr-B 15mg; d versus trained SHR vehicle; e versus trained SHR Pyr-B 5 mg.