Toxicon 137 (2017) 144–149

Contents lists available at ScienceDirect

Toxicon

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

Short communication

Pulmonary mechanic and lung histology induced by *Crotalus durissus cascavella* snake venom

Joselito de Oliveira Neto [a, *], João Alison de Moraes Silveira [b], Daniel Silveira Serra [c], Daniel de Araújo Viana [a], Diva Maria Borges-Nojosa [d], Célia Maria Souza Sampaio [e], Helena Serra Azul Monteiro [b], Francisco Sales Ávila Cavalcante [c], Janaina Serra Azul Monteiro Evangelista [a]

[a] Postgraduate Program in Veterinary Sciences, Faculty of Veterinary, State University of Ceará, Fortaleza, Ceará, Brazil
[b] Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, Fortaleza, Ceará, Brazil
[c] Postgraduate Program in Physics, State University of Ceará, Fortaleza, Ceará, Brazil
[d] Department of Biology, Federal University of Ceará, Fortaleza, Ceara, Brazil
[e] Department of Biology, State University of Ceará, Fortaleza, Ceará, Brazil

ARTICLE INFO

Article history:
Received 15 September 2016
Received in revised form
23 July 2017
Accepted 26 July 2017
Available online 28 July 2017

Keywords:
Crotalus durissus cascavella
Pulmonary mechanics
Histopathology
Venom
Balb/C

ABSTRACT

This study have analyzed the pulmonary function in an experimental model of acute lung injury, induced by the *Crotalus durissus cascavella* venom *(C. d. cascavella)* (3.0 μg/kg - *i.p*), in pulmonary mechanic and histology at 1 h, 3 h, 6 h, 12 h and 24 h after inoculation. The *C. d. cascavella* venom led to an increase in Newtonian Resistance (R_N), Tissue Resistance (G) and Tissue Elastance (H) in all groups when compared to the control, particularly at 12 h and 24 h. The Histeresivity (η) increased 6 h, 12 h and 24 h after inoculation. There was a decrease in Static Compliance (C_{ST}) at 6 h, 12 h and 24 h and inspiratory capacity (IC) at 3 h, 6 h, 12 h and 24 h. *C. d. cascavella* venom showed significant morphological changes such as atelectasis, emphysema, hemorrhage, polymorphonuclear inflammatory infiltrate, edema and congestion. After a challenge with methacholine (MCh), R_N demonstrated significant changes at 6, 12 and 24 h. This venom caused mechanical and histopathological changes in the lung tissue; however, its mechanisms of action need further studies in order to better elucidate the morphofunctional lesions.

1. Introduction

The subspecies *Crotalus durissus cascavella* (*C. d. cascavella*) is found in the Caatinga from Northeastern of Brazil (Pinho and Pereira, 2001; Barraviera, 1989). Its venom is presented as a toxic-enzymatic complex, mainly composed of phosphodiesterase, L-amino oxidase, 5-nucleotidase and toxins such as crotoxine, convulxin, crotalin and gyroxin (Rangel-Santos et al., 2004; Fonseca et al., 2006; Spinosa et al., 2008). This complex substance leads to systemic functional changes, being responsible for the primary cause of death after a snakebite (Evangelista et al., 2008), among which the acute respiratory failure related to neuromuscular paralysis can be highlighted (Damico et al., 2005).

Mechanical properties of the respiratory system are well defined; however, the characterization of lung structures and quantification of inflammation generated by *C. d. cascavella* venom are little known. Therefore, it was necessary to study the effects of possible changes in lung architecture so that, in any involvement, therapeutic intervention may be the earliest and most efficient as possible. This study aimed to analyze the pulmonary function in an experimental model of acute lung injury induced by *C. d. cascavella* venom.

2. Methods

2.1. Venom, chemicals and reagents

The *C. d. cascavella* venom was obtained and kindly provided by Professor Dr. Diva Maria Borges Nojosa, from Núcleo Regional de Ofiologia (NUROF) of the Department of Biology from Federal University of Ceará, Fortaleza, Brazil. The venom was lyophilized and diluted in saline (0.9% w/v NaCl solution) at the time of use. All

* Corresponding author.
E-mail address: joselitoneto@yahoo.com.br (J. Oliveira Neto).

http://dx.doi.org/10.1016/j.toxicon.2017.07.023
0041-0101/

chemicals were purchased from Sigma-Aldrich® (St Louis, MO, USA).

2.2. Animals

The Local Ethics Committee on the Use of Animals approved the experimental protocol (12773584-4). 36 Balb/C mice (males, 20–30 g) were randomly divided into 6 groups. According to Miller and Tainter (1944) apud Feitosa (1996), the calculation of the lethal dose was done according to the method of the probit in which it was observed that the LD50 for venom of C.d. Casvella is 3.0 ± 0.34 mg/kg.

All experimental groups received inoculation of 3.0 μg/kg of venom, diluted in 0.1 mL of saline (NaCl 0.9%) (i.p.). The animals were analyzed after 1 h of inoculation (group 1 h, n = 6), 3 h after inoculation (group 3 h, n = 6), 6 h after inoculation (group 6 h, n = 6), 12 h after inoculation (12 h group, n = 6) and 24 h after inoculation (group 24 h, n = 6). The Ctrl group (n = 6) received only 0.1 mL of saline (i.p).

2.3. Respiratory system mechanics

After the inoculation period of the venom in each group, the animals were sedated (diazepam, 1 mg/kg, i.p.), and anesthetized with sodium pentobarbital (50 mg/kg, i.p., Hypnol® 3%, Syntect, Brazil) and tracheotomized. The animals were intubated with a 18-gauge cannula (Eastern Medikit, Delhi, India) that was then connected to a computer-controlled ventilator for small animals (Scirec© -*flexVent*®, Montreal, QC, Canada). The animals were ventilated at baseline settings: respiratory frequency of 150 breaths/min, tidal volume of 10 mL/kg, limiting pressure of 30 cmH_2O, and positive end-expiratory pressure (PEEP) of 3 cmH_2O. Mices were then paralyzed with pancuronium bromide (0.5 mL/kg, i.p., Cristália, Lindoia, MG, Brazil).

Initially we standardized the mechanical history of the respiratory system with two deep inflations (DI, 6-s long, peak pressure: 30 cmH_2O), followed by a period of 5min of ventilation. Soon after, the impedance of the respiratory system (Z_{rs}) was measured with the forced oscillation technique (Hantos et al., 1992), 12 sequential 10-s sampling intervals, for a total of 2 min.

The experimental Z_{rs} was fitted to the constant phase model as previously described (Hirai et al., 1999):

$$Z_{rs} = R_N + I(2\pi f)i + \frac{G - Hi}{(2\pi f)^{\alpha}} \quad (1)$$

$$\alpha = \frac{2}{\pi}\tan^{-1}\left(\frac{H}{G}\right) \quad (2)$$

where R_N is the Newtonian resistance, which represents the central airways resistance, $i = \sqrt{-1}$, f is the frequency (Hz), I represents airway inertance, and G and H are respectively the dissipative and elastic properties of lung tissue (Hantos et al., 1992). Hysteresivity ($\eta = G/H$) was also calculated (Fredberg, 2001).

Thereafter, starting at the functional residual capacity (FRC) defined by the PEEP, the flexiVent delivered 7 inspiratory pressure steps for a total pressure of 30 cmH_2O, followed by 7 expiratory steps, pausing at each step for 1 s. At each step plateau pressure (P) was recorded and related to the total volume (V) delivered to produce a quasi-static PV (pressure-volume) curve. Static compliance (C_{ST}) was calculated as the slope of the curve (Salazar and Knowles, 1964). Two quasi-static PV curves were obtained to measure C_{ST}, an estimate of inspiratory capacity (IC), and PV loop area.

2.4. Hyperresponsiveness of airway smooth muscle

Immediately after measurements of respiratory system mechanics, two DIs (Deep Inflation) were done, followed by 5 min of ventilation with baseline settings. Airway smooth muscle hyperresponsiveness was evaluated by inhalation of methacholine (MCh) (Sigma-Aldrich, St. Louis, MI, USA) delivered by aerosol produced by an ultrasonic nebulizer (Inalasonic, NS Indústria de Aparelhos Médicos, São Paulo, SP, Brazil) coupled to the inspiratory line of the ventilator. For such purpose, 4 mL of MCh solution (3, 6, 12,5, 25 and 50 mg/mL) were added to the nebulizer container. The nebulization was carried out during 30 s under mechanical ventilation (Xue et al., 2008).

After nebulization, the same previous analysis was repeated (forced oscillation, 10-s sequential intervals for 2 min). Data regarding airway hyperresponsiveness collected after nebulization of MCh are presented as ΔR_N, where Δ means the parameter after nebulization minus its value before MCh challenge. All Δ values were normalized by the pre-nebulization values.

2.5. Histological analysis

After euthanasia or natural death of animals from the experiments, lungs were collected and stored in fixative solution (10% formaldehyde) for 72 h. Then tissues were washed and progressively dehydrated in an ascending series of alcohols (70%, 80%, 90%, 100%, 100%; 1 h at each concentration); diaphanized in xylene (2x, 1 h each); and bathed in paraffin (2x, 1h30 each bath). Then, tissues were embedded in paraffin and 4 μm sections were obtained using a manual microtome (Leica RM2025®). After a deparaffinization process (56 °C for 24 h), tissues were mounted on slides, rehydrated with decreasing concentration of alcohol (90%, 80% and 70%; 3 min at each solution) and stained by hematoxylin-eosin (HE) for further analysis in light microscopy (Microscope Nikon Eclipse Nis, Nis Software 4.0®).

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad, San Diego, CA, USA). To compare the results gathered from each group, initially the normality of the data (Kolmogorow–Smirnov test with Lilliefor's correction), and the homogeneity of variances (Levene median test) were evaluated. If both conditions were satisfied, one-way ANOVA was used. In the negative case, Kruskal–Wallis ANOVA was selected instead. The significance level was always set at 5%.

3. Results and discussion

3.1. Respiratory system mechanics

In R_N, a good estimate of the total airway resistance (Bates, 2009), statistical significance values were observed in all experimental groups, being higher in groups 12 h and 24 h (Fig. 1A). These results represent a greater narrowing or an increase in the stiffness of airways smooth muscle in these experimental groups when compared to the control group. By reducing the lung elastic recoil, breaking fibers and destroying alveolar attachments, there was a limitation of the air flow, leading to increased airway resistance. The air trapping and new lung morphology acted reducing the surface area available for gas exchange (Saetta et al., 2001; Ishizawa et al., 2004).

G and H are related to intrinsic properties of the tissue and both parameters showed significant increases in all experimental