Toxicon 207 (2022) 52–59

Contents lists available at ScienceDirect

Toxicon

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

Local envenomation caused by a bioactive peptide fraction of *Bothrops jararaca* snake venom induces leukocyte influx in the lung and changes in pulmonary mechanics

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ARTICLE INFO

Handling Editor: Glenn King

Keywords:
Bothrops jararaca
FT-IR spectroscopy
Lung inflammation
Peptide fraction
Pulmonary mechanic
Snake venom

ABSTRACT

The crude venom of the *Bothrops jararaca* snake (Bj-CV) is a complex mixture of biologically active proteins that includes a variety of peptides in the low molecular weight fraction (Bj-PF). We investigated how an intramuscular injection of Bj-CV (1.2 mg kg^{-1}) and Bj-PF (0.24 mg kg^{-1}) influenced lung mechanics and lung and muscle inflammation in male Swiss mice 15 min, 1, 6, and 24 h after inoculation. Pressure dissipation against lung resistive components (ΔP1) rose significantly from 1 to 24 h after Bj-CV and 6–24 h after Bj-PF inoculation. Both Bj-CV and Bj-PF increased the total pressure variation of the lung (ΔPtot) 24 h after injection. Lung static elastance increased significantly after injection in all time periods investigated by Bj-CV and from 6 to 24 h by Bj-PF. Lung static elastance increased significantly after injection in all time periods investigated by Bj-CV and from 6 to 24 h by Bj-PF. Furthermore, intramuscular inoculation of Bj-CV and Bj-PF resulted in an increase in muscle and pulmonary inflammation, as evidenced by an increase in leukocyte influx when compared to the control group. Finally, both Bj-CV and Bj-PF cause acute lung injury, as shown by pulmonary inflammation and decreased lung mechanics. Furthermore, the fact that Bj-PF produces mechanical alterations in the lungs and muscular inflammation implies that non-enzymatic compounds can cause inflammation.

1. Introduction

Snakebite envenoming is a severe public health issue that the World Health Organization (WHO) considers a neglected disease (Loi, 2014). *Bothrops* is a medically significant Viperidae snake in Central and South America (Gutiérrez et al., 2013), accounting for higher than 21,000 snakebites in Brazil each year (Ministerio da Saúde, 2016).

Proteinases (metalloproteinases and serine proteinases), phospholipase A2, cysteine-rich secretory protein, lectin-like protein, C-type lectin, and L-amino acid oxidase are among the physiologically active proteins found in the venom of the *Bothrops jararaca* snake (Fox and Serrano, 2008). Apart from them, the peptide fraction (PF) of the venom has yielded a number of active peptides, such as disintegrins (Niewiarowski et al., 1994) and bradykinin potentiating peptides (BPPs) (Hayashi and Camargo, 2005) found from the PF of the venom. Disintegrins are among the most potent antagonists of several integrins (Niewiarowski et al., 1994), as RGD-peptides are known to be inhibitors of ADP-induced rabbit and human platelet aggregation (Kuo et al., 2019). The BPPs are efficient natural angiotensin-converting enzyme (sACE) inhibitors and their structure-activity studies were the basis for the

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https://doi.org/10.1016/j.toxicon.2022.01.001
Received 23 October 2021; Received in revised form 2 January 2022; Accepted 3 January 2022
Available online 7 January 2022

development of anti-hypertensive drugs such as Captopril (Cushman and Ondetti, 1980). BPPs contain 5 to 13 amino acid residues with a pyroglutamic residue (pGlu) at the N-terminus and a proline residue at the C-terminus. BPPs longer than seven amino acids share similar features, including a high content of proline residues and the tripeptide sequence Ile–Pro-Pro at the C-terminus (Hayashi et al., 2003).

Bothrops envenomation is complex, involving both the direct action of venom components on the tissues and the release of various endogenous mediators (Russell et al., 1997), which cause prominent local tissue damage and systemic disturbances such as hemorrhage, coagulopathies, cardiovascular shock, and renal alterations (Clissa et al., 2001). Despite extensive studies on the effects of snake venom on different biological systems, relatively little is known about their effects on pulmonary mechanical properties that allow studying the behavior of the respiratory system in response to the injury generated by this venom.

Respiratory impedance, as measured at airway opening, is widely regarded as a sensitive indicator of pulmonary pathology in lung disease (Bates, 2009), including the elastic, resistive, and viscoelastic components of the lung tissue and the chest wall (Bates and Smith, 2018). The alterations in respiratory system components lead to altered lung mechanics, characterized by histological abnormalities, edema, hemorrhaging, inflammation, and increased deposition of matrix extracellular proteins (Massa et al., 2017). In a previous study, the hypothesis that the enzymes present in *B. jararaca* crude venom could induce a pulmonary inflammatory process was tested (Silveira et al., 2004). Indeed, *B. jararaca* venom led to pulmonary mechanical changes, moderate inflammation, and acute lung injury after intravenous (i.v.) injection in mice. However, the envenomation model in this study could not represent the most frequently bitten area in victims because the lower limbs, followed by the upper limbs, were reported as the main affected body areas (Nicoleti et al., 2010), where the venom is injected into skeletal muscle, explaining myonecrosis and dermonecrosis effects as the main local effects at a snakebite site. Therefore, in the present study, we investigated the effects of *B. jararaca* crude venom (Bj-CV) and biologically active peptide fraction (Bj-PF) on their effects on pulmonary mechanics and the pulmonary and muscular inflammatory processes generated by intramuscular administration in mice.

2. Material and methods

All chemicals used in the present study were of analytical reagent grade (purity higher than 95%) and purchased from Calbiochem-Novabiochem Corporation (USA), Gibco BRL (USA), Fluka Chemical Corp. (Switzerland) or Sigma-Aldrich Corporation (USA).

2.1. Crude venom and peptide fraction of B. jararaca venom

The Butantan Institute's Laboratory of Herpetology (São Paulo, Brazil) donated crude venom (Bj-CV), which was kept at −20 °C until use. Bj-CV (2 g) was diluted in 20 mL 0.1 M ammonium acetate, pH 5.0, and lyophilized after filtering through a Millipore (Billerica, MA, USA) centrifugal filter system having a molecular weight cut-off of 10 kDa. The ratio between the initial amount (g) of Bj-CV and Bj-PF lyophilized was used to assess the yield of the percentage of low molecular weight. According to our previous study, the filtrate containing the peptide fraction was previously examined by different methodological strategies (SDS-PAGE in 15% polyacrylamide gel stained with silver nitrate, gelatinolytic activity, Fourier transform Infrared spectroscopy, and mass spectrometry) to confirm the lack of proteolytic enzymes or other proteins (>10 kDa) in the venom (Alberto-Silva et al., 2020; Querobino et al., 2017).

2.2. Animals

The Federal University of ABC (São Bernardo do Campo, Brazil) raised male Swiss mice (body weight: 18–20 g). Animals were kept at a constant temperature of 22 °C with a relative humidity of 50–60%, with free access to water and food, and a light-dark cycle of 12 h each. The experimental methods were carried out in accordance with the standards for the use of laboratory animals in biochemical research and were approved by local authorities (protocol number 09/2013).

2.3. Experimental procedure

Albino male mice (Swiss) were divided into groups and treated with Bj-CV (1.2 mg kg^{-1}) or Bj-PF (0.24 mg kg^{-1}) diluted in sterile 0.5% NaCl (w/v). The dose injected of crude venom and peptide fraction was done according to previous studies (Gonçalves and Mariano, 2000; Santoro et al., 2008). Samples were injected into the gastrocnemius muscle (i.m.) using a 0.5-mL syringe and a 30-gauge needle (Ultra-Fine Short Needle, BD, Canada). The control group (n = 5) received only sterile 0.5% NaCl (w/v), under the same conditions reported earlier. At 15 min, 1, 6 and 24 h after injection (n = 6 for each treatment and time), mice were sedated [pentobarbital sodium (50 mg kg^{-1})]. A snugly fitting metal cannula (1.1 mm ID) was introduced into the trachea and then connected to a computer-controlled mechanical ventilator (Samay MVR16xp, Montevideo, Uruguai). The anterior chest wall was surgically removed. Blood samples from animals treated for 24 h were collected by puncture of the abdominal aorta and immediately dispensed in plastic bottles without anticoagulants. After samples were maintained at room temperature for 1 h, centrifuged at 1500×*g* for 5 min, and plasma stored at −80 °C for biochemical evaluation, Animals were euthanized by CO_2 asphyxiation and samples of gastrocnemius from animals treated for 24 h were taken by keeping the site of saline or venom injection in the middle and 0.5 cm of tissue around. The samples were fixed in 4% paraformaldehyde for histological analysis, while the other was snap-frozen in liquid nitrogen and stored at −80 °C until the FT-Raman spectra were recorded.

2.4. Histopathological analysis of gastrocnemius

Samples of gastrocnemius muscle were taken by keeping the site of area injection in the middle and adding 0.5 cm of tissue around it. The samples were fixed in 4% paraformaldehyde (for 24 h) and then processed for embedding in paraffin. Cross-sections (5-μm thick) were obtained (Leica® rotative microtome), transferred to histological slides, and double-stained with hematoxylin and eosin (H&E). The sections were examined using a photomicroscope (Axioskop 2, Zeiss, Germany), and the images were captured with a Pixera digital camera system (Pixera Corporation, USA) attached to the photomicroscope and a microcomputer (Intel® Pentium®) using the software Adobe Photoshop version 7.0.1 (Adobe Systems, USA). The qualitative analysis of the gastrocnemius morphology was based on the muscle fiber profile, pathological state, leukocyte infiltration, connective tissue status, and indistinctness of muscle fiber bundles.

2.5. Creatine kinase (CK) assay

The myotoxic effect of the Bj-CV or Bj-PF obtained from animals treated for 24 h was determined by measuring the enzyme creatine kinase (CK) using 5 μL of plasma incubated for 5 min at 37 °C with 200 μL of a reagent according to the kinetics of CK-DAS InVitro (Minas Gerais, Brazil). The values of CK activity were expressed as $U.L^{-1}$, with one unit corresponding to the production of 1 mmol of NADH per minute at 25 °C.

2.6. Fourier-transformed infrared (FT-IR) spectroscopy of gastrocnemius

To generate the spectra of the muscles, a Bruker RFS 100/S FT-Raman spectrometer (Bruker, Inc., Karlsruhe, Germany) was used with a Nd: YAG laser operating at 1064 nm as an excitation light source in the mid-infrared spectral range from 4000 to 400 cm^{-1}. The laser power at

the sample was kept constant at 230 mW, and the spectrometer resolution was 4 cm^{-1} in transmission mode with a temperature controlled range of 18–20 °C. The spectra were recorded with 100 scans. For FT-Raman data collection, samples were brought to room temperature and kept moistened in a 0.9% physiological solution to preserve their structural characteristics, and then placed in a windowless aluminum holder for the Raman spectra collection. The spectra were preprocessed with the aid of the OPUS software (Bruker, Inc., Karlsruhe, Germany), followed by baseline corrections, spectral smoothing, normalization, and placing in absorbance.

2.7. Mechanical ventilation parameters

Mechanical ventilation with a frequency of 100 breaths min^{-1} and an adequate amount of positive end-expiratory pressure (PEEP = 2 cmH_2O) was applied right before the pleural cavity was entered, according to previous literature (Oliveira Neto et al., 2017; Silveira et al., 2004). Briefly, after pleural incision, there was an increase in transpulmonary pressure (PL), and the same pressure was applied to the lung as PEEP. A pneumotachograph was attached to the tracheal cannula to measure airflow velocity (V′) and lung volume changes (VT). The airflow resistance of the equipment (Req), tracheal cannula included, was constant up to airflow rates of 26 mL s^{-1} and amounted to 0.12 cmH_2O mL^{-1} s. The equipment resistive pressure (Req.V′) was subtracted from the pulmonary resistive pressure to obtain intrinsic values. Airflow and pressure signals were measured by transducers connected to the pneumotachograph and then sent to a signal conditioner (EMG System do Brasil, São José dos Campos, Brazil), where signals were amplificated by 1000x, sampled at 250 Hz with a 12-bit analog-to-digital converter, and stored on a microcomputer for off-line analysis. WindaqTM 2.81 software (DATAQ Instruments, Akron, Ohio, USA) was used to collect all data.

2.8. Pulmonary mechanics measurement

Pulmonary mechanics were measured, according to previous literature (Oliveira Neto et al., 2017; Silveira et al., 2004). Tracheal pressure reflects transpulmonary pressure (PL) in an open chest preparation. There is an initial fast drop in PL (ΔP1) from the preocclusion value down to an inflection point (Pi) followed by a slow pressure decay (ΔP2) until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung (Pel). ΔP1 selectively reflects pressure dissipated against pulmonary resistance in normal animals and humans, and ΔP2 reflects viscoelastic properties (stress relaxation) and/or in homogeneities of lung tissues together with a tiny contribution of pendelluft in normal situations. Total pressure drop (ΔPtot) is equal to the sum of ΔP1 and ΔP2. Lung static elastance (Est) was calculated by dividing Pel by VT. Pulmonary mechanics measurements were performed 10 to 15 times in each animal. The experiments did not last more than 40 min.

2.9. Bronchoalveolar lavage fluid analyses

After the measurement of pulmonary mechanics, animals were euthanized (60 mg kg^{-1} pentobarbital, i.p.), the lungs were washed with 1 mL of sterile saline, and bronchoalveolar lavage fluid (BALF) was centrifuged at 750×*g* for 6 min at 4 °C and the supernatant removed. The cell pellets were pooled for differential cell counts using 100 μL of the resuspended cells. Cytospins were prepared, air-dried, and stained (Instant Prov). A differential cell count was determined on a minimum of 100 cells.

2.10. Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test Dunnett post-test (GraphPad Prism 4.0, GraphPad Software, Incorporation) (parametric data) and one-way analysis of variance on Ranks (ANOVA on Ranks) followed by Dunn's *post hoc* test (non-parametric data) were used for comparisons of the different parameters between groups, using the GraphPad Prism 4.0, GraphPad Software, Incorporation. Values were expressed as mean ± SD for parametric data and as mean ± SEM for nonparametric data. The criteria for statistical significance were set at $p < 0.05$.

3. Results

3.1. Histopathological analysis

The local and systemic effects of envenomation induced by Bj-CV and Bj-PF are demonstrated in the present study (Fig. 1). The control gastrocnemius muscle was histologically normal in aspect. The muscle fibers exhibited crossed striations and were juxtaposed in well-organized fascicles whose perimysium showed no evidence of invasion by inflammatory cells. The histological sections of the muscle region injected with Bj-CV showed that the muscle fibers reached by venom exhibited different stages of degeneration, including myonecrosis (m), hyper contracted fibers (f), edema (e) and leukocyte infiltrate (star) (Fig. 1B), in contrast to control (Fig. 1A). The group of mice treated with Bj-PF showed similar features to the control group. Interestingly, infiltrating leukocytes were found in spread all over the numerous muscle fibers in the muscle treated with Bj-PF (arrow) (Fig. 1C).

3.2. Creatine kinase (CK) level analysis

Myonecrosis quantification by serum CK levels showed a marked serum level of the enzyme in Bj-CV, therefore indicating that venom-induced rupture of the sarcolemma in a great number of cells compared to saline controls or Bj-PF (Fig. 1D).

3.3. Characterization of gastrocnemius muscle by FT-IR

FT-IR spectroscopy was used to further analyze the structural and biochemical changes induced by the intramuscular injection of B. jararaca venom and its peptide fraction in the gastrocnemius muscle. The spectra obtained from all experimental groups were divided into low-wavenumber (LW; 500–2500 cm^{-1}) (Fig. 2A) and high-wavenumber regions (HW; 2500–3600 cm^{-1}) (Fig. 3A), and the comparison of average spectra of muscle samples treated with Bj-CV, Bj-PF and control indicated the typical FT-Raman spectra for biological tissue. All samples demonstrated good reproducibility with homogeneous distribution around the mean, but intensity variations were identified in some spectral regions between groups. In the LW spectra (Fig. 2B), we assigned the bands: 939 cm^{-1} [C–C stretching mode of proline and valine and protein backbone (a-helix conformation)/glycogen]; 1002 cm^{-1} [Symmetric ring breathing mode of phenylalanine]; 1049 cm^{-1} [Glycogen band (due to OH stretching coupled with bending)]; 1083 cm^{-1} [C–N stretching mode of proteins (and lipid mode to lesser degree)]; 1118 cm^{-1} [C–O stretching vibration of C–OH group of ribose (RNA)]; 1332 cm^{-1} [amide III - collagen]; 1452 cm^{-1} [Stretching (CH2) - lipids, glycosaminoglycans, metalloproteinases, collagens, and residues]; and 1658 cm^{-1} [amide I (C═O stretching mode of proteins, a-helix conformation)/C═C lipid stretch]. In the HW spectra (Fig. 3B), we identified three bands: 2933 cm^{-1} [CH stretch of lipids and proteins]; 3193 cm^{-1} [NH2 symmetric stretch]; and 3058 cm^{-1} [CH stretch of aromatic rings]. Calculations of the area and relative intensities between the bands identified in the LW region demonstrated that Bj-CV decreased the intensities bands of C–C stretching mode of proline and valine (939 cm^{-1}), symmetric ring breathing mode of phenylalanine (1002 cm^{-1}), and amide III (1332 cm^{-1}) concerning the Bj-PF and control groups (Fig. 2B). Also, Bj-CV reduced the glycogen (1049 cm^{-1}) and amide I (1658 cm^{-1}) in relation to the control group. On the other hand, Bj-PF treatment reduced only the amide I and amide II bands compared to the control group. Furthermore, Bj-CV decreased the NH_2 symmetric

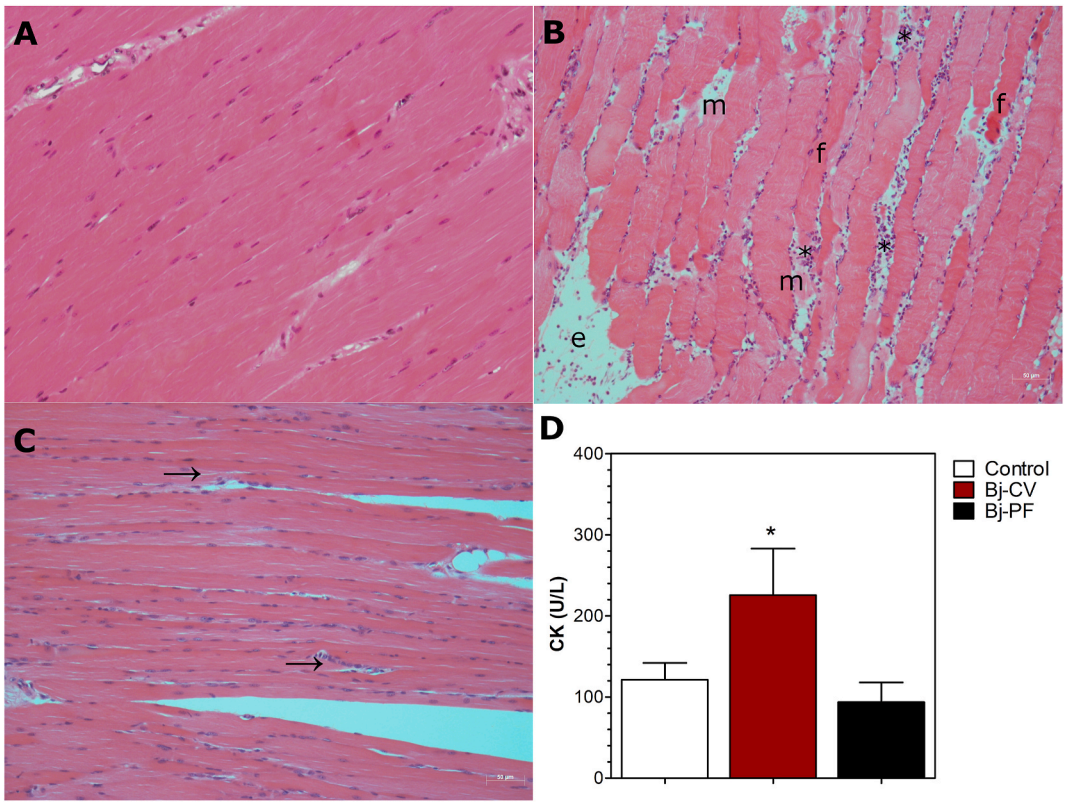

Fig. 1. Local and systemic effects of envenomation induced by crude venom (Bj-CV) and peptide fraction (Bj-PF) from *B. jararaca* snake venom. (A–C) Photomicrograph of the gastrocnemius muscle at the injection site 24 h after treatment. Animals were treated with saline, Bj-CV, and Bj-PF (Fig. 1B). Normal-looking muscle fibers are demonstrated in animals treated with saline (Panel A). Damage cells were seen in Bj-CV, myonecrosis (m), edema (e), inflammatory infiltrate (*) hypercontracted fibers (f) (Panel B). Influx leukocytes increased in spread all over the numerous muscle fibers in animals treated with Bj-PF (arrow) (Panel C). (D) Total creatine kinase (CK) in blood samples. Data are presented as the mean ± SD and significant differences as assessed through one-way ANOVA followed by Tukey post hoc test (*$p < 0.05$). Staining: hematoxylin and eosin (H&E). Scale bar: 50 μm.

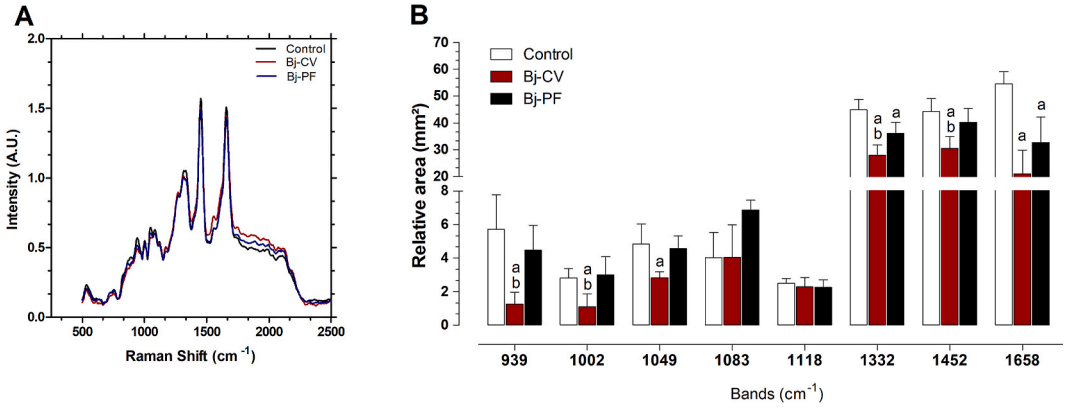

Fig. 2. FT-IR analyses of low-wavenumber (500–2500 cm^{-1}) obtained from gastrocnemius samples at the site of injection after 24 h of treatment. Representative spectra from muscle sample injected with 0.91% NaCl (black line), Bj-CV (red line), and Bj-PF (blue line) **(A)**. Relative area and intensity of low-wavenumber bands among groups **(B)**. Data are presented as the mean ± SD and significant differences as assessed through one-way ANOVA followed by Tukey post hoc test. a: $p < 0.05$ in relation to the Control group; b: $p < 0.05$ when compared with Bj-PF. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stretch intensity in the HW region (Fig. 3B).

3.4. Pulmonary mechanics

We did not observe significant differences in air flow, volume, and PEEP administered during mechanical ventilation among animals as well as groups, and they were not superior to 10% and 30%, respectively (Table 1). Thus, the respiratory mechanics parameters presented here were due to the effects of Bj-CV and Bj-PF on lung mechanical properties.

3.5. Elastic, resistive and viscoelastic properties

The ΔP1 values are increased in the Bj-CV and Bj-PF groups, which represent the resistive properties of pulmonary tissue (Fig. 4A). In the Bj-CV group, ΔP1 increased after 15 min of injection and remained

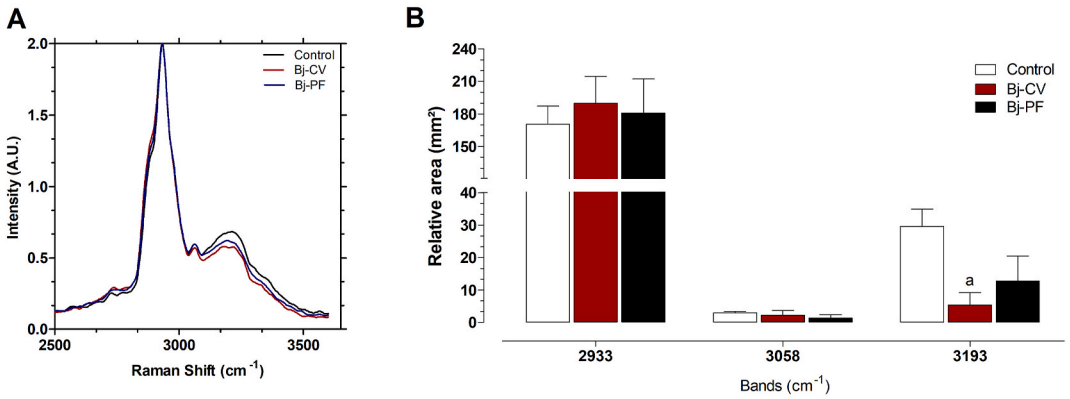

Fig. 3. FT-IR analyses of high-wavenumber (2500–3600 cm^{-1}) obtained from gastrocnemius samples at the site of injection after 24 h of treatment. Representative spectra from muscle sample injected with 0.91% NaCl (black line), Bj-CV (red line), and Bj-PF (blue line) **(A)**. Relative area and intensity of high-wavenumber bands among groups **(B)**. Data are presented as the mean ± SD and significant differences as assessed through one-way ANOVA followed by Tukey post hoc test. a: $p < 0.05$ in relation to the Control group; b: $p < 0.05$ when compared with Bj-PF. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Parameters for pulmonary mechanic evaluation. Control, injected with 0.5% NaCl (w/v) sterile; Bj-CV, mice were treated with crude venom treated with 1.2 mg kg^{-1} by 15 min, 1, 6, and 24 h; Bj-PF, mice were treated with peptide fraction obtained from crude venom with 0.24 mg kg^{-1} by 15 min, 1, 6, and 24 h; PEEP, positive end-expiratory pressure.

N = 54		Flow (mL/s)	Volume (mL)	PEEP (cmH_2O)
Control	15 min	1.03 ± 0.074	0.19 ± 0.015	2,52 ± 0.012
	1 h	1.05 ± 0.054	0.19 ± 0.005	2.42 ± 0.011
	6 h	1.08 ± 0.044	0.19 ± 0.012	2.41 ± 0.001
	24 h	1.03 ± 0.068	0.19 ± 0.015	2.39 ± 0.022
Bj-CV	15 min	1.01 ± 0.010	0.19 ± 0.005	2.50 ± 0.001
	1 h	1.05 ± 0.008	0.19 ± 0.014	2.49 ± 0.031
	6 h	1.02 ± 0.036	0.19 ± 0.005	2.51 ± 0.017
	24 h	1.99 ± 0.013	0.19 ± 0.005	2.17 ± 0.011
Bj-PF	5 min	1.08 ± 0.015	0,20 ± 0.004	2.36 ± 0.018
	1 h	0.95 ± 0.029	0.19 ± 0.007	2,52 ± 0.020
	6 h	1.03 ± 0.023	0.19 ± 0.007	2,36 ± 0.040
	24 h	0.99 ± 0.044	0.19 ± 0.006	2.42 ± 0.014

Control, injected with 0.5% NaCl (w/v) sterile; Bj-CV, mice were treated with crude venom treated with 1.2 mg/kg by 15 mi, 1, 6, and 14 h; Bj-PF, mice were treated with peptide fraction obtained from crude venom with 0.24 mg/kg by 15 min, 1, 6, and 14 h; PEEP, positive end-expiratory pressure.

elevated in all measured periods (Fig. 4A). In contrast, ΔP1 of the Bj-PF group increased only after 6 h of injection and remained elevated after 24 h. After 15 min and 1 h, ΔP1 of Bj-CV was higher than that found in Bj-PF (Fig. 4A). The pressure dissipation against the viscoelastic component, represented by ΔP2 values, decreased only after 15 min in the Bj-PF group; there were no changes in ΔP2 values after Bj-CV injection (Fig. 4B). The values for ΔPtot represent the total lung pressure variation (Fig. 4C). Bj-PF injection decreased ΔPtot value at 15 min, and both Bj-CV and Bj-PF injection increased ΔPtot at 24 h (Fig. 4C). The lung elastic elastance of the Bj-CV group was elevated in all measurement periods. In contrast, the lung static elastance of the Bj-PF group increased after 6 and 24 h of its injection (Fig. 4D).

3.6. Leukocyte influx in bronchoalveolar lavage fluid

In the present study (Fig. 5), the time-course of leukocyte influx into the lung after injection of Bj-CV, Bj-PF, or saline solution was demonstrated (Fig. 5). Both crude venom and Bj-PF evoked a significant cell accumulation in the lung from 6 to 24 h following the injection (Fig. 5A). Differential cell counts demonstrated that BALF cells from Bj-CV mice showed significant differences in the number of neutrophils that infiltrated the lung at 6 h (Fig. 5B). Bj-PF significantly increased the number of neutrophils at 6 h as well as mononuclear cells at 6 h and 24 h (Fig. 5B and C). No significant differences in specific cell types were seen at 15 min and 1 h after crude venom or Bj-PF injection.

4. Discussion

This study investigated the ability of Bj-CV and Bj-PF to cause changes in mechanical properties and inflammation in the lungs of mice. We show that *B. jararaca* crude venom and its peptide fraction without enzymatic components can alter the resistive and viscoelastic properties of the lung and cause inflammation, as evidenced by an increase in leukocyte influx. To the best of our knowledge, this is the first demonstration of lung changes caused by biologically active peptides as well as leukocyte influx into the lung and muscle, both of which may play important roles in the pathological manifestations caused by crude venom.

Vibrational spectroscopy, namely the FT-IR method, was used to characterize biochemical modifications that occur during the pathogenesis of the gastrocnemius muscle injected with Bj-CV and Bj-PF, giving a diagnostic tool termed as “optical molecular pathology” (Zezell et al., 2015). Studies have established that FT-IR spectra found in the spectral range from 500 to 3300 cm^{-1} absorbance bands comprise a biochemical fingerprint of the molecular structure and composition of cells or tissue samples (Movasaghi et al., 2008), and can be applied to differentiate normal and inflammatory tissues (Rodrigues et al., 2018). In the present study, spectral differences were obtained in samples of gastrocnemius muscle treated with Bj-CV, specifically in the low-wavenumber regions, when compared to the control or Bj-PF. Bj-CV promoted biochemical alterations in the injected muscle region by reducing the regions of the spectra and collagen content (Movasaghi et al., 2008). These biochemical alterations can be explained by the action of phospholipases A2 and metalloproteinase hemorrhagins present in Bj-CV that damage the muscle cytoarchitecture, in contrast to Bj-PF which does not contain proteolytic enzymes. Our results are in accordance with the literature, which demonstrated the changes in the different molecules (lipids, amide I, and amide III bands) that occur throughout the pathogenesis of the gastrocnemius muscle injected with *Bothrops jararacussu* venom (Vieira et al., 2018). Bj-CV also induced an increase in CK levels and the morphologic examination demonstrated intense myonecrosis, with interstitial edema and a massive leukocyte influx, characteristic of *Bothrops* venom administration (Gutiérrez et al., 2009), which is corroborated by biochemical alterations shown by FT-IR analyses.

Bj-PF obtained from *B. jararaca* crude venom comprises a series of bioactive peptides, especially BPPs, and their biological properties have

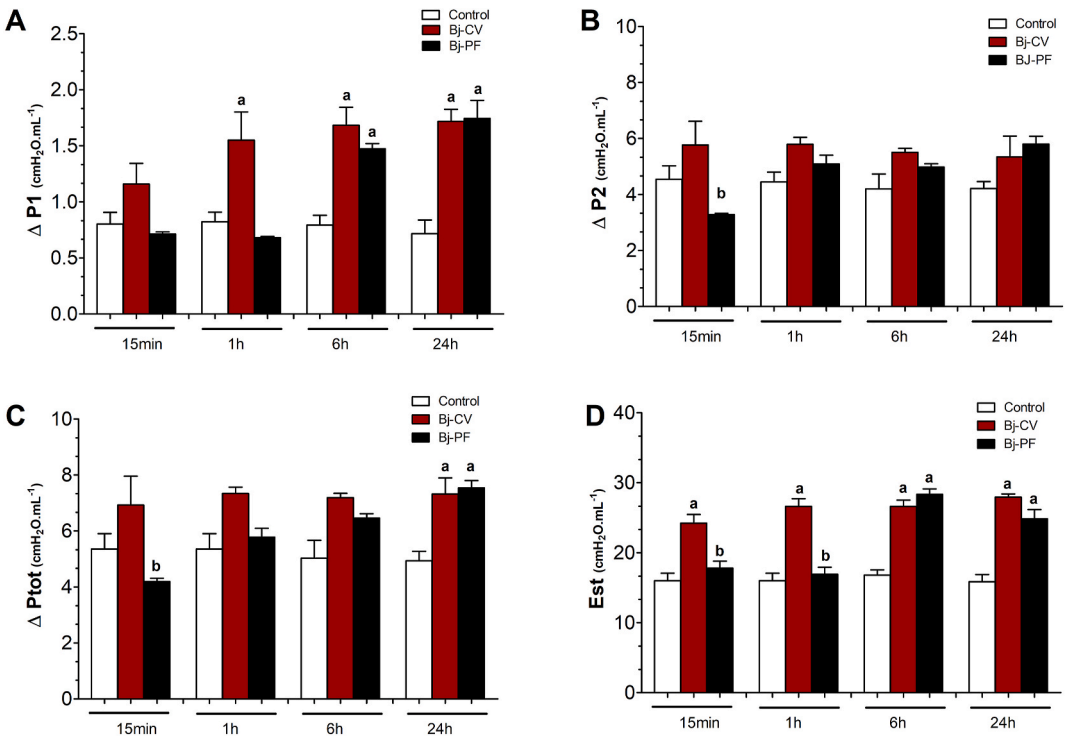

Fig. 4. Effect of *B. jararaca* crude venom (Bj-CV) and peptide fraction (Bj-PF) on mean values of ΔP1 (A), ΔP2 (B), ΔPtot (C), and static elastance (Est) after 15 min, 1, 6, and 24 h of treatment. Bj-CV (1.2 mg kg^{-1}), Bj-PF (0.24 mg kg^{-1}) or vehicle were injected intramuscularly. Values are expressed as mean ± SD and analyzed by ANOVA one-way followed by Tukey post hoc test. a: $p < 0.05$ in relation to the Control group; b: $p < 0.05$ when compared with Bj-PF. ΔP1: pressure dissipated against pulmonary resistance; ΔP1: pressure spent against viscoelastic/inhomogeneous components; ΔPtot: total dissipated pressure.

been studied in different biological systems (Querobino et al., 2018). Here, we demonstrated that Bj-PF did not cause morphological alterations in gastrocnemius muscle or CK levels; however, a leukocyte infiltrate was observed in the muscle region injected and also reduced the intensity of the I and III amide bands in FT-IR analyses. The literature demonstrated that after the administration of a biologically active peptide named BPP-11e (pGlu-Ala-Arg-Pro-Pro-His-Pro-Pro-Iso-Pro--Pro) leukocyte rolling and adhesion were increased by fivefold in post-capillary venules in the cremaster muscle of rats (Rioli et al., 2008), demonstrating a pro-inflammatory action of the BPPs, highly representative in the Bj-PF studied here.

In the present study, both Bj-CV and Bj-PF led to pulmonary mechanical changes that lasted up to 24 h. Bj-CV resulted in an increase in ΔP1 from 1 to 24 h and Bj-PF from 6 to 24 h, suggesting an increment in the resistive properties, mainly in airways, with accumulation of secretion. This finding corroborates a study by Silveira et al. (2004), who demonstrated that *B. jararaca* crude venom leads to pronounced mechanical pulmonary alterations with elevated ΔP1, indicating airway flooding. Bj-CV had no effect, and Bj-PF presented a decrease at 15 min after the injection on the values of viscoelastic pressures in our study, represented by ΔP2. In contrast, a previous study that analyzed an intravenous *B. jararaca* crude venom administration in mice resulted in increased lung viscoelastic pressures after 1 up to 72 h (Silveira et al., 2004). The discrepancies between the results of the present study and those conducted by Silveira et al. (2004) could be due to the route of administration since we used intramuscular injection, and the amount of venom that reached the lung was not enough to cause viscoelastic alterations. The values of the total pressure variation of the lung, represented for ΔPtot, demonstrate that in 24 h period, an increase of the total pressure for the displacement of airflow in the lung occurs by both Bj-CV and Bj-PF. ΔPtot is the sum of ΔP1 and ΔP2, which is before the elastic retraction pressure has been established. In this study, ΔPtot increased due to the increase in ΔP1, which can indicate hypersensitivity reactions and accumulation of secretion. Our results agree with the literature, which shows increased ΔPtot in the lungs of animals injected with *B. jararaca* venom (Silveira et al., 2004). Lung static elastance increased significantly in all periods analyzed by Bj-CV and from 6 to 24 h by Bj-PF following injection, which is in accordance with the literature that shows an increase in lung static elastance after venom injection (Silveira et al., 2004). Therefore, the results found in this study confirmed that *B. jararaca* venom causes mechanical alterations in the pulmonary tissue, and these effects were not reversible until 24 h. Moreover, Bj-PF also induces mechanical alterations that can amplify the action of the crude venom.

Bj-CV has been shown to cause an increase in inflammatory cells in the lung (Silveira et al., 2004). The pulmonary modifications by the Bj-CV and Bj-PF reported in our study are related to leukocyte influx into the lung, which causes edema and, as a result, changes in pulmonary mechanical characteristics, by increasing alveolar-capillary permeability. As shown here, Bj-CV and Bj-PF were able to induce a pronounced leukocyte influx in the bronchoalveolar lavage fluid with neutrophil predominance at 6 h and mononuclear cells at 24 h. Leukocyte migration to the tissue requires the activation of adhesion molecules responsible for the rolling, firm adhesion, and transmigration, which explains the leukocyte influx observed after Bj-CV and Bj-PF injections. It can be related to the effect on events related to the activation of adhesion molecules. This hypothesis is based on the study by Rioli and its collaborators (Rioli et al., 2008) who demonstrated that these peptides induce leukocyte rolling and adhesion in the cremaster muscle of mice. The action of Bj-CV and Bj-PF on the inflammatory process in the lung may also be related to modulation of the production of inflammatory cytokines, released by cells present in the tissue and by cells that migrate to the lung. In this approach, it has been shown that *B jararaca* venom can boost the release of cytokines that are critical for leukocyte recruitment as well as trigger the expression of adhesion molecules (Zamuner and Teixeira, 2002).

In conclusion, we demonstrated for the first time that Bj-PF, containing a complex and very rich mixture of bioactive peptides obtained

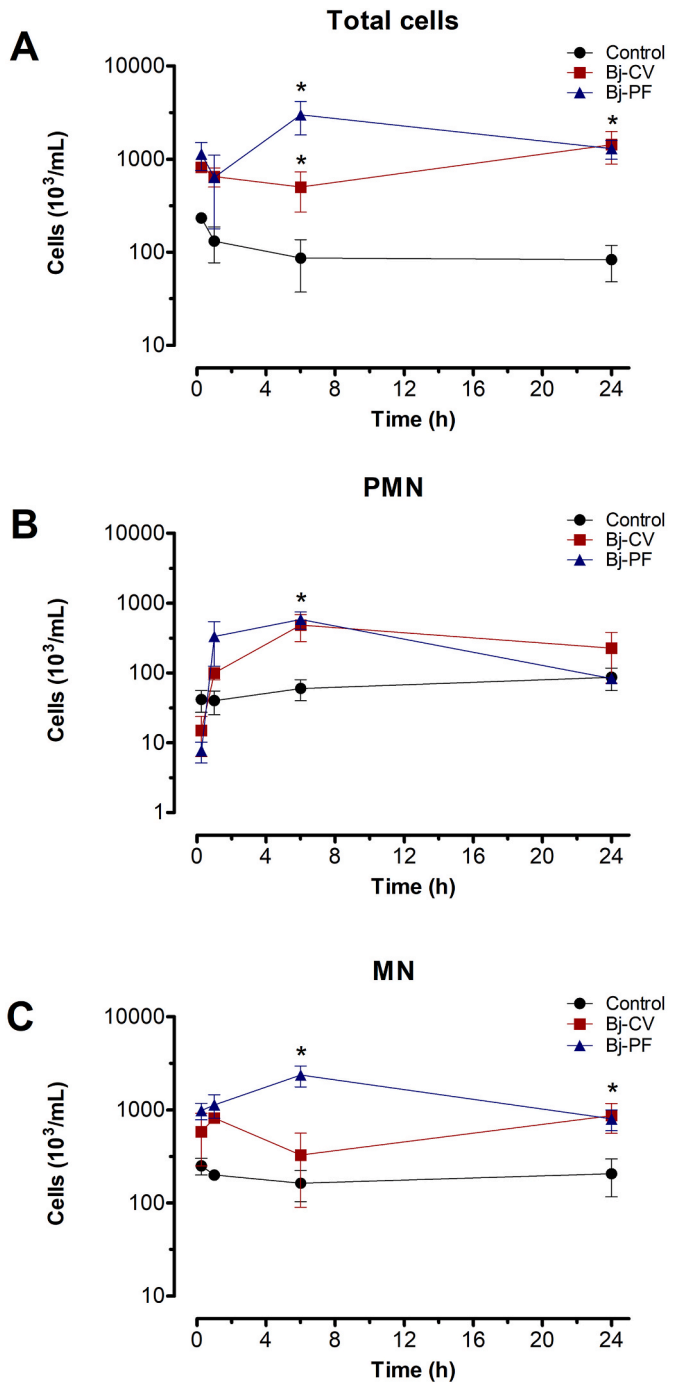

Fig. 5. Time-course of leukocyte influx into the lung induced by *B. jararaca* crude venom (Bj-CV) and peptide fraction (Bj-PF). The venom of Bj-CV or BJ-PF or sterile saline (control group) were injected i.m. Animals were anesthetized and the BAL was collected for cells counts at the time intervals indicated in material e methods. Panel A represents the number of total leukocytes, Panel B the number of polymorphonuclear cells (PMN), and panel C the number of mononuclear cells (MN). Values are expressed as mean ± SD and analyzed by ANOVA one-way followed by Tukey post hoc test. *$p < 0.05$ in relation to the Control group.

from *B. jararaca* snake venom, causes mechanical pulmonary alterations as well as lung and muscle leukocyte infiltration. The severity of lung inflammation induced by Bj-CV or Bj-PF, as measured by histopathology and bronchoalveolar lavage fluid studies, was linked to the viscoelastic and elastic changes found in this investigation. Surprisingly, crude venom and its low molecular weight fraction have similar pro-inflammatory activities in response to lung leukocyte migration, suggesting that non-enzymatic components are responsible for these effects.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Funding

This work was supported by the State of São Paulo Research Foundation (FAPESP) and the Coordination for the Improvement of Higher Education Personnel (CAPES) (Finance Code 001).

Ethics approval

All experimental protocols were performed following the guidelines of the human use of laboratory animals of the Ethics Committee on Animal Use of Federal University of ABC (CEUA/UFABC) and were approved under the protocol number 009/2013.

Ethical statement

The authors declare that this material has not been published in whole or in part elsewhere; the manuscript is not currently being considered for publication in another journal; all authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

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.

Acknowledgments

The authors would like to thank the technical group of the Experimental Morphophysiology Laboratory for assistance in the histological procedures. Thanks are also due to the administrative-technical group of Natural and Humanities Sciences Center for secretarial assistance.

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