Chronic Stress Improves the Myocardial Function without Altering L-type Ca+2 Channel Activity in Rats

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Abstract

Background: Chronic stress is associated with cardiac remodeling; however the mechanisms have yet to be clarified.

Objective: The purpose of this study was test the hypothesis that chronic stress promotes cardiac dysfunction associated to L-type calcium Ca^{2+} channel activity depression.

Methods: Thirty-day-old male Wistar rats (70 - 100 g) were distributed into two groups: control (C) and chronic stress (St). The stress was consistently maintained at immobilization during 15 weeks, 5 times per week, 1h per day. The cardiac function was evaluated by left ventricular performance through echocardiography and by ventricular isolated papillary muscle. The myocardial papillary muscle activity was assessed at baseline conditions and with inotropic maneuvers such as: post-rest contraction and increases in extracellular Ca^{2+} concentration, in presence or absence of specific blockers L-type calcium channels.

Results: The stress was characterized for adrenal glands hypertrophy, increase of systemic corticosterone level and arterial hypertension. The chronic stress provided left ventricular hypertrophy. The left ventricular and baseline myocardial function did not change with chronic stress. However, it improved the response of the papillary muscle in relation to positive inotropic stimulation. This function improvement was not associated with the L-type Ca^{2+} channel.

Conclusion: Chronic stress produced cardiac hypertrophy; however, in the study of papillary muscle, the positive inotropic maneuvers potentiated cardiac function in stressed rats, without involvement of L-type Ca^{2+} channel. Thus, the responsible mechanisms remain unclear with respect to Ca^{2+} influx alterations. (Arq Bras Cardiol 2012;99(4):907-914)

Keywords: Stress, physiological / complications; stress, physiological / physiopathology; cardiovascular diseases / psychology; rats; papillary muscles.

Introduction

Stresses play in integral role in our daily lives and are often related to issues with marital issues, health, work, and low socioeconomic status[1]. Selye[2] defined stress as a state characterized by a uniform response pattern, regardless of the particular stressor, that could lead to long-term pathological changes such as: chronic anxiety, depression, obesity, immunologic disorders, inflammation, insulin resistance and cardiovascular disease[3,4].

The most well-defined cardiovascular disorders related to chronic stress in humans are arterial hypertension, heart rate variability, left ventricular systolic and diastolic dysfunction and vascular alterations[5-8]. In addition, this variable stress for 15 days was able to produce hypertrophic cardiac and permanent cardiac structural changes[9]. Zhao et al.[10] also showed that rats subjected to the chronic stress of restraint for 21 days resulted in cardiac dysfunction and to structural injury of the heart.

In addition, experiments performed in rats subjected to emotional-pain stress demonstrated increased contraction and relaxation velocity of isolated papillary muscles[11]. Meerson et al.[12] observed how rat-isolated heart and papillary muscle adaptation to short-term stresses increased myocardial resistance to arrhythmogenic and contractile effects of excessive Ca^{2+}.

Furthermore, the rats that underwent chronic stress of immobilization for 7 days, 2 hours per day, showed an increase of type 1 and 2 inositol-1,4,5-trisphosphate receptors in their left ventricles[13]. In experiments with a 21-day restraint stress rat model, Zhao et al.[14] determined that stress increases the expression of α1c subunit of the L-type calcium channel and calcium current in ventricular myocytes of rats.

The L-type calcium channel plays a major role in maintenance of normal cardiac function. The influx of Ca^{2+} ions through the L-type calcium channel is crucial for excitation–contraction coupling in the heart[15]. Alterations

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in density or role of the L-type calcium channel have been implicated in a variety of cardiovascular diseases[16,17].

Given this information, the objective was to test the hypothesis that chronic stress promotes cardiac dysfunction associated to L-type calcium Ca^{2+} channel activity depression. One of the novel features of this study is the time in which the rats were subjected to stress, 15 weeks, considering there is a lack of studies evaluating chronic stress for prolonged periods of time with parameters encompassing cardiac structure and activity. This study will contribute to the understanding of cardiac alterations caused by chronic stress.

Materials and methods

Animal care

Thirty-day-old male *Wistar* rats (70 - 100 g) obtained from the Animal Center of Botucatu Medical School (Botucatu, São Paulo, Brazil) were housed in individual cages. The environment was controlled in terms of light (12 h light/dark cycle starting at 6 am), clean-air room temperature (23 ± 3°C), and relative humidity (60 ± 5%). After 7 days of acclimatization, the rats were distributed into two groups: control (C, n = 8) and chronic stress (St, n = 8). The animals were weighted weekly. All experiments and procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Research Council and were approved by the Ethics Committee of the Instituto de Biociências UNESP-Botucatu (protocol nº 95/08-CEEA).

Chronic stress

After 30 days, the members of the St group were immobilized individually in metal capsules at room temperature 25°C, 1 hour per day, 5 days a week for 15 weeks. During the session stress, C group remained in their respective cage at room temperature 25°C, without receiving food and water. At the end of the session, St group members were reintroduced to their original cage.

Nutritional profile

Weekly calorie intake (CI) was calculated by average weekly food consumption *x* dietary energetic density. Feed efficiency (FE), the ability to transform calories consumed into body weight (BW), was determined by following the formula: mean body weight gain (g)/total calorie intake (kcal).

Comorbidities associated with chronic stress

Noting that chronic stress may develop some comorbidities, such as hypertension and hypercorticosteronemia, the following evaluations were performed in all groups. The animals were killed and the hypertrophy of adrenals glands were assessed. The glands were removed, dissected and weighed.

Systolic blood pressure (SBP)

The evaluation of SBP was assessed every three weeks during the 15 weeks. The assessment was by the non-invasive tail-cuff method with a Narco BioSystems® Electro-Sphygmomanometer (International Biomedical, Austin, TX, USA). The average of two pressure readings were recorded for each animal.

Corticosterone level

At the end of the experimental period, animals were subjected to 12–15 h of fasting, anesthetized with sodium pentobarbital (50 mg/kg i.p.), and euthanized by decapitation. Blood samples were collected in heparinized tubes, and the serum was separated by centrifugation at 3000 × g for 15 minutes at 4°C and stored at −80°C until further analysis. Corticosterone levels were measured by radioimmunoassay using specific kit (Coat-A-Count Rat Corticosterone – Diagnostic Products Corporation).

Echocardiography

The rats were weighed, and anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (1 mg/kg), administered intramuscularly.

The animals were positioned in the left lateral position for the echocardiography, which was performed with a HDI 5000 Phillips ultrasound machine, equipped with a 12 MHz electronic transducer. For the measurement of heart structures, monodimensional mode (M-mode) images were obtained, with two-dimensional mode images guiding the ultrasound beam, and the transducer placed in a parasternal location in the short axis. The image of the left ventricle (LV) was obtained by positioning the M-mode cursor just below the mitral valve plane at the level of the papillary muscles. The images of the aorta and the left atrium were obtained with the M-mode cursor positioned at the level of the aortic valve. The images were recorded in a Sony Co. UP-890 printer model. Subsequently, heart structures were manually measured, with a precision caliper.

When the diameter of the ventricular cavity was maximal, the LV diastolic diameter (LVDD), the LV posterior wall diastolic thickness (LVPWDT) and the interventricular septum diastolic thickness (IVSDT) were measured. When the diameter of the cavity was minimalized, the LV systolic diameter (LVSD), the LV posterior wall systolic thickness (LVPWST) and the interventricular septum systolic thickness (IVSST) were measured. The left atrium (LA) was measured at its maximal diameter. The LV mass (LVM) was calculated using the following formula: $LVM = [(LVDD + LVPWDT + IVSDT)^3 – (LVDD)^3] \times 1.04$. The following variables were derived from the dimensions described above: LV relative thickness (LVPWDT/LVDD), LVDD/BW, LA/BW and LVM index (LVMI, LVM/BW).

The LV systolic function was assessed by the following indexes: Percentage of mesocardial shortening (% Meso. Short.): [(LVDD + ½ LVPWDT + ½ IVSDT) – (LVSD + ½ LVPWST + ½ IVSST)] / (LVDD + ½ LVPWDT + ½ IVSDT); percentage of endocardial shortening (% Endo. Short.): [(LVDD - LVSD) / LVDD]; posterior wall shortening velocity (PWSV). The diastolic function was assessed by the ratio index between the peak of initial inflow velocity (E wave) and the atrial contraction (A wave) of the transmitral flow (E/A), the deceleration time of the E wave (DTE) and the isovolumetric relaxation time (IVRT). After the euthanasia, the atria (A), the right ventricle (RV) and the LV were weighed in absolute values and corrected by BW.

Myocardial function

Myocardial role was evaluated by studying isolated papillary muscles from the LV. This preparation allows us to measure the capacity of the cardiac muscle to shorten and develop force independently of influences that can modify *in vivo* mechanical performance of the myocardium, such as heart rate, preload, and after load. The rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and sacrificed by decapitation. The hearts were removed and placed in oxygenated Krebs-Henseleit solution at 28°C. LV papillary muscles were dissected, mounted between two spring clips, and placed in a chamber containing Krebs-Henseleit solution (118.5 mM NaCl; 4.69 mM KCl; 2.5 mM $CaCl_2$; 1.16 mM $MgSO_4$; 1.18 mM KH_2PO_4; 5.50 mM GL, and 24.88 mM $NaCO_3$) maintained at 28°C with a thermostatic water circulator. The bathing solution was bubbled with 95% oxygen and 5% carbon dioxide, with a pH of 7.4. The lower spring clip was attached to a 120T-20B force transducer (Kyowa, Tokyo, Japan) by a thin steel wire (1/15,000 inch). The upper spring clip was connected by a thin steel wire to a rigid lever arm, above which a micrometer stop was mounted for adjusting the muscle length. The muscle preparation was placed between two platinum electrodes (Grass E8, GRASS Technologies, An Astro-Med, Inc. Product Group, West Warwick, RI, USA) and stimulated at a frequency of 0.2 Hz (12 pulses/min) by using square-wave pulses of 5 ms in duration.

The muscles were contracted isotonically with light loads for 60 min and then loaded (50 g) to contract isometrically and stretched to the maximum of their length-tension curves. After a 5-min period during which preparations underwent isotonic contractions, muscles were again placed under isometric conditions, and the peak of the length-tension curve (L_{max}) was carefully determined. A 15-min period of stable isometric contraction was imposed prior to the experimental period, during which one isometric contraction was then recorded. Conventional mechanical parameters at L_{max} were calculated from isometric contraction: maximum developed tension normalized per cross-sectional area (DT [g/mm^2]), peak of the positive (+dT/dt [g/mm^2/s]) and negative (-dT/dt [g/mm^2/s]) tension derivatives normalized per cross-sectional area.

The papillary muscles were evaluated under the baseline condition of 2.5 mM Ca^{2+} and after inotropic and lusitropic maneuvers: increases in extracellular Ca^{2+} concentration and post-rest contraction (PRC). Inotropic responses were recorded 5 min after the addition of each concentration of extracellular Ca^{2+} to the bathing solution. PRC was studied at an extracellular Ca^{2+} concentration of 0.5 mM, where the stimulus was paused for 10, 30, and 60 s before restarting the stimulation. During rest in the rat myocardium, the SR accumulates Ca^{2+} above and beyond that accumulated during regular stimulation, and the first beat after the rest interval (B1) is stronger than the beat before the rest interval (B0).

The evaluation of L-type Ca^{2+} channel activity was performed using a specific inhibitor, *Diltiazem hydrochloride* (10^{-4} M), in the presence of cumulative Ca^{2+} concentrations. Twenty minutes after diltiazem addition to the solution, each concentration of Ca^{2+} was separately added to the bathing solution for 10 min, and muscle activity was assessed.

At the end of the study, the parameters used to characterize the papillary muscle were length (mm), weight (mg), and CSA (mm^2). The CSA was calculated from the length and weight of papillary muscle, assuming uniformity and a specific gravity of 1.0. The muscle length at L_{max} was measured with a cathetometer (Gartner Scientific Corporation, Chicago, IL, USA), and the muscle between the two clips was blotted dry and weighed.

Statistical analysis

Data were reported as means ± standard deviation. Comparisons between groups were performed using Student's t-test for independent samples. The body weight and blood pressure between groups were compared by analysis of variance (ANOVA) for repeated measures. When significant differences were found ($p \leq 0.05$), Bonferroni's post-hoc test for multiple comparisons was carried out. The maneuvers of papillary muscle study were analyzed with repeated-measures of two-way analysis of variance (ANOVA) and complemented by Tukey's post-hoc test for specific differences. The comparison of post-rest contraction was performed using the Student's t-test. The level of significance considered was 5 %.

Results

Body weight, food and calorie intake in relation to corticosterone levels and blood pressure

The chronic stress did not change the BW (C = 438 ± 49 vs. St = 421 ± 33, p > 0,05), while CI (C = 68,7 ± 5,1 vs. St = 69,2 ± 8,6, p > 0.05) and FE (C = 2.24 ± 0.25 vs. St = 2.25 ± 0.32, p > 0.05) increased the corticosterone level [C = 59.32 ± 19.2 vs. St = 98.02 ± 23.0, $p \leq 0.05$] and mass of adrenal glands [C = 0.57 ± 0.09 vs. St = 0.73 ± 0.08, $p \leq 0.05$]. The stress increased the blood pressure after three weeks of exposure; this lift persisted until the end of the experimental protocol (Figure 1).

Morphology post-mortem study

In the *post-mortem* study, chronic stress stimulated increase in the LV/BW ratio (C = 1.71 ± 0.24 vs. St = 1.86 ± 0.37, $p \leq 0.05$). Conversely, there was no difference between groups in other variables: AT/BW ratio (C = 0.19 ± 0.04 vs. St = 0.18 ± 0.02, p > 0.05) and RV/BW ratio (C = 0.56 ± 0.97 vs. St = 0.57 ± 0.84, p > 0.05).

Echocardiography study

The Table 1 shows the study of cardiac structure analyzed by echocardiography. The St group had increase of left ventricular mass index. However, in other variables, there was a lack of significant differences between groups. In relation to functional parameters analyzed by echocardiography, the chronic stress did not produce any alteration (Table 2).

Papillary muscle function

Table 3 summarizes the mechanical properties of isolated papillary muscle from C and St groups at baseline condition. No substantial difference was noted between groups.

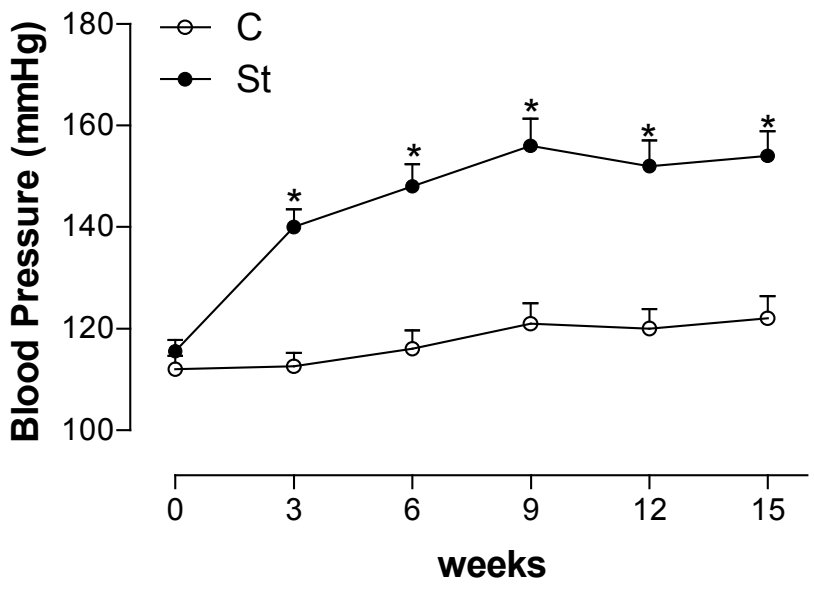

Figure 1 - *Development of blood pressure of control rats (C) or rats subjected to chronic stress (St). Chronic stress: immobilization stress 1h/day, 5 times/week, during 15 weeks. Number of animals: 8; *p ≤ 0.05; ANOVA and Bonferroni test.*

Table 1 - Structural cardiac assessment

Variables	Groups	
	C	St
LVDD (mm)	8.33±0.41	8.28±0.42
LVPWDT (mm)	1.38±0.1	1.35±0.1
IVSDT (mm)	1.41±0.1	1.37±0.1
LVSD (mm)	3.79±0.23	3.92±0.11
LVPWST (mm)	2.98±0.3	2.46±0.1
IVSST (mm)	2.81±0.2	2.36±0.2
LA (mm)	5.45±0.49	5.92±0.61
LA/BW (mm/Kg)	12.0±2.17	13.0±1.89
LVM (mm)	0.81±0.12	0.79±0.11
LVMI (mm/Kg)	1.67±0.11	1.78±0.15*

*Values in mean ± standard deviation; LV: ventricle left. LA: left atrium. BW: body weight. LVDD: left ventricle diastolic diameter. LVPWDT: left ventricle posterior wall diastolic thickness. IVSDT: interventricular septum diastolic thickness. the LVSD: left ventricle systolic diameter. LVPWST: left ventricle posterior wall systolic thickness. IVSST: interventricular septum systolic thickness. LVM: left ventricular mass. LVMI: left ventricular mass index. Number of animals: 8; *p ≤0.05; ANOVA and t-student.*

Table 2 - Cardiac functional assessment

Variables	Groups	
	C	St
HR (bpm)	294±55	287±31
% Meso. Short	33.49±4.0	32.54±1.8
% Endo. Short	54.51±5.8	52.72±1.6
PWSV (mm/s)	37.23±3.5	37.38±3.3
Mitral E (cm/s)	90.1±11.5	87.3 ± 5.2
Mitral A (cm/s)	59.9± 10.9	55.7 ±6.6
E/A	1.49 ± 0.4	1.58 ± 0.1
DTE (ms)	48.7±6.3	45.2±4.1
IVRT (ms)	23.14±4.3	22.90±2.3

Values in mean ± standard deviation; HR: heart rate; % Meso. Short: relative mesocardial shortening fraction; % Endo. Short: relative endocardial shortening fraction; PWSV: velocity of shortening of the posterior wall; Mitral E: mitral E wave; Mitral A: mitral A wave; E/A: ratio of E and A waves; DTE: deceleration time of E wave; IVRT: isovolumetric relaxation time of the left ventricle. Number of animals: 8.

After inotropic and lusitropic maneuvers, the application of stress induced several differences. In the PRC, animals that were exposed to stress evinced increases of DT, +dT/dt and -dT/dt in relation to control group (Figure 2). As the number of maneuvers increased extracellular Ca^{2+} concentration, the DT, +dT/dt and -dT/dt parameters were significantly raised in stressed rats (Figure 3).

However, the blockade of L-type Ca^{2+} channel by diltiazem did not lead to any changes as the increase in maneuvers expectedly increased extracellular Ca^{2+} concentration in all parameters analyzed (Figure 4).

Discussion

The stress response is contingent upon intensity, duration and type of stressor[18]. In this study, the model of chronic stress did not produce significant changes on body weight, caloric intake and feeding efficiency. These results challenge other investigations which attempt to corroborate the notion that stress induces anorexia and body weight loss via rat models[19,20]. The literature illustrates that a 30% minority of humans, lose weight during or after stress, while most individuals gain body weight due to increases in their food intake[21,22].

In this work, the stress increased systemic corticosterone levels. Authors underscored that stress is able to enhance levels of this hormone, consistent with the putative function of the corticosterone stress hormone, involving the hypothalamus pituitary adrenal (HPA) axis and the sympathetic–adrenomedullary system. Corticotrophin-releasing hormone (CRH) released by the hypothalamus, stimulates the secretion of Adrenocorticotropic Hormone (ACTH) from the anterior pituitary. Circulating ACTH acts on the fasciculate zone of the adrenal cortex where it stimulates the release of cortisol in humans and corticosterone in rats[18,23].

This increase of corticosterone is associated with adrenal glands hypertrophy. Various investigators have used the fresh weight of the adrenal gland, an organ that responds to stress, as indicative of stressogenic conditions[8]. The weight of the adrenal gland may be reduced or may remain unchanged after exposure to acute stress, but is often increased by chronic stress[24,25].

In the present study, animals undergoing stress had arterial hypertension. Blood pressure is controlled via several systems, among them the sympathetic nervous system, renin-angiotensin aldosterone system, and oxidative stress, which causes peripheral vasoconstriction[26-28]. This hypertension observed in rats subjected to stress is probably related to systems mentioned above; however, these mechanisms were not assessed in this particular study. This result corroborates findings characterized in the literature, which observed these type of alterations in stressed humans[5]. These results strongly support the chronic stress model as an exceptional model to study chronic stress, as several disorders linked to stress including hypercorticosteronemia, adrenal glands hypertrophy and arterial hypertension may be investigated via this model. This stress also generated left ventricular cardiac hypertrophy assessed by echocardiography and *post-mortem* studies; these findings corroborate the literature[9]. The proliferation of cardiac mass is related to neurohumoral activations, involving the sympathetic nervous system, renin angiotensin aldosterone system and oxidative stress and mechanical factors, as well as arterial hypertension[29].

Table 3 - Baseline data from isolated LV papillary muscle function

Variables	Groups	
	C	St
DT(g/mm^2)	6.64±0.79	6.92±1.31
+dT/dt (g/mm^2/s)	71.6±11.1	75.1±13.9
-dT/dt (g/mm^2/s)	19.9±2.1	21.1±7.4

Values in mean ± standard deviation; DT: maximum developed tension; +dT/dt: peak of the positive tension derivatives; -dT/dt: peak of the negative tension derivatives. Number of animals: 8.

The echocardiography study did not show any significant alterations in diastolic and systolic left ventricular function. However, the myocardial role assessed in baseline conditions also did not show any change; the stressed papillary muscle presented improvement of inotropic response to post rest-contraction and an increase of extracellular calcium concentration. Currently, several investigations have used these maneuvers to identify changes in phases of contraction and relaxation, which may eventually not be observed under basal conditions; in addition, they assist in the understanding of possible mechanisms responsible for alterations in cardiac activity[30].

The mechanism responsible for this improvement of myocardial function remains unclear; therefore, we can only speculate about the intrinsic mechanism responsible for these results. The better response of the stressed rats may be due to increase in myofilament calcium-sensitivity[31,32] and increased sarcoplasmic reticulum calcium uptake[33,34] induced by chronic stress. These postulations are extrapolated from a previous study[35] that observed better inotropic response to calcium in rats subjected to exercise training. The exercise and stress are similar in respect to several neuroendocrine characteristics, such as increased level of vasopressin, ACTH, corticosterone, aldosterone and catecholamines[36]. The adrenal gland hypertrophy observed in this investigation is in concordance with the underlying assumption that the sympathetic–adrenomedullary system could participate in the heart's enhanced response to calcium.

Our group's studies also assessed the involvement of L-type Ca^{2+} channel using the calcium blocker, diltiazem; the inclusion of this drug decreased supply of Ca^{2+} to the tissue[17]. The result with diltiazem did not change the papillary muscle's function between groups, suggesting that the activity of L-type Ca^{2+} channel was similar in both groups. The literature has yet to showcase the use of a similar methodology. Zhao et al. [14] observed increase of L-type Ca^{2+} channel density and calcium current in rats subjected to restraint stress for 21 consecutive days in ventricular myocyte. Contrasting this result, our investigations resulted in commensurate L-type Ca^{2+} channel density and calcium current between groups. This dissimilarity may be due to the extensive period of stress exposure indicating an adaptive response by cardiac cells.

In conclusion, the data produced in this study conflict with our initial hypotheses. The chronic stress did not

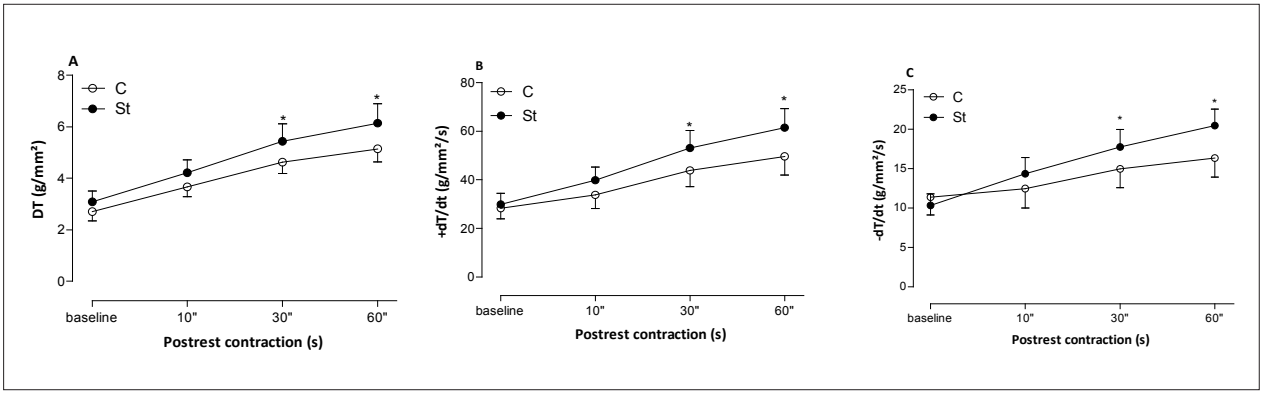

Figure 2 - *Post-rest contraction of control rats (C) or rats subjected to chronic stress (St). Chronic stress: immobilization stress 1h/day, 5 times/week, during 15 weeks. A: maximum developed tension normalized per cross-sectional area (DT); B: peak of the positive derivatives normalized per cross-sectional area positive (+dT/dt); C: peak of the negative tension derivatives normalized per cross-sectional area positive (-dT/dt). Number of animals: 8; *p ≤ 0.05; ANOVA and Tukey test.*

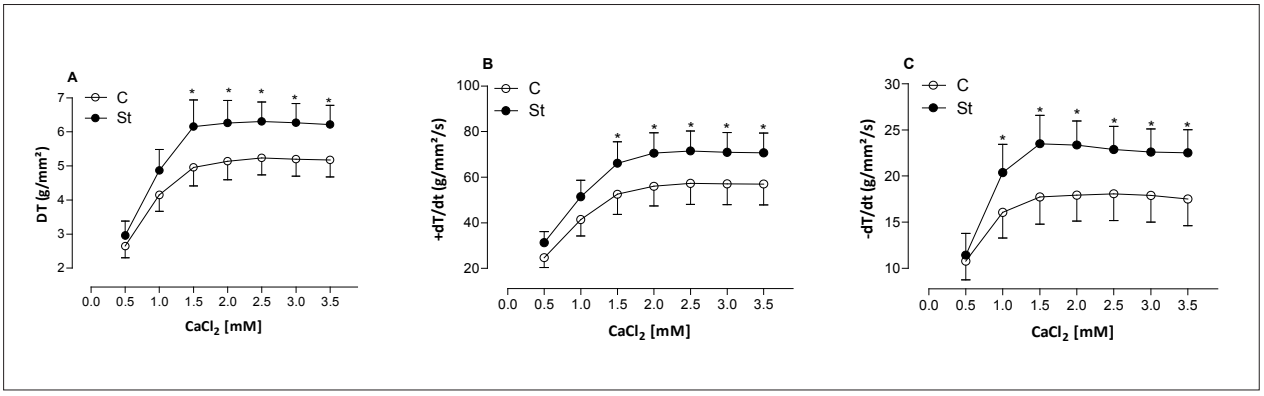

Figure 3 - *Increase in extracellular Ca^{2+} concentration of control rats (C) or rats subjected to chronic stress (St). Chronic stress: immobilization stress 1h/day, 5 times/week, during 15 weeks. A: maximum developed tension normalized per cross-sectional area (DT); B: peak of the positive derivatives normalized per cross-sectional area positive (+dT/dt); C: peak of the negative tension derivatives normalized per cross-sectional area positive (-dT/dt). Number of animals: 8; *p ≤ 0.05; ANOVA and Tukey test.*

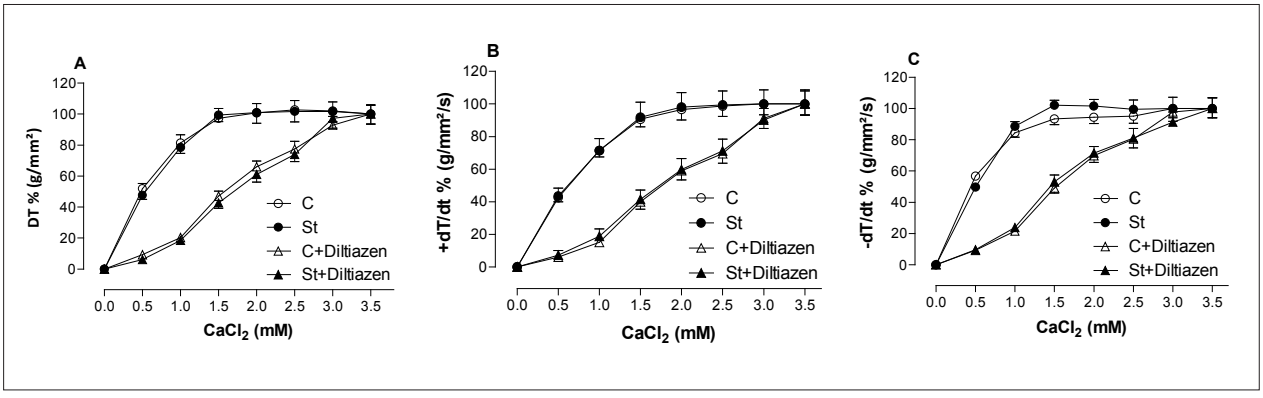

Figure 4 - *Increase in extracellular Ca^{2+} concentration in presence or absence of diltiazem (10−4 M) for control rats (C) or rats subjected to chronic stress (St). Chronic stress: immobilization stress 1h/day, 5 times/week, during 15 weeks. A: maximum developed tension normalized per cross-sectional area (DT); B: peak of the positive derivatives normalized per cross-sectional area positive (+dT/dt); C: peak of the negative tension derivatives normalized per cross-sectional area positive (-dT/dt). Number of animals: 8; *p ≤ 0.05; ANOVA and Tukey test.*

depress the cardiac function under basal conditions and improved myocardial response to inotropic stimulation. The results demonstrate that the L-type Ca^{2+} channel is not involved in improved myocardial function to stress stimulus. Further studies are necessary to better understand the effect of stress on cardiac performance. These results suggest that cardiac alterations of stressed individuals are observed only after exposure to extrinsic stimuli and this response may be an adaptive response to stress conditions with the aim of protecting the individual from cardiovascular disease. This study contributes to the body of knowledge concerning cardiac alterations associated to stress and, consequently, could help physicians proffer useful advice to patients since stress is one of the major cause of cardiovascular diseases.

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Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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