Toxicology Letters 337 (2021) 121–133

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Cardiac effect induced by *Crotalus durissus cascavella* venom: Morphofunctional evidence and mechanism of action

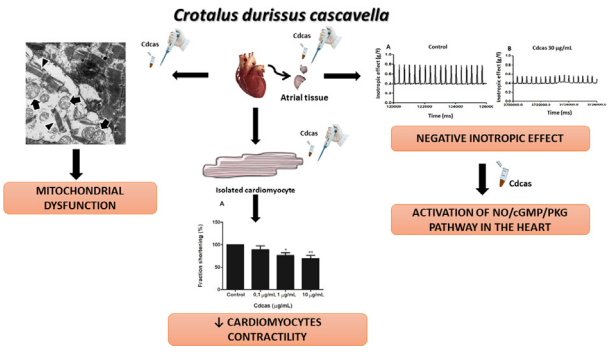
Letícia O. Simões[a], Quiara L. Alves[a], Samuel B. Camargo[a], Fênix A. Araújo[a], Viviane R.S. Hora[a], Rafael L.C. Jesus[a], Breno C. Barreto[b], Simone G. Macambira[b,c], Milena B.P. Soares[b], Cássio S. Meira[b], Márcio C. Aguiar[d], Ricardo. D. Couto[e], Bruno Lomonte[f], José Evaldo Menezes-Filho[g], Jader S. Cruz[g], Marcos A. Vannier-Santos[h], Luciana L. Casais-e-Silva[a,*], Darizy.F. Silva[a]

[a] Department of Bioregulation, Federal University of Bahia, Salvador, BA, 40110-902, Brazil
[b] Gonçalo Moniz Institute, FIOCRUZ, Salvador, BA, Brazil
[c] Department of Biochemistry and Biophysics, Federal University of Bahia, Salvador, BA, 40110-902, Brazil
[d] Department of Biomorphology, Institute of Health Sciences, Federal University of Bahia, Salvador, BA, 40110-902, Brazil
[e] Department of Clinical and Toxicological Analysis, Federal University of Bahia, Salvador, BA, 41170290, Brazil
[f] Clodomiro Picado Institute, Faculty of Microbiology, University of Costa Rica, San José, 11501, Costa Rica
[g] Department of Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, MG, 30161970, Brazil
[h] Oswaldo Cruz Institute, Oswaldo Cruz Foundation – IOC-Fiocruz, Rio de Janeiro, Brazil

HIGHLIGHTS

- Animal toxins are natural resources for pharmacological studies.
- *Crotalus durissus cascavella* (Cdcas) venom reduced cardiomyocytes contractility.
- Venom induced negative inotropic effect through of NO/cGMP/PKG pathway activation.
- Cdcas venom demonstrated absence of the cardiotoxic action.
- Cdcas induced focal ultrastructural changes suggestive of mitochondrial dysfunction.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 July 2020
Received in revised form 28 October 2020
Accepted 18 November 2020
Available online 22 November 2020

Keywords:
Crotalus durissus cascavella
Cardiotoxicity
Atrial

ABSTRACT

Envenoming, resulting from snake bites, is a global public health problem. The present study was undertaken to investigate the influence of *Crotalus durissus cascavella* (Cdcas) venom on cardiac activity and the mechanisms of action underlying its effect. To investigate the inotropic and chronotropic effects induced by Cdcas, studies were performed on the left and right atria. A series of tests were conducted to investigate whether the negative inotropic effect, induced by Cdcas, was related to cardiac damage. Cdcas venom (0.1–30 μg/mL) elicited a significant negative inotropic effect. The addition of Cdcas crude venom (7.5, 15 and 30 μg/mL) did not induce significant alterations in cell proliferation, nor in the enzymatic activity of total-CK and CK—MB. Ultrastructural evaluation demonstrated that cardiac cells from isoproterenol and Cdcas groups revealed discreet swelling and displaced intermyofibrillar mitochondria

* Corresponding author at: Laboratory of Neuroimmunoendocrinology and Toxinology, Department of Bioregulation, Institute of Health Sciences (ICS), Federal University of Bahia, Salvador, Bahia, 40110-902, Brazil.
E-mail address: luciana.casais@ufba.br (L.L. Casais-e-Silva).

http://dx.doi.org/10.1016/j.toxlet.2020.11.019

Negative inotropism effect
Nitric oxide
Soluble guanyly cyclase

with disorganization of the cristae. No change was observed in cardiac electrical activity in perfused isolated rat hearts with Cdcas. In addition, Cdcas reduced contractility in isolated cardiomyocytes from the rat left ventricle. The negative inotropic effect of Cdcas was reduced by L-NAME (100 μM), PTIO (100 μM), ODQ (10 μM) and KT5823 (1 μM), suggesting the participation of NO/cGMP/PKG pathway due to Cdcas. In non-anesthetized rats, Cdcas induced hypotension followed by bradycardia, the latter was also observed by ECG (anesthetized animals). Our results suggest that the negative inotropic effect induced by Cdcas venom is unrelated to cardiac toxicity, at least, at the concentrations tested; and occurs through of NO/cGMP/PKG pathway, likely leading to hypotension and bradycardia when administered *in vivo*.

1. Introduction

Envenoming, resulting from snake bites, is a global public health problem, particularly in Latin American, African and Asian countries, affecting mainly low income populations residing in rural areas (Gutiérrez et al., 2006; Kasturiratne et al., 2008; Harrison et al., 2009; Gutiérrez et al., 2010). Approximately 4.5–5.4 million people are bitten by snakes annually, among which 1.8–2.7 million develop clinical illness (envenoming) after the snakebite, with the number of deaths ranging from 81,000 to 138,000 (WHO, 2018).

In the Americas, the *Viperidae* and *Elapidae* families comprise a clinically important group of venomous snakes (Campbell and Lamar, 2004; Gutiérrez, 2011; Bucaretchi et al., 2016). The *Viperidae* family is responsible for the majority of envenomation, and the genus *Crotalus* causes the highest number of deaths from snakebites in Brazil (Warrell, 2004; Gutiérrez et al., 2009; Gutiérrez, 2011).

The *Crotalus* genus venom accounts for almost 10 % of ophidian accidents recorded in Brazil and has the highest lethality ratio (1.5 %) due to the frequency in with which venom exposure progresses to acute renal failure (Brazil, 1999). In Brazil, the Crotalus genus is represented by a single species, *Crotalus durissus*, and by five subspecies. *Crotalus durissus cascavella* (Cdcas) is mainly found in the "Caatinga" areas of the northeast of Brazil, although its presence has been recorded in the northeast coastal region as well (Boldrini-Franca et al., 2010). *Crotalus durissus* venom is characterized by its neurotoxicity and systemic myotoxicity (rhabdomyolysis), accompanied by thrombin-like activity and acute renal insufficiency, but lacks significant pain and local pathology (Azevedo-Marques et al., 2009).

The effects of Cdcas venom or its isolated toxins on the cardiovascular system have already been demonstrated in previous studies. A natriuretic peptide isolated from Cdcas venom induced renal and vascular effects, likely acting by increasing nitric oxide production (Evangelista et al., 2008). Injection of crude venom intravenously resulted in a decrease in both heart rate and respiratory frequency in rats (Evangelista et al., 2011). In addition, previous studies by our group have demonstrated a vasorelaxant effect caused by Cdcas in the mesenteric artery, through the actions of nitric oxide/guanylyl cyclase/protein kinase G pathway and vascular smooth muscle hyperpolarization through K^+ channels activation (Santos et al., 2017). However, there were no demonstrations of a direct cardiac effect of Cdcas venom, as assessed by toxicity or potential therapeutic effects in the heart. Therefore, the present study aimed to investigate the impact of Cdcas venom on cardiac activity by assessing its therapeutic and/or cardiotoxicity effects and the underlying mechanism of action.

2. Material and methods

2.1. The venom of Crotalus durissus cascavella

The vacuum-dried *Crotalus durissus cascavella* (Cdcas) venom was obtained from adult snakes from Ibiquera, Bahia, Brazil. The venom samples were extracted by manual pressure of the gland from several adult specimens of both sexes. After extraction, the samples were vacuum dried and immediately stored at −20 °C. After preparation of the solutions, the venom was maintained at 4 °C until use. Fresh stock solutions were prepared daily in distilled water and stored on ice until used.

2.2. Drugs and solutions

Ketamine, xylazine, heparin, isoproterenol, epinephrine, propranolol, L-NAME (Nω-Nitro-L-arginine methyl ester), PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide), ODQ (1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one), KT5823, protease type XXIII, porcine pancreas trypsin, bovine serum albumin and ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Collagenase type II was purchased from Worthington Biochemical Co. (Freehold, NJ, USA). Krebs Henseleit solution was used.

2.3. Animals

Male Wistar rats (250−300 g) were obtained from the Neurosciences Laboratory of the Health Sciences Institute at Federal University of Bahia (ICS/UFBA). The animals were housed under controlled temperature (21 ± 1 °C) and day/night cycles (6:00−18:00 h), with free access to food and water. The tests were performed in accordance with National Institutes of Health (NIH) guide for the care and use of Laboratory animals and were approved for use by the Ethics Committee on Animal Use from the Institute of Health Sciences, Federal University of Bahia (CEUA-ICS protocol No. 072/2014).

2.4. Venom characterization

A 500 μg Cdcas sample diluted in milli-Q water was centrifuged at 10,000 rpm for 5 min at room temperature and the supernatant was collected. Trifluoroacetic acid solution (Solvent A, TFA, 0.1 %) was added to the supernatant until achieving the final volume of 2 mL. This mixture was applied to a 2 mL loop, and separated by high-performance liquid chromatography (HPLC) using a reverse-phase (RP) column Teknokroma Europa protein C18 (25 cm × 0.4 cm, 5 mm particle size, 300 Å pore size) in a Shimadzu 10AVP system. The column was equilibrated for 20 min with solvent A, prior to the sample injection. The protein elution fractions were performed by a gradient of solvent B (0.1 % TFA in 100 % of acetonitrile) at a flow rate of 1 mL/ min. Initially the elution was performed at 0% solvent B for 5 min, 0–15 % solvent B for 10 min, 15–45 % solvent B for 60 min, 45–70 % solvent B for 10 min, and 70 % solvent B for 9 min. The elution was monitored at 214 nm, and the experiments were performed in duplicate. In addition, the pooled protein content was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA) and 15 and 30 μg of this material was denatured and reduced in SDS loading buffer for

5 min, for further analysis by 1D polyacrylamide gel (SDS-PAGE 15 %) using a BioRad system under the following conditions: 120 V, 30 mA, 1:30 h (Laemmli, 1970; Santoro et al., 1999). The gel was stained with 0.1 % of Coomassie brilliant blue G (CBB-G-250) for visualization.

2.5. Atrial tissue preparation

Rats were euthanized in a CO_2 chamber, with the heart was carefully removed and the isolated left and right atria were maintained in an organ bath with Krebs-bicarbonate solution at 37 °C, and gassed with a carbogenic mixture (95 % O_2 and 5 % CO_2). The resting tension of each atrium was adjusted to 500 mg and the tissues were equilibrated for at least 60 min before experiments. The left atrium was electrically driven through two parallel platinum electrodes by rectangular voltage pulses with a frequency of 3 Hz, 3 ms duration and voltage at 1.5-fold threshold level. The isometric tension was recorded through a force transducer (FORT-10; WPI, Sarasota, FL, USA) connected to an amplifier-recorder (Miobath-4, WPI, Sarasota, FL, EUA). Cumulative concentration–response curves to Cdcas venom (0.1–30 μg/mL) were performed. In some experiments, the rate of spontaneous beating of right atrium was measured, which was defined as atrial rate, in order to assess the venom-induced chronotropic effects. The inotropic effect induced by venom was studied in the electrically stimulated left atrium.

To investigate the mechanism involved on Cdcas negative inotropic response, the preparations were incubated with different pharmacological agents: epinephrine (10 μM); propranolol (10 μM), a nonselective β-adrenergic receptor antagonist; L-NAME (100 μM), non-selective inhibitor of nitric oxide synthase NOS; PTIO (100 μM), a stable radical scavenger for nitric oxide; ODQ (10 μM), a soluble guanylyl cyclase (sGC) inhibitor; KT5823 (1 μM), a selective PKG inhibitor. For this, after a stabilization period, blockers were added separately and then concentrations of Cdcas (10 and/or 30 μg/mL) were added cumulatively. The responses obtained in the presence or absence of each blocker were compared.

2.6. Cytotoxicity to mammalian cells

Cytotoxicity of Cdcas venom on rat cardiomyocytes was determined using the resazurin assay (Lancaster and Fields, 1996; Naumann et al., 2011). H9c2 heart myoblast line cell were plated onto 96-well plates at a cell density of 5×10^3 cells/well in Dulbecco's modified Eagle medium (DMEM; Life Technologies, GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (FBS; GIBCO), and 50 μg/mL of gentamycin (Novafarma, Anápolis, GO, Brazil) and incubated for 24 h and maintained at 37 °C and 5 % CO_2. Following this, each sample was added at five concentrations of venom (0.37–30 μg/mL) in triplicate and incubated for 72 h. Twenty μL/well of AlamarBlue (Invitrogen, Carlsbad, CA) were added to the plates during 10 h. Colorimetric readings were performed at 570 and 600 nm. The concentration that reduced cell viability by 50 % (CC_{50}) was calculated using data-points gathered from three independent experiments. Gentian violet was used as positive control.

2.7. Determination of creatine kinase activity (CK)

To determine the direct myotoxic activity of Cdcas venom in cardiac tissue, the left atrium was incubated with 30 μg crude venom/mL, vehicle or 10 μM isoproterenol (ISO) for 20 min. Prior to this, samples of organ bath solution were obtained (control condition before the addition of Cdcas venom, vehicle or ISO). Total-CK and CK-MB activity was determined by a UV kinetic method in a Beckman DU 800 spectrophotometer (LabMax 560, clinical chemistry Analyzer, MG, Brazil) with commercial kits, according to the manufacturer's instructions. The results were expressed in U/mL.

2.8. Morphological and ultrastructural analysis of rat atria after Cdcas treatment

After 20 min of treatment with Cdcas (30 μg/mL), vehicle or ISO (10 μM), the left atrium was collected, processed and subjected to morphological analysis by light microscopy and transmission electron microscopy. Samples were fixed in 2.5 % formaldehyde buffered in 0.1 M sodium phosphate, pH 7.4 for 48 h. Next, samples were washed for one hour, dehydrated in a series of increasing concentrations of ethanol, cleared in xylol and embedded in paraffin for histological analysis. Sections were cut (5 μm thick) on a microtome (RM2125RT, Leica®, Nussloch, Germany), stained with hematoxylin-eosin (HE). The images of the histological sections were captured by Analysis Get It software, linked to a digital camera (DP71, Olympus®, Tokyo, Japan) and a light microscope.

The results were described and compared to the control (vehicle), emphasizing the presence of inflammation and hemorrhage in the connective tissue, as well as cellular alterations (hypereosinophilia, decreased cell volume, presence of undulated muscle fibers, absence of transverse striations, sarcoplasmic granulation, contraction bands, nuclear alterations and loss of continuity between muscle fibers), suggestive of myocyte injury.

For cell ultrastructural analysis, samples were fixed for 1 h at room temperature in 2.5 % glutaraldehyde solution in 0.1 M sodium cacodylate buffer, pH 7.4, washed and post-fixed in 1% osmium tetroxide and 0.8 % potassium ferrocyanide and 5 mM calcium chloride in the same buffer at room temperature, in the dark. After washing, samples were dehydrated in acetone and embedded in PolyBed resin (Polyscience, Warrington PA). Ultrathin sections obtained with a diamond knife were collected in 400-mesh copper grids, contrasted with 5 % uranyl acetate and 3 % lead citrate, in aqueous solutions for 20 and 5 min., respectively, and observed in a Jeol JEM 1230 transmission electron microscope.

2.9. Langendorff heart preparation

To assess whether the cardiac effects induced by the venom were possibly due to cardiotoxicity, studies were conducted to evaluate cardiac electrical activity using a Langendorff isolated heart system. Rats were heparinized (300–400 IU) and anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A bilateral thoracotomy was performed, and the hearts were quickly excised. Aortas were cannulated and the hearts were retrograde perfused at constant perfusion pressure with gassed (5 % CO_2, 95 % O_2) Krebs-Henseleit buffer (pH 7.4). An epicardial electrocardiogram (ECG) was continuously recorded using two fine stainless steel electrodes, one attached to the apex of the heart and the other placed on the right atrium.

After stabilization for 15 min, Krebs solution was replaced by Krebs solution containing Cdcas venom (0.1, 1 and 10 μg/mL). Hearts were excluded from the study if they were mechanically unstable or arrhythmic. The electrical functions of the heart were continuously monitored with a computer-based data acquisition system (ML870 PowerLab 8/35, Castle Hill, Australia). Electrocardiograph records were evaluated, and variety of parameters were analyzed, including arrhythmias, heart rate, PR interval, intrinsic deflection, wave amplitude and duration of QT interval.

2.10. Contractility measurement technique in cardiomyocytes

Left ventricular cardiomyocytes were isolated from rats as described by Shioya (2007). The cardiomyocyte contractile function was measured by means of cardiomyocyte length change

technique using an edge motion detection system (Ionoptix, Milton, MA, USA) mounted on an inverted microscope (Nikon Eclipse TS100®, Tokyo, Japan), as previously described by Novaes et al. (2011) and Penitente et al. (2014). The cardiomyocytes were placed in an experimental chamber with glass base and bathed in Tyrode solution.

Cardiomyocytes were field stimulated at a frequency of 1 Hz (10 V/5 min duration) using a pair of steel electrodes and an electrical stimulator field (Myopacer, Ionoptix, MA, USA). Cardiomyocytes was visualized on a personal computer monitor with an NTSC camera (MyoCam, Ionoptix, MA, USA) attached to the microscope using an image detection program (Ionwizard, Ionoptix, MA, USA). This image was used to measure cell shortening in response to electrical stimulation using a video motion edge detector (IonWizard; IonOptix, MA, USA). The longitudinal movements of cardiomyocytes were captured by the edge detection system and stored for subsequent analysis. Only cardiomyocytes that presented sarcomeres with well-defined edges and streaks, relaxed at rest, and without voluntary contractions were used for contraction measures.

The cells from the left ventricles were incubated with Cdcas at three different concentrations (0.1, 1 and 10 μg/mL) to evaluate the effects venom on the percent shortening (bl % peak h), time to maximal departure velocity (dep v t) and time to 10 % of the peak (TP 10 %). The percent shortening was calculated by using the relaxed sarcomere length as 100 %.

2.11. Measurement of mean arterial pressure (MAP) and heart rate (HR) in non-anesthetized normotensive rats

The day before the experimental session, a catheter (PE50) filled with heparinized saline solution (1000 U/mL) was inserted into the aorta artery through femoral artery from rats under ketamine/xylazine anesthesia and exteriorized at the nape of the animal's neck to permit blood pressure recording. An additional catheter was placed in the right femoral vein to allow intravenous drug administration. After 24 h, arterial pressure was continuously monitored through the arterial catheter connected to a blood pressure transducer (ADInstruments, New South Wales, Australia) whose signal was amplified and digitally recorded by an analog-to-digital interface (Power/Lab 8.35, application for data acquisition, LabChart, New South Wales, Australia) and recorded (1 kHz) on a microcomputer for posterior analysis. The MAP and HR were recorded before (baseline values) and after i.v. administration of Cdcas (0.1, 0.3, 1 e 3 μg/mL, i.v, randomly).

2.12. Effect of cdcas on in vivo ECG

Assays for electrocardiographic measurements were performed according to Gondin et al. (2017). Briefly, rats were anesthetized by intraperitoneal administration of 80 mg/kg ketamine and 10 mg/Kg xylazine and were then heparinized (200 U). The administration of Cdcas (0.1, 0.3, 1 and 3 μg/Kg) was performed through the vena cava after 15 min of stabilization. To record the electrocardiogram, animals were maintained in the supine position and three stainless steel electrodes were subcutaneously implanted. Then, the ECG signals were amplified and digitized (Dataq DI400, DI 205, Windaq PRO Acquisition). For each dose of Cdcas, the measured heart rate, PR interval (PRi), QT interval (QTi), RR interval and duration of the QRS complex were measured. For QT correction, the corrected Bazett's formula normalized for rodents was used (QTc - B = QT/ $\sqrt{}$ RR/ f (Kmecova and Klimas, 2010).

2.13. Statistical analysis

Values were expressed as mean ± S.E.M. Statistical significance was determined by Student's *t*-test or One-way ANOVA followed by Bonferroni's post-test, when appropriate. Two-sided $p < 0.05$ was considered statistically significant. All statistical calculations were performed with Prism software version 5.0 (GraphPad Software Inc. La Jolla, CA, USA).

3. Results

3.1. Chromatography and densitometric profile of Cdcas venom

Reduced SDS-PAGE showed protein separation with different molecular weights, especially those at 15, 16 and 40 kDa. Remarkably, Cdcas venom fractions detected by chromatography also resulted in three major proteins peaks (Fig. 1).

3.2. Cdcas venom induces negative inotropic effect

The force and rhythmicity of atrial contraction was assessed in the left and right atriums, respectively, and Cdcas -mediated inotropic and chronotropic effects were determined. The beating

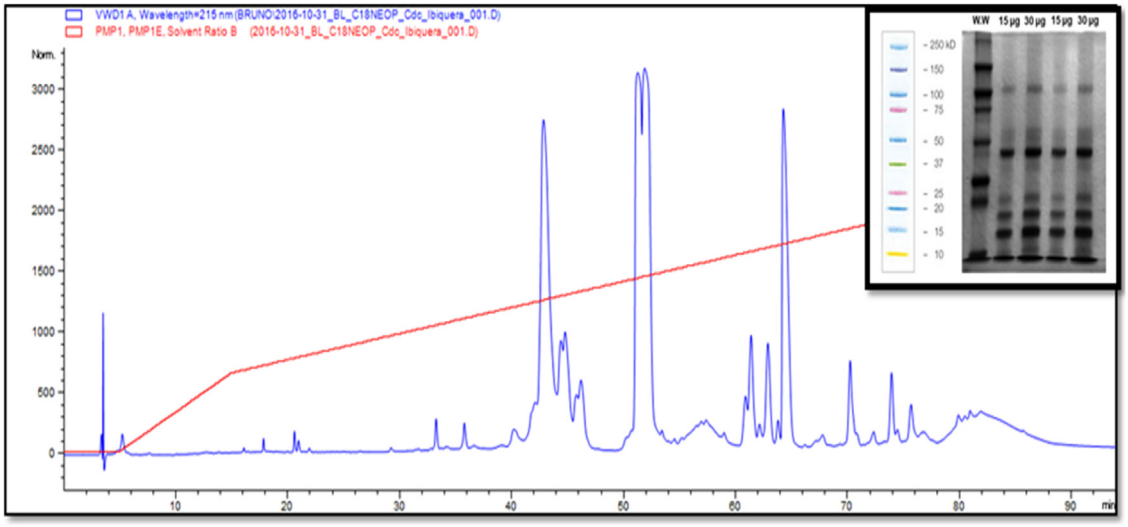


Fig. 1. SDS-PAGE and reverse-phase chromatography analysis of Cdcas. The panel on the right shows the SDS-PAGE (15 %) analysis of Cdcas with distribution of the proteins present in the Cdcas sample.

frequency of the spontaneous right atrium was quantified and defined as atrial rate in order to assess the venom-induced chronotropic effects. Six cumulative concentrations of venom (0.1–30 μg/mL) were added to the right atrium preparation, with a 10 min interval, and no effects on cardiac chronotropism were observed, when compared to control (Fig. 2C). To analyze the effect of Cdcas on the strength of cardiac contraction, cumulative concentrations were added to the left atrium preparation. In this situation, Cdcas (Fig. 2B and D) caused a significant negative inotropic effect (94.7 ± 7.4 %; 80.8 ± 4.2 %; 72.0 ± 5.3 %; 65.8 ± 8.9 %; 58.3 ± 5.5 %; 54.4 ± 7.7 %, p^{**} <0.01, p^{***}<0.001, n = 5) when compared to the control.

3.3. Lack of cytotoxicity in H9c2 heart myoblast line cell treated by Cdcas venom

Fig. 3 shows that addition of Cdcas, at concentrations of 7.5, 15 and 30 μg/mL, did not result in alterations of H9c2 cell proliferation. Gentian violet (GV) was used as positive control and caused a significant loss of cell viability (Fig. 3A).

3.4. Creatine kinase activity in Cdcas -treated atria

The presence of cardiac injury induced by the venoms was assessed by the release of total creatine-kinase (total-CK) and isoenzyme creatine-kinase MB (CK—MB), marker enzymes for cardiac lesions. Isoproterenol (ISO-positive control, 10 μM), a known inducer of myocardial infarction, caused a significant increase in the activity of total-CK (7.6 ± 0.5; 15.4 ± 0.9 U/L, before and after ISO, respectively, n = 5) and CK—MB (5.0 ± 2.2; 16.4 ± 3.0 U/L, before and after ISO, respectively, n = 5). Total-CK and CK—MB values were significantly increased after treatment with ISO compared with vehicle group control. The addition of Cdcas did not induce changes in enzymatic activity of total-CK (7.0 ± 1.2; 9.8 ± 1.3 U/L before and after Cdcas, respectively, n = 5) or CK—MB (3.0 ± 1.5; 3.2 ± 0.6 U/L, before and after Cdcas, respectively, n = 5) (Fig. 3B and 3C).

3.5. Effect of Cdcas on the tissue morphology and cell ultrastructure

In order to evaluate cardiac cell damage, morphological and ultrastructural cell analysis were performed. Data from light microscopy showed that the incubation of rat atria with Cdcas (30 μg/mL) for 20 min, did not induce any significant morphological changes in cardiac tissue when compared to control (Fig. 4).

In all experimental groups, degenerate cardiac myofibrils were observed, but focal electrolucent mitochondrial damage was more common in the ISO-treated group, accompanied by the presence of edema. In addition, ultrastructural evaluation showed fiber disorganization in Cdcas -treated animals. Cardiac cells from all groups revealed swollen and displaced intermyofibrillar mitochondria with disorganized cristae and structural damage, however this effect was mostly observed in the Cdcas group (Fig. 5).

3.6. Absence of Cdcas influences the electrical activity of isolated hearts

The influence on electrical activity induced by the Cdcas venom was assessed using a Langendorff system. Rat hearts perfused by

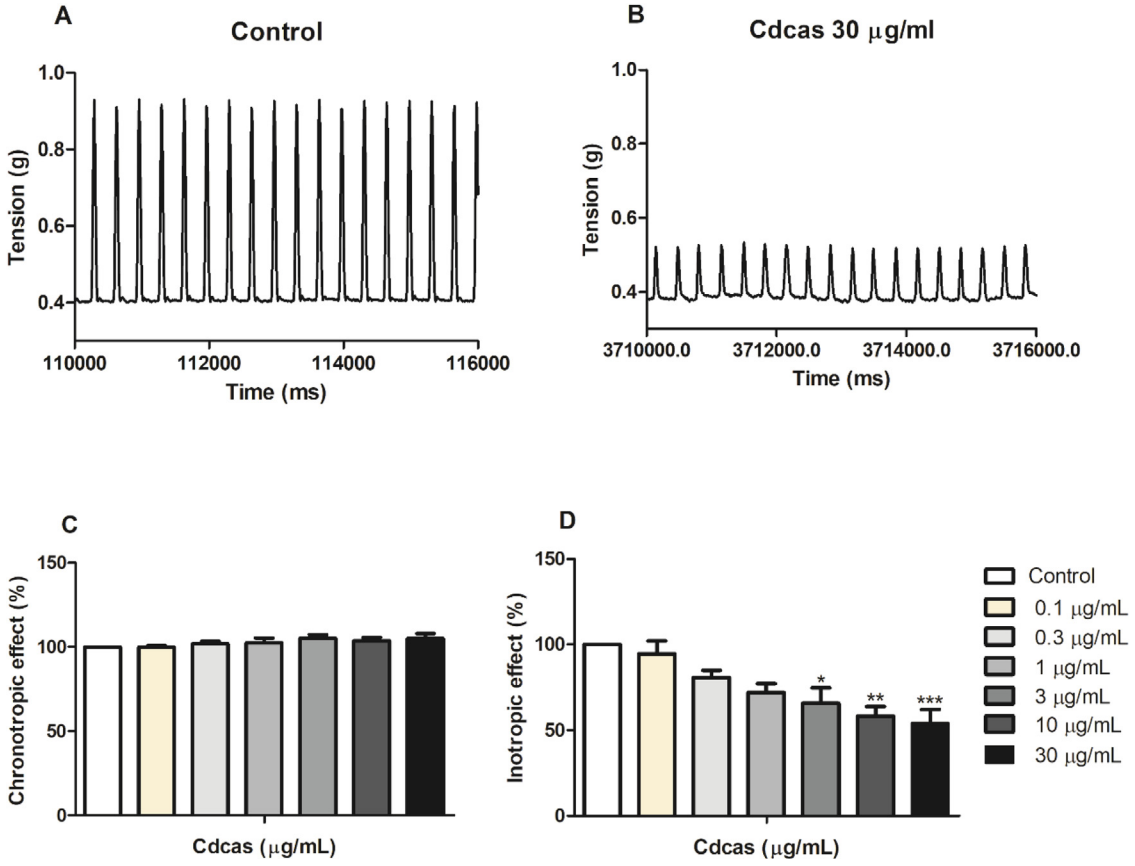


Fig. 2. Cdcas effect on cardiac chronotropism and inotropism in atria isolated from rat hearts. A) Representative tracings showing left atrium without addition of any Cdcas concentration; B) Representative tracings showing the effects of Cdcas (30 μg/mL) on left atrial force contraction. Effects of Cdcas over right atrial beating rate (C) and on the left atrial contraction force (D). Values are expressed with mean ± S.E.M, n = 5, *p < 0.05, **p < 0.01 and ***p < 0.001 *vs.* control.

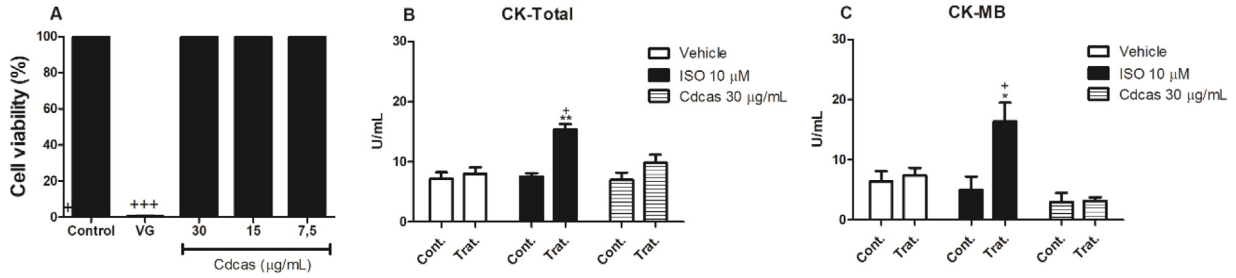


Fig. 3. Absence of cytotoxic effect of Cdcas on H9c2 cells and atria isolated from rat hearts. A) Bar Graph showing H9C2 cell treated with different concentrations of Cdcas (7.5, 15 and 30 μg/mL, n = 9) for 72 h. Cell viability was determined by the Alamar Blue assay and gentian violet (GV) was used as a positive control. B) Bar chart, in concentration of Total-CK, demonstrating increased activity of total-CK induced by isoproterenol (ISO, 10 μM, n = 5) and the absence of influence of Cdcas venom (30 μg/mL, n = 5) on the activity of total-CK in Krebs solution containing rat atria; C) Bar chart, in concentration of CK-MB on rat atria, demonstrating increased activity of CK-MB induced by isoproterenol (ISO - 10 μM) and the absence of Cdcas venom (30 μg/mL) on the activity of CK-MB in Krebs solution. Data are expressed as mean ± SEM; n = 5, *p < 0.05 and **p < 0.01 *vs* treated vehicle; +p < 0.05 and +++ p < 0.001 *vs* control.

Cdcas venom (0.1, 1 and 10 μg/mL) did not show any alterations in electrical activity (Table 1).

3.7. Cdcas effect on the contractility of ventricular cardiomyocytes isolated from rats

To determine whether Cdcas acts directly on cell contractility, cardiomyocytes from rats were incubated with different concentrations of venom (0.1, 1 and 10 μg/mL) for 10 min. Cdcas at 1 and 10 μg/mL induced a significant reduction in cell contractility (76.2 ± 5.9 % and 68.9 ± 7.2 %, respectively, * p < 0.05 and ** p < 0.01, n = 50) when compared to control (100 ± 0.0 %, n = 50; Fig. 6A).

As observed in Fig. 6B, Cdcas significantly decrease departure velocity, only at 10 μg/mL of (67.7 ± 12.6 %, *p < 0.05, n = 50 *versus* 100 ± 0.0 %; Fig. 6B). No changes were observed in the TP 10 % after incubation with Cdcas (104.5 ± 7.1 %, 124.0 ± 6.6 %, 110.6 ± 17.0 %, for concentrations of 0.1, 1 and 10 μg/mL, respectively, n = 50; Fig. 6C)

3.8. The role of adrenergic receptors in inotropic effects induced by Cdcas

In order to evaluate if Cdcas venom is capable of reversing the positive cardiac inotropism induced by adrenergic receptor

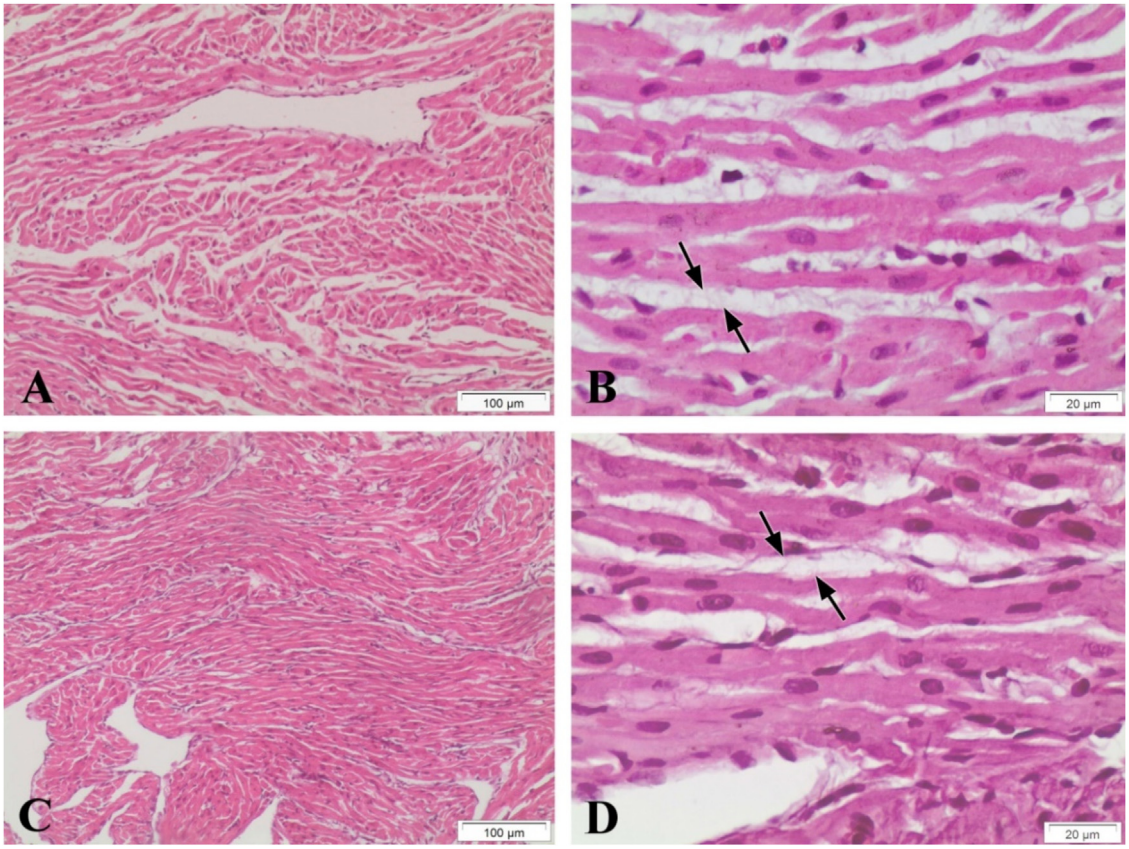


Fig. 4. Photomicrographs showing longitudinal sections of the atrial myocardium of control (A and B, bar size 100 and 20 μm, respectively) and specimens treated with Cdcas (C and D, bar size 100 and 20 μm, respectively). Myocardium from both groups present a slight enlargement of the connective tissue sheath between myocytes (B and D, arrows). Hematoxylin and Eosin staining.

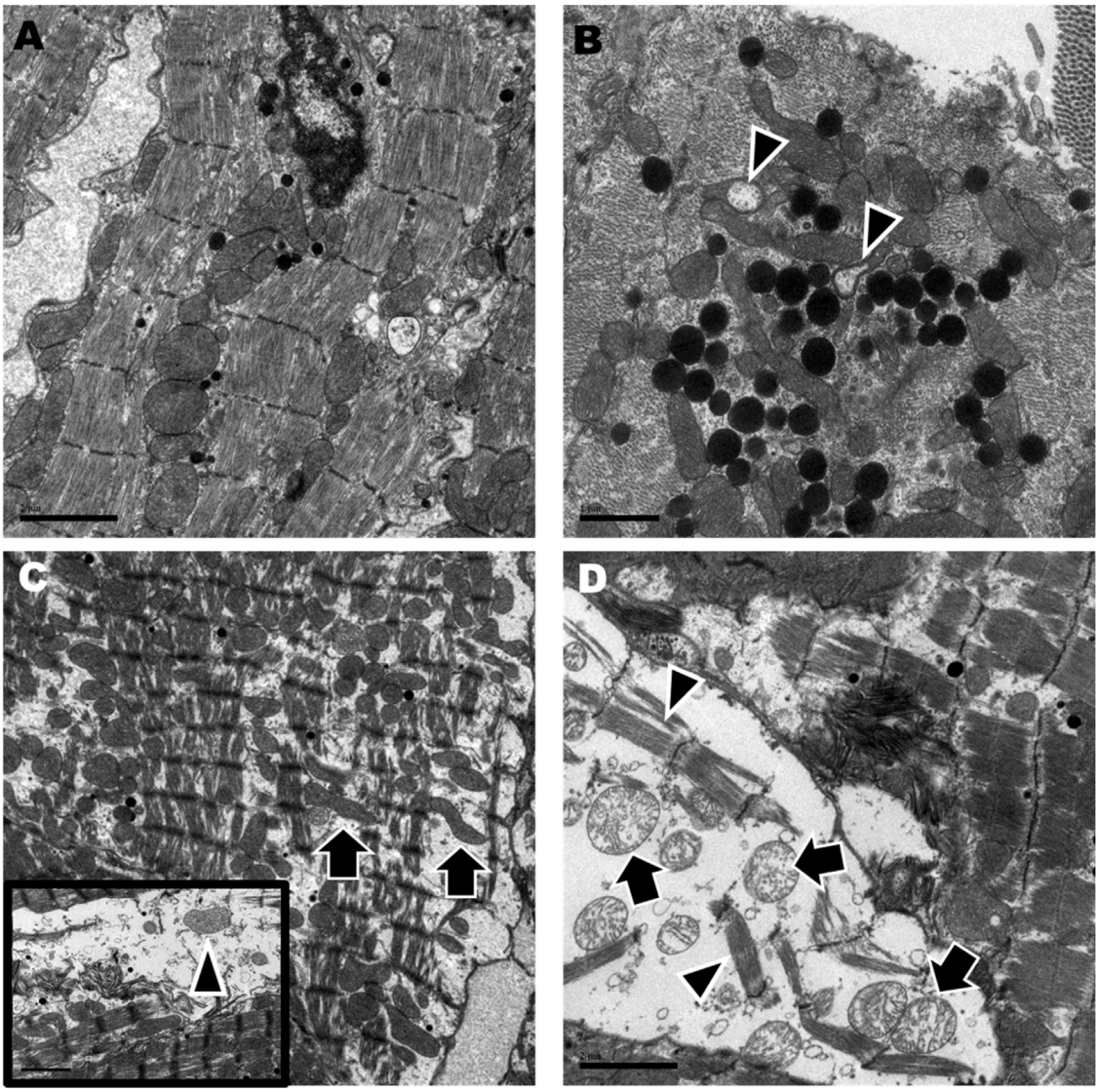


Fig. 5. Transmission electron microscopy of cardiac tissue. Saline-treated myocardium (A) displaying well-organized myofibrils with interposed mitochondria. Myocardium treated with 10 μM Isoproterenol mostly showing preserved fibers, with sporadic mitochondrial lesions (B, arrowheads). Cdcas -treated (30 μg/mL) myocardia (C and D) often presented loosely arranged fibers in the vicinity of randomly distributed mitochondria (C, arrows). Some fibers appeared disrupted releasing mitochondria (inset arrowhead). Several Cdcas -treated samples displayed disorganized sarcomeres (D, arrowheads) and dilated mitochondria presenting reduced electrondensity (D, arrows). The bar size in B is 1 μm. The remaining bars are 2 μm.

activation, experiments were performed using isolated rat atria, which was incubated with epinephrine (10 μM). As observed in Fig. 7A, the addition of Cdcas at 30 μg/mL abolished the positive inotropic effect induced by epinephrine (Epinephrine = 184.6 ± 4.8 %; Epinephrine + Cdcas = 107.8 % ± 13.0, n = 5, ** $p < 0.01$). To assess the participation of adrenergic receptors in the Cdcas -induced negative inotropic effect, left atria was incubated with propranolol (10 μM), a non-selective adrenergic receptor antagonist. In the presence of propranolol, Cdcas (10 and 30 μg/mL) abolished the atrial contractility (Fig. 7B).

3.9. The role of NO/cGMP/PKG in inotropic effects induced by Cdcas

L-NAME (100 μM), PTIO (100 μM), ODQ (10 μM) and KT5823 (1 μM) were used to evaluate the participation of the NO/GMPc/PKG pathway in Cdcas -induced negative inotropic effect. Fig. 7 demonstrates that the Cdcas (30 μg/mL) effects were abolished in the presence of pharmacological inhibitors (L-NAME: 83.6 ± 4.9 %; PTIO: 109.2 ± 3.4 %; ODQ: 135.4 ± 10.0 %; KT5823: 101.8 ± 3.2 %; ** $p < 0.01$ and *** $p < 0.001$ *versus* Cdcas 30 μg/mL (Fig. 7C, D, E and F).

3.10. Effects of Cdcas on mean arterial pressure (MAP) and heart rate (HR) in rats

Intravenous injections of Cdcas (0.1, 0.3, 1 and 3 μg/kg, i.v) resulted in a significant reduction in blood pressure (MAP = -6.5 ± 1.3, -18.2 ± 2.9, -22.9 ± 1.2, -36.0 ± 2.3 %) in a dose-dependent manner (n = 5). This hypotensive effect was associated with bradycardia (HR = -18.8 ± 2.6, -13.0 ± 4.0, -19.2 ± 3.7, -5.0 ± 2.5 %) when compared to the vehicle (Fig. 8 A and B).

3.11. Bradycardic effect of Cdcas in anesthetized animals

To evaluate the influence of Cdcas on cardiac electrical activity, *in vivo* electrocardiographic measurements were performed after administration of different doses of Cdcas (0.1, 0.3, 1 and 3 μg/kg, i.v) in anesthetized rats.

Table 1
Absence of Cdcas effects on cardiac electrogenesis. Heart rate (HR), IRR (RR interval), IPR (PR interval), ventricular activation time (TAV), QT interval (IQT), QRS amplitude (QRS amp). Values are expressed as mean ± S.E.M.

Cdcas						
	IRR	IQT	TAV	QRS Amp	HR	IPR
Control	219 ± 38.8	71 ± 0.4	17 ± 0.4	1.3 ± 0.5	261 ± 34.7	29 ± 2.1
0.1 μg/mL	224 ± 33.3	71 ± 0.0	18 ± 0.8	1.1 ± 0.3	266 ± 34.1	29 ± 2.1
Washing 1	212 ± 33.8	71 ± 0.8	17 ± 1.25	0.9 ± 0.2	269 ± 29.3	29 ± 2.5
1 μg/mL	210 ± 31.6	72 ± 0.8	18 ± 1.70	0.9 ± 0.2	270 ± 26.2	30 ± 2.6
Washing 2	207 ± 25.6	72 ± 0.8	18 ± 1.41	1.1 ± 0.7	279 ± 22.0	29 ± 2.5
10 μg/mL	211 ± 20.3	72 ± 0.4	18 ± 1.41	0.8 ± 0.2	279 ± 19.1	30 ± 2.6
Washing 3	209 ± 17.0	71 ± 0.8	18 ± 1.25	0.8 ± 0.2	266 ± 31.0	29 ± 2.8

The Cdcas reduced heart rate at doses of 0.1, 0.3 and 1 μg/Kg (173.5 ± 5.6, 184.7 ± 4.9, 181.3 ± 7.7 bpm, respectively, n = 6) compared to the control (210.5 ± 3.7 bpm, n = 6) (Fig. 8 C, D, E and F). No significant change was observed in QRS, PR and QTc times after Cdcas administration.

4. Discussion

The crotalic venom found in some species can trigger neurotoxic, myotoxic and coagulant actions. Systemic reactions may be due to stimuli of various origins, including malaise, prostration, sweating, nausea, drowsiness or restlessness and dry mouth (Brasil, 1999). Neurological actions may appear in the first few hours after a bite (Fernandes et al., 2008). Systemic neuro- and myotoxicity upon *C. durissus* ssp. bites are mainly attributable to the high concentration of crotoxin, a heterodimeric PLA_2 molecule (Warrell, 2004; Boldrini-franca et al., 2010). Biological differences between the subspecies of snakes are already reported in the literature, since differences in the composition of the venom have previously been demonstrated, as well as composition changes within the same subspecies according to the geographical variation (Boldrini-Franca et al., 2010). The present study demonstrates, for the first time, experimental evidence that Cdcas venom induced negative inotropic effect unrelated to cardiac tissue damage or cell death, but that may be associated with focal injury to the cell ultrastructure, especially inducing mitochondrial dysfunction. In addition, this study demonstrates the participation of NO/cGMP/PKG pathway in the negative inotropic effect induced by Cdcas. These results support a better understanding of the cardiac mechanisms induced by Cdcas that may assist both, in the clinical management during ophidian accident by Cdcas, as well as by demonstrating the potential source of new drugs present in the Cdcas venom.

Initially, in order to characterize crude Cdcas venom, experiments were performed using RP-HPLC and SDS-PAGE electrophoresis. The results demonstrated a similar chemical profile described by Boldrini-França et al., 2010. In addition, electrophoresis results demonstrated that Cdcas contains predominantly low molecular weight proteins, mainly in the range of 10–50 kDa. The molecular weight band intensity was strongest at approximately 14.4 kDa, which may correspond to the dissociation of certain subunits, including an acidic subunit (CA) and basic subunit (CB) of crotoxin, that tends to occur in the presence of SDS and appears in gel, in the form of monomer and dimers (Rangel-Santos et al., 2004).

The hemodynamic outcome induced by Cdcas during snakebite has already been described (Brasil, 1999). However, the knowledge about direct cardiac actions caused by Cdcas venom remains incomplete. Thus, we directly investigated the cardiac effects of the crude venom in the atrium isolated from rats. Our study demonstrated that the Cdcas venom induced a negative inotropic effect, due to the reduced contractile force, without altering rhythmicity. It is likely that Cdcas is acting on the contractile machinery since the automated cardiac rhythm was not altered, which may be indicative of an absence of action on the sinoatrial node. The findings are similar to studies performed in isolated pig heart in the presence of venom from another snake subspecies, *Crotalus durissus terrificus*, where negative inotropic effects were also observed, without significant changes in heart rate (Santos et al., 1990).

Some snake venom has been described as capable of causing injury in skeletal muscular tissue, as similarly observed in cardiac muscle, following ophidian envenomation (Dias et al., 2012; Lomonte et al., 2003; De Paola and Rossi, 1993; Mebs and Ownby, 1990; Ownby, 1998; Gutiérrez and Lomonte, 1995). Thus, experiments were performed to assess whether the negative inotropic effect observed *in vitro* assays could be due to tissue damage.

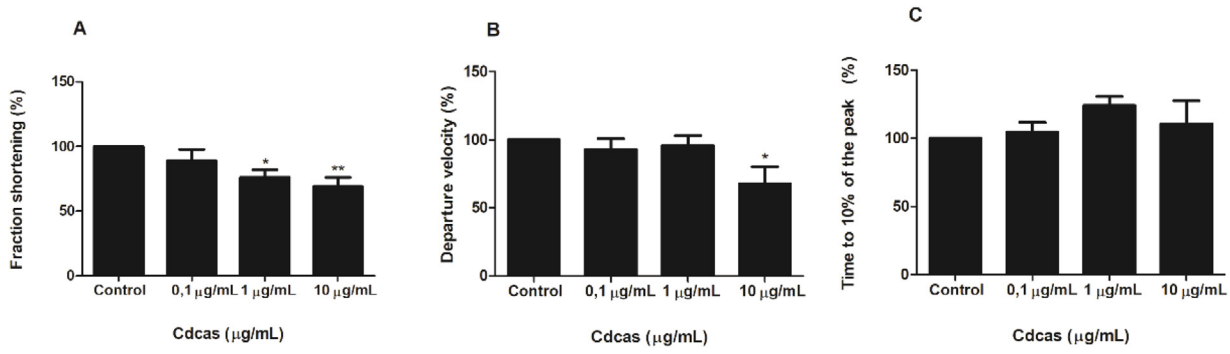


Fig. 6. Influence of Cdcas on the left ventricular cardiomyocyte contractility. A) Bar graph showing the fraction of shortening (A), the departure velocity – dep v t (B) and the time of 10 % of cardiomyocyte peak (C) in absence (control) or in the presence of Cdcas (0.1, 1 and 10 μg/mL). Values are expressed as mean ± SEM. (n = 50 cells). * $p < 0.05$, ** $p < 0.01$ *vs* control.

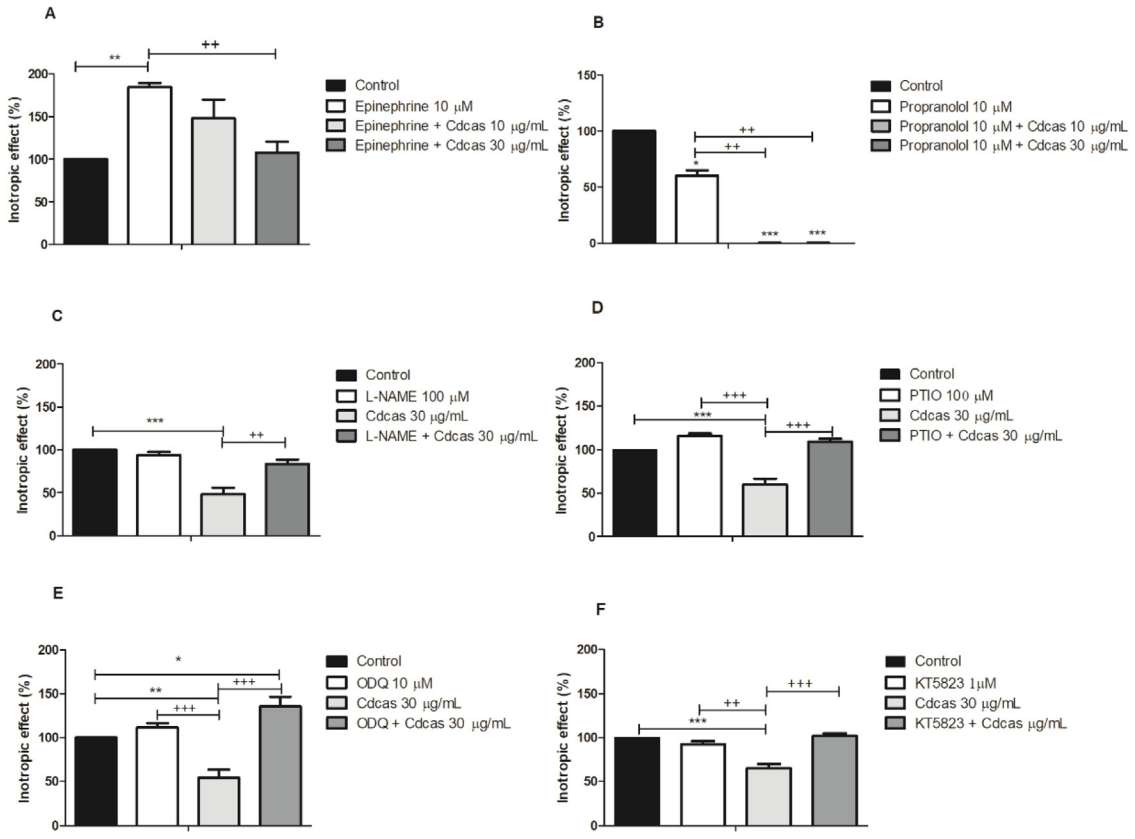


Fig. 7. Participation of the NO/cGMP/PKG pathway in the negative inotropic effect of CDC. Bar graphs demonstrating the effects induced by Cdcas in the presence of epinephrine (A, 10 μM), propranolol (B, 10 μM), L-NAME (C, 100 μM), PTIO (D, 100 μM), ODQ (E, 10 μM) and KT5823 (F, 1 μM) compared with control. Values are expressed as mean ± SEM. (n = 5). * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ *vs* control; + $p < 0.05$; ++$p < 0.01$ and +++ $p < 0.001$ *vs* epinephrine, propranolol, or Cdcas 30 μg/mL.

Initially, the effect of venom on cell viability was determined by a colorimetric Alamar Blue assay. Treatment of cardiomyocytes with Cdcas venom did not change cell viability, even at the highest concentration, suggesting a possible absence of cytotoxic effects. It is worth mentioning that there are no previous publications of venom trials from the *Crotalus* genus in cardiomyocytes from the H9c2 cell line.

The literature describes myotoxic action in skeletal muscle of *Crotalus durissus* venoms (Lomonte et al., 2003). Serial measurements of serum CK, lactate dehydrogenase (LD) and their isoenzymes CK-MB and LD1–5 in victims of *C. durissus terrificus* bites demonstrated a similar pattern to that observed in acute myocardial infarction (AMI), although the clinical course, as well as electro-(ECG) and echocardiographic data (ECHO) did not suggest cardiac involvement (Cupo et al., 1990). In order to investigate if the cardiac effect induced by the venom is associated with any form of tissue damage, quantification of creatine kinase enzyme activity (Total-CK and CK-MB) was performed. Creatine kinase (CK) is an important enzyme regulating the production and use of high energy phosphates in contractile tissues. It is found in skeletal muscles, heart muscle and the brain. CK-MB is present in the myocardium, where it represents about 20 % of the total CK. In AMI, total CK begins to increase within 6 h following the onset of myocardial infarction (Hørder et al., 1990). In the present study, we observed that Cdcas venom did not induce any significantly changes in total-CK or CK-MB levels in bath solutions of left atrial samples, suggesting that this venom is not myotoxic for the cardiac tissue, at least at the concentrations tested.

Additionally, in order to approach eventual Cdcas-induced cell damage, morphological and ultrastructuctural analyses of atrial tissue treated with venoms were performed. In these experiments, histological analysis did not demonstrate important morphological alterations after treatment with Cdcas, when compared to control, while the ultrastructural analysis, Cdcas and ISO produced some effects indicating cell damage, at least at the highest concentration used. However, these effects on the cellular ultrastructure were not able to permanently alter cardiac function. Furthermore, this apparent discrepancy between focal damage, observed in electron microscopy (EM), and the effect of non-toxicity observed in different experiments, such as histological assays, CK-MB dosage, cellular viability analysis, as well as maintenance of the contractile profile of cardiac tissue, even after treatment with Cdcas (functional experiments), may be due to the high sensitivity and resolution of EM images.

In rat cardiac tissue receiving a high dose of venom from another subspecies, *Crotalus durissus terrificus* (80 μg in 0.2 mL of saline i.p), swollen muscle fibers, contraction bands and myocytolytic necrosis were observed, which were associated with mild to moderate edema in the interstitial space, as well as infiltration of mononuclear cells and a large number of mast cells (De Paola and Rossi, 1993). The presence of crotamine and high PLA_2 activity in *Crotalus durissus terrificus* venom may explain, at least in part, greater myotoxic activity when compared to other venoms of the same subspecies (Santoro et al., 1999).

Furthermore, the effects of venom on cardiac electrical activity and evidence of cardiotoxicity were performed in isolated hearts using the Langendorff technique. The results observed in this study suggested that Cdcas venom has no effect on cardiac electrogenesis, evidenced by the non-alteration of measured electrical parameters, such as heart rate, QT interval and RR interval. These

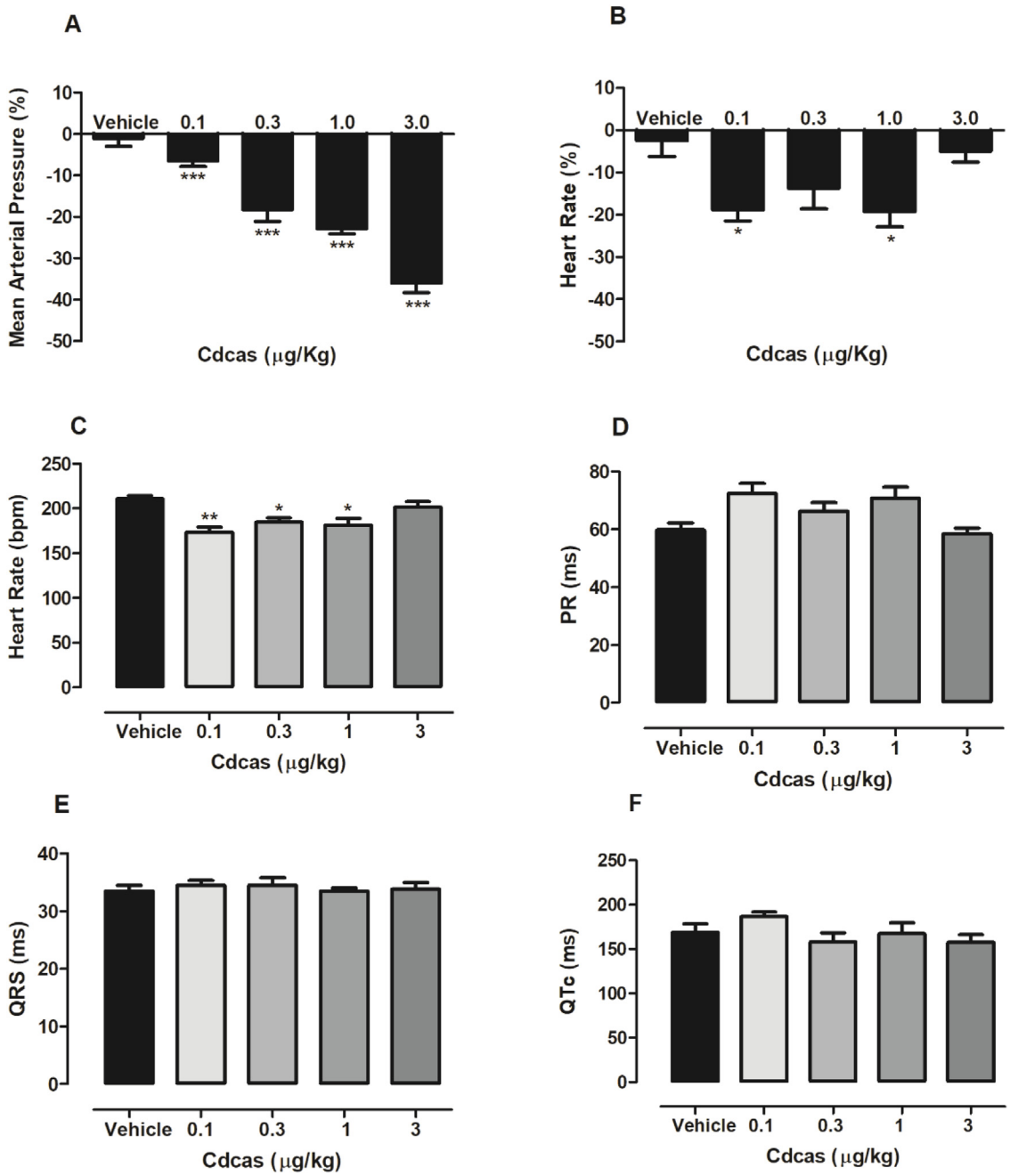


Fig. 8. Hypotensive and bradycardic effect of Cdcas in rats. Bar graphs showing the effects of intravenous administration of Cdcas (0.1, 0.3, 1 and 3 μg/kg, iv) on Mean Blood Pressure (A, MAP) and Heart Rate (B, HR) in non-anesthetized rats. Bar graphs showing the effect of Cdcas (0.1, 0.3, 1 and 3 μg/kg, iv) on HR (C), duration of PR segment (D, PR), QRS interval (E), QTc segment (F) in anesthetized rats. Values are expressed as mean ± SEM, n = 6, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ *vs* vehicle.

data are similar to experiments performed by Breithaupt (1976), that demonstrated no cardiotoxic effect in the Langendorff preparation of rat hearts perfused with phospholipase A alone (6×10^{-6} M) or together with crotapotin (10^{-5} M), isolated from *Crotalus durissus terrificus*. In contrast, the study by Kempsford and Hawgood (1987) demonstrated in Langendorff system perfused isolated rat heart, which examined the effects of *Crotalus horridus horridus* venom, that there was a progressive loss of the RST component of the ECG, which, together with the absence of any effects on sinus frequency and P and Q waves, suggests a specific ventricular action by this venom. It should be noted that the differences in the venom of the *Crotalus* species in South and North Americas, in which the North American species are more proteolytic than Brazilian ones.

To better understand the mechanism of action involved in Cdcas -induced response in cardiac tissue, several agonists and antagonists were used. Initially, the effect of Cdcas was studied in the presence of epinephrine (10 μM), which is known to induce significant increases in cardiac contractile force. In this situation, Cdcas (10 and 30 μg/mL) reversed the epinephrine-induced response, suggesting that Cdcas may reduce the inotropic effect in situations of sympathetic nervous system hyperactivity.

To evaluate whether Cdcas venom induced a negative inotropic effect through adrenergic receptor antagonism in cardiac tissue,

propranolol (10 μM) was used (Jeong et al., 2018). In the presence of β-adrenergic receptors antagonist, the atrial contractility was reduced, as expected. However, Cdcas induced additional negative inotropic effects when incubated with propranolol. This result suggests that β-adrenergic receptors are not required for Cdcas -induced cardiac effects.

Out research group has demonstrated, the vasorelaxant effect induced by Cdcas in the mesenteric artery, in which NO/cGMP/PKG pathway and vascular smooth muscle hyperpolarization through K^+ channels activation was involved in the mechanism of action induced by Cdcas (Santos et al., 2017). Thus, we aimed to more completely understand whether this signaling is important for cardiac activity of Cdcas.

Therefore, L-NAME and PTIO, a non-selective inhibitor of NOS and a NO scavenger respectively, were initially used. As result, the addition of L-NAME and PTIO were observed to induce a significant attenuation of the negative inotropic effect induced by Cdcas, suggesting the involvement of NO in the cardiac response. It is well described that NO is a principal signaling molecule in the cardiovascular and nervous system. The several studies using cardiomyocytes have identified the role of NO in the regulation of cardiac growth and remodeling, contractile performance, rhythmicity and metabolic rates (Smiljić et al., 2014). NO also induces inotropic biphasic effects. The positive inotropic response to NO in isolated cardiomyocytes has been demonstrated previously (Kojda et al., 1997; Vila-Petroff et al., 1999). However, there are also some reports showing NO causing negative inotropic effects at high concentrations (Brady et al., 1993; Mayourian et al., 2018).

The mechanisms of NO in the cardiac cell are mediated by increased cGMP concentration, by direct effects of NO on the target proteins or by indirect effects mediated by NO derivatives. It is generally accepted that soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)-dependent phosphorylation are predominant mechanisms that mediate the effects of NO on the cardiac cell (Zhang, 2017).

Therefore, in order to evaluate the participation of soluble guanylyl cyclase (sGC) in Cdcas effect, ODQ, a selective inhibitor of sGC, was used. In this experiment the GC/cGMP pathway was demonstrated as being important for the venom negative inotropic action, since Cdcas effect was significantly reduced by ODQ.

NO has consistently been shown to reduce Ca^{2+} influx through the L-type Ca^{2+} channel (Cav) and mediated by both S-nitrosylation and PKG phosphorylation (Wang et al., 2015). Additionally, some studies have shown that NO increases cGMP/PKG-dependent phosphorylation of cardiac troponin I and cardiac myosin binding protein C, promote myocyte relaxation and has a negative inotropic effect on cardiac tissue (Jin et al., 2013). Based on these previous observations, the participation of PKG in the Cdcas -induced cardiac effect was evaluated using a selective PKG inhibitor, KT5823. In the presence of this inhibitor, the Cdcas effect was abolished. Taken together, these results suggest that the cardiac effect induced by Cdcas venom may be due to NO release and consequent activation of the sGC and PKG.

In another set of experiments, we investigated the effect of Cdcas *in vivo*, allowing for the observation of the influence of various biological systems, such as the nervous and endocrine systems, on the cardiovascular effect of Cdcas. These experiments were performed to evaluate the hemodynamic effects of the venom in non-anesthetized rats. Cdcas induced a dose-dependent hypotensive response followed by bradycardic effect. The hypotensive effect caused by Cdcas may be related to the decrease in peripheral vascular resistance, as demonstrated by Santos et al. (2017). The bradycardic effect may be related to the direct effect of venom on cardiac tissue, as demonstrated in this study, with an important role of NO. A significant reduction in blood pressure was also observed by Evangelista et al. (2011) following intravenous administration of Cdcas in normotensive rats. The venom evoked a dose-dependent decrease on mean arterial pressure, heart rate, and respiratory rate, with increased plasma nitrite levels. In addition, L-NAME (10 mg/kg) blocked both hypotension and increased nitrite production after venom administration, suggesting NO participation in the hypotensive effect of Cdcas, corroborating the data presented in this study.

Finally, to evaluate electrogenic changes *in vivo* induced by Cdcas, ECG was obtained in animals treated with Cdcas. It was possible to detect changes in both time and intensity of cardiac electrical waves, as well as to identify lesions in the heart muscle itself using electrocardiographic measurements (Nolte and Mckee, 2004). Its effectiveness is shown to be reliable in the diagnosis, since functional alterations reflect changes in the propagation of the cardiac action potentials, consequently causing changes in the ECG's own waves (Arnolds et al., 2011; Schwarz, 2009). In this study, only significant changes in HR were observed, which was similar to that observed in the previous *in vivo* experiment, with no change in PR, QRS and QTc interval duration, suggesting a direct cardiac effect of Cdcas and a possible absence of tissue toxicity.

To the best of our knowledge, this is the first experimental demonstration of the negative inotropic effect induced by Cdcas, unrelated to cardiac toxicity, but possibly related to subcellular disorganization or mitochondrial dysfunction. Furthermore, our data demonstrated that even in the absence of venous-induced cardiotoxic action, focal ultrastructural changes have been identified and may, depending on the dose or exposure time of the venom to cardiac tissue, induce cardiotoxic activity. Finally, additional experiments were performed demonstrating the participation of NO/cGMP/PKG pathway on the Cdcas -induced negative inotropic effect, which may be responsible for the hypotensive and bradycardic effect observed in unanesthetized rats treated with Cdcas. In addition, this study may highlight the biotechnological potential of Cdcas venom in the development of new therapeutic agents for cardiovascular diseases, since cardiac effects of the venom were observed, accompanied by the sparce evidence of possible toxic effects.

Funding

This work was supported by the Bahia State Research Support Foundation [FAPESB grant number 5198/2014], National Council for Scientific and Technological Development (CNPq) and Coordination for the Improvement of Higher Education Personnel (CAPES) - Finance Code 001. JSC, DFS, MBPS and MAVS are CNPq Research Fellows.

CRediT authorship contribution statement

Letícia O. Simões: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Quiara L. Alves:** Investigation, Formal analysis, Methodology. **Samuel B. Camargo:** Investigation, Formal analysis, Methodology. **Fênix A. Araújo:** Investigation, Methodology. **Viviane R.S. Hora:** Investigation, Methodology. **Rafael L.C. Jesus:** Investigation, Methodology. **Breno C. Barreto:** Investigation, Formal analysis. **Simone G. Macambira:** Formal analysis, Methodology, Resources. **Milena B.P. Soares:** Formal analysis, Resources. **Cássio S. Meira:** Investigation, Formal analysis, Methodology. **Márcio C. Aguiar:** Formal analysis, Methodology, Writing - review & editing, Resources. **Ricardo. D. Couto:** Methodology, Resources. **Bruno Lomonte:** Methodology, Formal analysis. **José Evaldo Menezes-Filho:** Investigation, Methodology. **Jader S. Cruz:** Methodology, Resources. **Marcos A. Vannier-Santos:** Formal analysis, Methodology, Writing - review & editing. **Luciana L. Casais-e-Silva:** Formal analysis, Resources,

Writing - review & editing. **Darizy.F. Silva:** Formal analysis, Funding acquisition, Methodology, Project administration, Validation, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Arnolds, D.E., Chu, A., McNally, E.M., Nobrega, M.A., Moskowitz, I.P., 2011. The emerging genetic landscape underlying cardiac conduction system function. Birth Defects Res. Part A Clin. Mol. Teratol. 91 (6), 578–585. doi:http://dx.doi.org/10.1002/bdra.20800.

Azevedo-Marques, M.M., Hering, S.E., Cupo, P., 2009. Acidente crotálico, In: Cardoso, J.L.C., França, F.O.S., Wen, F.H., Málaque, C.M.S., Haddad Jr, V. (Eds.), Animais Peçonhentos No Brasil. Biologia, Clínica E Terapêutica Dos Acidentes, 2ª ed. , pp. 108–115 Sarvier, São Paulo, Brasil.

Boldrini-França, J., Corrêa-Netto, C., Silva, M.M., Rodrigues, R.S., De La Torre, P., Pérez, A., Soares, A.M., Zingali, R.B., Nogueira, R.A., Rodrigues, V.M., Sanz, L., Calvete, J.J., 2010. Snake venomics and antivenomics of *Crotalus durissus* subspecies from Brazil: assessment of geographic variation and its implication on snakebite management. J. Proteomics 73 (9), 1758–1776. doi:http://dx.doi.org/10.1016/j.jprot.2010.06.001.

Brady, A.J., Warren, J.B., Poole-Wilson, P.A., Williams, T.J., Harding, S.E., 1993. Nitric oxide attenuates cardiac myocyte contraction. Am. J. Physiol. Heart Circulat. Physiol. 265 (1), H176–H182. doi:http://dx.doi.org/10.1152/ajpheart.1993.265.1.H176.

Brasil, S., 1999. Manual De Diagnóstico E Tratamento De Acidentes Por Animais Peçonhentos. Fundação Nacional De Saúde. CENEPI, Brasilia, Brasil, pp. 55–61.

Breithaupt, H., 1976. Neurotoxic and myotoxic effects of Crotalus phospholipase A and its complex with crotapotin. Naunyn Schmiedebergs Arch. Pharmacol. 292 (3), 271–278. doi:http://dx.doi.org/10.1007/BF00517389.

Bucaretchi, F., De Capitani, E.M., Vieira, R.J., Rodrigues, C.K., Zannin, M., Da Silva Jr, N. J., Casais-e-Silva, L.L., Hyslop, S., 2016. Coral snake bites (Micrurus spp.) in Brazil: a review of literature reports. Clin. Toxicol. 54 (3), 222–234. doi:http://dx.doi.org/10.3109/15563650.2015.1135337.

Campbell, J.A., Lamar, W.W., 2004. The Venomous Reptiles of the Western Hemisphere. Cornell University Press, Ithaca, USA.

Cupo, P., Azevedo-Marques, M.M., Hering, S.E., 1990. Acute myocardial infarction-like enzyme profile in human victims of *Crotalus durissus terrificus* envenoming. Trans. R. Soc. Trop. Med. Hyg. 84 (3), 447–451. doi:http://dx.doi.org/10.1016/0035-9203(90)90358-L.

De Paola, F., Rossi, M.A., 1993. Myocardial damage induced by tropical rattlesnake (*Crotalus durissus terrificus*) venom in rats. Cardiovasc. Pathol. 2 (1), 77–81. doi:http://dx.doi.org/10.1016/1054-8807(93)90016-U.

Dias, L., Rodrigues, M.A., Smaal, A., Rennó, A.L., Mello, S.M., Moreno, H., Hyslop, S., 2012. Cardiovascular responses to *Bothrops alternatus* (Urutu) snake venom in anesthetized dogs. Cardiovasc. Toxicol. 12 (3), 243–257. doi:http://dx.doi.org/10.1007/s12012-012-9163-1.

Evangelista, J.S., Martins, A.M., Nascimento, N.R., Sousa, C.M., Alves, R.S., Toyama, D. O., Evangelista, J.J.F., de Menezes, D.B., Fontele, M.C., Moraes, M.E., Monteiro, H.S. A., 2008. Renal and vascular effects of the natriuretic peptide isolated from *Crotalus durissus cascavella*venom. Toxicon 52 (7), 737–744. doi:http://dx.doi.org/10.1016/j.toxicon.2008.08.014.

Evangelista, J.S., Evangelista, J.J., Evangelista, I.L., Nojosa, D.M., Nascimento, N.R., Souza, M.H., Alves, R.S., Martins, A.M., Moraes, M., Monteiro, H.S., 2011. Hypotensive effects of the *Crotalus durissus cascavella* venom: involvement of NO. Nat. Prod. Commun. 6 (6), 871–874. doi:http://dx.doi.org/10.1016/j.toxicon.2008.08.014.

Fernandes, T.A., Aguiar, C.N., Daher, E.F., 2008. Envenenamento Crotálico: epidemiologia, insuficiência renal aguda e outras manifestações clínicas. Revista Eletrônica Pesquisa Médica 2 (2), 1–10.

Gutiérrez, J.M., 2011. Envenenamientos por mordeduras de serpientes en América Latina y el Caribe: una visión integral de carácter regional. Boletín de malariología y salud ambiental 51 (1), 1–16.

Gutiérrez, J., Lomonte, B., 1995. Phospholipase A2 myotoxins from Bothrops snake venoms. Toxicon 33 (11), 1405–1424. doi:http://dx.doi.org/10.1016/0041-0101(95)00085-Z.

Gutiérrez, J.M., Theakston, R.D.G., Warrell, D.A., 2006. Confronting the neglected problem of snake bite envenoming: the need for a global partnership. PLoS Med. 3 (6), e150. doi:http://dx.doi.org/10.1371/journal.pmed.0030150.

Gutiérrez, J.M., Fan, H.W., Silvera, C.L., Angulo, Y., 2009. Stability, distribution and use of antivenoms for snakebite envenomation in Latin America: report of a workshop. Toxicon 53 (6), 625–630. doi:http://dx.doi.org/10.1016/j.toxicon.2009.01.020.

Gutiérrez, J.M., Williams, D., Fan, H.W., Warrell, D.A., 2010. Snakebite envenoming from a global perspective: towards an integrated approach. Toxicon 56 (7), 1223–1235. doi:http://dx.doi.org/10.1016/j.toxicon.2009.11.020.

Harrison, R.A., Hargreaves, A., Wagstaff, S.C., Faragher, B., Lalloo, D.G., 2009. Snake envenoming: a disease of poverty. PLoS Neglected Tropical Diseases, 3(12), e 569. head of bacteriophage T4. Nature 227 (5259), 680–685. doi:http://dx.doi.org/10.1371/journal.pntd.0000569 1970.

Hørder, M., Elser, R.C., Gerhardt, W., Mathieu, M., Sampson, E.J., 1990. International Federation of Clinical Chemistry (IFCC): Scientific Division, Committee on Enzymes. IFCC methods for the measurement of catalytic concentration of enzymes. Part 7. IFCC method for creatine kinase (ATP: creatine (N-phosphotransferase, EC 2.7. 3.2). IFCC Recommendation. J. Anal. Methods Chem. 12 (1), 22–40. doi:http://dx.doi.org/10.1155/S1463924690000049.

Jeong, T.Y., Asselman, J., De Schamphelaere, K.A., Van Nieuwerburgh, F., Deforce, D., Kim, S.D., 2018. Effect of β-adrenergic receptor agents on cardiac structure and function and whole-body gene expression in Daphnia magna. Environ. Pollut. 241, 869–878. doi:http://dx.doi.org/10.1016/j.envpol.2018.06.026.

Jin, C.Z., Jang, J.H., Kim, H.J., Wang, Y., Hwang, I., Sadayappan, S., Park, B.M., Kim, S.H., Jin, Z.H., Seo, E.Y., Kim, K., Kimn, Y., Kim, S.J., Zhang, Y.H., 2013. Myofilament Ca^{2+} desensitization mediates positive lusitropic effect of neuronal nitric oxide synthase in left ventricular myocytes from murine hypertensive heart. J. Mol. Cell. Cardiol. 60, 107–115. doi:http://dx.doi.org/10.1016/j.yjmcc.2013.04.017.

Kasturiratne, A., Wickremasinghe, A.R., de Silva, N., Gunawardena, N.K., Pathmeswaran, A., Premaratna, R., Savioli, L., Lalloo, D.G., de Silva, H.J., 2008. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. PLoS Med. 5 (11), e218. doi:http://dx.doi.org/10.1371/journal.pmed.0050218.

Kempsford, R., Hawgood, B.J., 1987. Characteristics of the action of *Crotalus horridus horridus* (timber rattlesnake) venom on the isolated, perfused rat heart. Toxicon 25 (12), 1311–1319. doi:http://dx.doi.org/10.1016/0041-0101(87)90009-2.

Kmecova, J., Klimas, J., 2010. Heart rate correction of the QT duration in rats. Eur. J. Pharmacol. 641 (2-3), 187–192. doi:http://dx.doi.org/10.1016/j.ejphar.2010.05.038.

Kojda, G., Kottenberg, K., Noack, E., 1997. Inhibition of nitric oxide synthase and soluble guanylate cyclase induces cardiodepressive effects in normal rat hearts. Eur. J. Pharmacol. 334 (2-3), 181–190. doi:http://dx.doi.org/10.1016/s0014-2999(97)01168-0.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (5259), 680. doi:http://dx.doi.org/10.1038/227680a0.

Lancaster, M.V. and Fields, R.D. (1996). Antibiotic and Cytotoxic Drug Susceptibility Assays using Resazurin and Poising Agents. U.S. Patent No. 5,501,959.

Lomonte, B., Angulo, Y., Calderón, L., 2003. An overview of lysine-49 phospholipase A2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. Toxicon 42 (8), 885–901. doi:http://dx.doi.org/10.1016/j.toxicon.2003.11.008.

Mayourian, J., Ceholski, D.K., Gonzalez, D.M., Cashman, T.J., Sahoo, S., Hajjar, R.J., Costa, K.D., 2018. Physiologic, pathologic, and therapeutic paracrine modulation of cardiac excitation-contraction coupling. Circ. Res. 122 (1), 167–183. doi:http://dx.doi.org/10.1161/CIRCRESAHA.117.311589.

Mebs, D., Ownby, C.L., 1990. Myotoxic components of snake venoms: their biochemical and biological activities. Pharmacol. Ther. 48 (2), 223–236. doi:http://dx.doi.org/10.1016/0163-7258(90)90081-C.

Naumann, G.B., Silva, L.F., Silva, L., Faria, G., Richardson, M., Evangelista, K., Kohlhoffa, M., Gontijo, C.M.F., Navdaev, A., Rezende, F.F., Eble, J.A., Sanchez, E.F., 2011. Cytotoxicity and inhibition of platelet aggregation caused by an L-amino acid oxidase from *Bothrops leucurus* venom. Biochimica et Biophysica Acta (BBA)-General Subjects 1810 (7), 683–694. doi:http://dx.doi.org/10.1016/j.bbagen.2011.04.003.

Nolte, E., McKee, M., 2004. Does health care save lives? Avoidable mortality revisited. Nuffield Trust. 139. doi:http://dx.doi.org/10.13016/74zg-n46j.

Novaes, R.D., Penitente, A.R., Gonçalves, R.V., Talvani, A., Neves, C.A., Maldonado, I.R., Natali, A.J., 2011. Effects of Trypanosoma cruzi infection on myocardial morphology, single cardiomyocyte contractile function and exercise tolerance in rats. Int. J. Exp. Pathol. 92 (5), 299–307. doi:http://dx.doi.org/10.1111/j.1365-2613.2011.00781.x.

Ownby, C.L., 1998. Structure, function and biophysical aspects of the myotoxins from snake venoms. J. Toxicol. Toxin. Rev. 17 (2), 213–238. doi:http://dx.doi.org/10.3109/15569549809009250.

Penitente, A.R., Novaes, R.D., Silva, M.E., Silva, M.F., Quintão-Júnior, J.F., Guatimosim, S., Cruz, J.S., Chianca-Jr, D.A., Natali, A.J., Neves, C.A., 2014. Basal and β-Adrenergic cardiomyocytes contractility dysfunction induced by dietary protein restriction is associated with downregulation of SERCA2a expression and disturbance of endoplasmic reticulum Ca2+ regulation in rats. Cell. Physiol. Biochem. 34 (2), 443–454. doi:http://dx.doi.org/10.1159/000363013.

Rangel-Santos, A., Dos-Santos, E.C., Lopes-Ferreira, M., Lima, C., Cardoso, D.F., Mota, I., 2004. A comparative study of biological activities of crotoxin and CB fraction of venoms from *Crotalus durissus terrificus*, *Crotalus durissus cascavella* and *Crotalus durissus collilineatus*. Toxicon 43 (7), 801–810. doi:http://dx.doi.org/10.1016/j.toxicon.2004.03.011.

Santoro, M.L., Sousa-e-Silva, M.C., Gonçalves, L.R., Almeida-Santos, S.M., Cardoso, D. F., Laporta-Ferreira, I.L., Saiki, M., Peres, C.A., Sano-Martins, I.S., 1999. Comparison of the biological activities in venoms from three subspecies of the South American rattlesnake (*Crotalus durissus terrificus, C. Durissus cascavella* and *C. Durissus collilineatus*). Comp. Biochem. Physiol. C, Pharmacol. Toxicol. Endocrinol. 122 (1), 61–73. doi:http://dx.doi.org/10.1016/S0742-8413(98)10079-8.

Santos, P.E.B., Souza, S.D., Freire-Maia, L., Almeida, A.P., 1990. Effects of crotoxin on the isolated guinea pig heart. Toxicon 28 (2), 215–224. doi:http://dx.doi.org/10.1016/0041-0101(90)90415-4.

Santos, S.S., Jesus, R.L.C., Simões, L.O., Vasconcelos, W.P., Medeiros, I.A., Veras, R.C., Casais-E-Silva, L.L., Silva, D.F., 2017. NO production and potassium channels activation induced by *Crotalus durissus cascavella* underlie mesenteric artery relaxation. Toxicon 133, 10–17. doi:http://dx.doi.org/10.1016/j.toxicon.2017.04.010.

Schwarz, L., 2009. Artigo de revisão: eletrocardiograma. Revista Ilha Digital 1, 3–19.

Shioya, T., 2007. A simple technique for isolating healthy heart cells from mouse models. J. Physiol. Sci. 57 (6), 327–335. doi:http://dx.doi.org/10.2170/physiolsci.RP010107.

Smiljić, S., Nestorović, V., Savić, S., 2014. Modulatory role of nitric oxide in cardiac performance. Med. Pregl. 67 (9-10), 345–352. doi:http://dx.doi.org/10.2298/MPNS1410345S.

Vila-Petroff, M.G., Younes, A., Egan, J., Lakatta, E.G., Sollott, S.J., 1999. Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. Circ. Res. 84 (9), 1020–1031. doi:http://dx.doi.org/10.1161/01.RES.84.9.1020.

Wang, Y., Youm, J.B., Jin, C.Z., Shin, D.H., Zhao, Z.H., Seo, E.Y., Jang, J.H., Kim, S.J., Jin, Z.H., Zhang, H.Y., 2015. Modulation of L-type Ca^{2+} channel activity by neuronal nitric oxide synthase and myofilament Ca^{2+} sensitivity in cardiac myocytes from hypertensive rat. Cell Calcium 58 (3), 264–274. doi:http://dx.doi.org/10.1016/j.ceca.2015.06.004.

Warrell, D.A., 2004. Snakebites in Central and South America: epidemiology, clinical features, and clinical management. In: Campbell, J.A., Lamar, W.W. (Eds.), The Venomous Reptiles of the Western Hemisphere. Comstock Publishing Associates, Ithaca and London, pp. 709–761.

World Health Organization, 2018. Prevalence of Snakebite Envenoming. Available: http://www.who.int/snakebites/epidemiology/en/. Accessed 30 October. .

Zhang, Y.H., 2017. Nitric Oxide Signalling and Neuronal Nitric Oxide Synthase in the Heart Under Stress. [version 1; peer review: 2 approved]. F1000Research, 6 (F1000 Faculty Rev):742. doi:http://dx.doi.org/10.12688/f1000research.10128.1.