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In vitro effects of *Crotalus atrox* snake venom on chick and mouse neuromuscular preparations

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ABSTRACT

The neuromuscular effect of venoms is not a major clinical manifestation shared between rattlesnakes native to the Americas, which showed two different venom phenotypes. Taking into account this dichotomy, nerve muscle preparations from mice and chicks were used to investigate the ability of *Crotalus atrox* venom to induce *in vitro* neurotoxicity and myotoxicity. Unlike crotalic venoms of South America, low concentrations of *C. atrox* venom did not result in significant effects on mouse neuromuscular preparations. The venom was more active on avian nerve-muscle, showing reduction of twitch heights after 120 min of incubation with 10, 30 and 100 μg/mL of venom with diminished responses to agonists and KCl. Histological analysis highlighted that *C. atrox* was myotoxic in both species of experimental animals; as evidenced by degenerative events, including edematous cells, delta lesions, hypercontracted fibers and muscle necrosis, which can lead to neurotoxic action. These results provide key insights into the myotoxicity and low neurotoxicity of *C. atrox* in two animal models, corroborating with previous genomic and proteomic findings and would be useful for a deeper understanding of venom evolution in snakes belonging to the genus *Crotalus*.

1. Introduction

The genus *Crotalus*, commonly known as rattlesnakes, belongs to the venomous Viperidae family and is composed of 39 extant species distributed from North to South America (Wallach et al., 2014; Arevalo-Paez et al., 2017). *Crotalus* snakes are the most common source of venomous snakebites in North America (Juckett and Hancox, 2002), while most of clinical case numbers in Central and South America are provoked by *Bothrops* species (Chippaux, 2017), with lower ophidic accidents caused by *Crotalus*. Regarding rattlesnakes, in the United States, the *Crotalus adamanteus and Crotalus atrox* are responsible for the majority of snakebite mortalities (Juckett and Hancox, 2002), while *Crotalus durissus* is the most commonly reported snakebite in South America (Boldrini-França et al., 2010; da Silva et al., 2011).

North American rattlesnake envenomings, such as those provoked by *Crotalus atrox*, have a toxicological profile that differs from those produced by venoms of South American rattlesnakes (*Crotalus durissus* ssp), which are highly neurotoxic owing to proteins that mediate the blockade of neuromuscular transmission (Bosak et al., 2014; Sant'Ana Malaque and Gutiérrez, 2017). Commonly, snakebites from *Crotalus atrox* rattlesnakes are characterized by local tissue injury (cytotoxicity, myotoxic and hemorrhagic), cardiovascular effects, coagulopathy, thrombocytopenia and platelet aggregation; with few reports of neurotoxic events, that can appear as fasciculations and paresthesias (Clark et al., 1997; Bosak et al., 2014). Although currently geographically separated, with distinct mechanisms of action and phenotype venom, *Crotalus atrox* and *Crotalus durissus* are believed to have originated from a monophyletic clade in the north-central region of the Mexican Plateau and which then dispersed across the Americas (Klauber, 1972; Knight et al., 1993).

C. atrox (western diamondback rattlesnake) is one of the more aggressive snakes of medical importance from North America and feeds on different preys, ranging from mammals to birds and lizards (Beavers, 1976; Calvete et al., 2009). The biological effects of *C. atrox* venom on distinct animal models have not been extensively studied or scientifically explored. Despite the descriptions of clinical manifestations, little is known regarding the effect of this venom in neuromuscular preparations *in vitro* and their mechanism of action (Harvey et al., 1994).

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Biological comparisons between the *in vitro* neurotoxic actions of *C. atrox* and other species of *Crotalus* have not yet been reported.

According to Ranawaka et al. (2013) the neurotoxic activity is a key and relevant element of envenomation induced by some snakes, which is still not fully understood. This review article highlighted that further study into the neurotoxic effects is essential to expand the knowledge of their molecular mechanisms and patterns. Thus, the present study aimed to investigate the complex interactions that occur between *C. atrox* components and avian and mammalian nerve-muscle preparations, as well as understanding the pathology of envenomation of cases reported in literature and compare the neurotoxicity described for *Crotalus* snake species from America searching for valuable insights into the evolutionary paths of *Crotalus* genus.

2. Material and methods

2.1. Venom and reagents

The snake venom of *Crotalus atrox* used in our biological experiments was purchased by the National Natural Toxins Research Center (NNTRC) of the Texas A&M University–Kingsville (USA). All reagents used were of sequencing or analytical grade.

2.2. Animals

For screening of *C. atrox* snake venom for myotoxic and neuromuscular action, male Swiss mice (25–30 g) and HY-Line W36 chicks (4 to 8 days old) were supplied by Multidisciplinary Center for Biological Investigation (CEMIB, the central animal house at UNICAMP) and Flamboyant Alimentos S/A (Mogi Mirim, SP, Brazil). The animals were maintained at 24–28 °C with food and water *ad libitum*. All biological assays were done in accordance with the general guidelines of the Institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, Protocol 3712-1).

2.3. Screening for neuromuscular action

The neuromuscular effect of whole *C. atrox* snake venom was performed using two different neuromuscular preparations: mouse phrenic nerve-diaphragm (PND) preparations and chick *biventer cervicis* (BC) nerve-muscle preparations.

2.3.1. Mouse nerve-muscle preparation

The phrenic nerve-diaphragm preparations were removed from mice euthanized with isoflurane. These preparations were mounted under a resting tension of 1 g in a 5 mL tissue bath, which was constantly bubbled (95% O_2 and 5% CO_2) in a Tyrode solution composed by: NaCl 137 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.49 mM, NaH_2PO_4 0.42 mM, $NaHCO_3$ 11.9 mM and glucose 11.1 mM) at 37 °C, as originally described by Bülbring (1946). Supramaximal stimuli (0.1 Hz and 0.2 ms for indirect stimulation) were delivered from a Grass S88 stimulator (Grass Instrument Co., Quincy, MA, USA). Isometric muscle tension was recorded with a Model MLT0201 Force transducer 5 mg–25 g (Panlab sl, AD Instruments Pty Ltd. Spain) connected to a PowerLab/4SP (Quad Bridge, AD Instruments, Barcelona, Spain). After a 20 min stabilization period, the preparations were exposed to varying amounts of *C. atrox* snake venom for 120 min. Twitch responses were recorded and compared to control preparations (Tyrode alone).

2.3.2. Chick biventer cervicis nerve-muscle preparation

The chick *biventer cervicis* (BC) nerve-muscle preparations were isolated and prepared as originally reported by Ginsborg and Warriner (1960); with some adaptations. Firstly, the chicks were euthanized with isoflurane and the nerve-muscle preparations were isolated and mounted under a resting tension of 1 g in a 5 mL tissue bath (Automatic organ multiple-bath LE01, Letica Scientific Instruments Barcelona, Spain) with Krebs solution, which was composed by (mM): NaCl, 118.7; KCl, 4.69; $CaCl_2$, 1.88; KH_2PO_4, 1.17; $MgSO_4$, 1.17; $NaHCO_3$ 25.0 and $C_6H_{12}O_6$ 11.65, constantly bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 37 °C. The BC preparations were stimulated with bipolar electrodes (field stimulation). Supramaximal pulses of 0.1 Hz frequency and 0.2 ms were applied (Grass S88 stimulator, Grass Instrument Co., Quincy, MA, USA). The maximum muscle contractions provoked by electrical stimuli and contraction in response to the addition of KCl (40 mM), carbachol (20 μM), and ACh (1 mM) were recorded using isometric transducers (Model MLT0201 Force transducer 5 mg - 25 g (Panlab sl, AD Instruments Pty Ltd. Spain) connected to a PowerLab/4SP (Quad Bridge AD Instruments, Barcelona, Spain). The contractions to CCh, KCl and ACh were monitored and recorded before venom addition (absence of electrical stimulation), during and after a 120 min incubation with different amounts of whole *C. atrox* snake venom.

2.4. Histological and quantitative analysis of myotoxicity

Myotoxicity was also studied on the basis of morphological alterations triggered after 120 min of incubation of PND and BC muscles with *C. atrox* venom. These preparations were fixed in 10% formaldehyde (overnight) and stored in 70% ethanol. The samples were dehydrated in ascending ethanol concentrations (80%, 95% and 100%), clarified with xylol (1:1 ethanol:xylol and 100% xylol), embedded (1:1 xylol:paraffin and 100% paraffin) and included in paraffin. Three sections per group (5 μm thick), separated by 100 μm from each other, were obtained. The thick sections were stained with hematoxylin–eosin (HE) and examined under a light microscope (Leica DM 5000 B, Leica, Germany). All images were captured and analyzed with Image Leica QWin Plus V3 software (Leica, Germany) and the muscle damage (index of myonecrosis) was calculated and expressed as a percentage of normal fibers based on the quantitative method described by Oshima-Franco et al. (2001).

2.5. Statistical analysis

The twitch-tension responses of BC and PND preparations were expressed as percentages relative to time zero values. Statistical comparisons were performed using the mean ± SEM from at least five experiments and analyzed by ANOVA followed by the Tukey-Kramer test, with $p < 0.05$ indicating significance, using GraphPad Prism 5 (San Diego, USA).

3. Results

3.1. Activity in mouse phrenic nerve-diaphragm preparation (PND)

C. atrox venom caused only mild neuromuscular effects in the mouse phrenic nerve-diaphragm preparations. Higher venom concentrations (100 μg/mL) produced partial time-dependent neuromuscular blockade of PND preparations, with twitch heights 51.4 ± 10.1% of control after a 120 min of incubation (Fig. 1). Following incubation with the venom, the preparations were washed several times with fresh Tyrode solution (Fig. S1), but no reversal of the neuromuscular blockade was observed. The incubation with 30 μg/mL (Fig. 1) and lower concentrations of venom (data not shown) were unable to produce any alterations in the twitch responses. No facilitation in twitch responses was observed at any concentration.

Despite not showing any neuromuscular blockade at 30 μg/mL of venom, the histological analysis of PND preparations revealed the presence of edematous cells, delta lesions, hypercontracted fibers and myonecrosis (Figs. 2 and 3). Higher concentrations induced similar, but more evident, histopathological alterations. Controls showed normal histological features, characterized mainly by parallel muscle fibers and peripheral nuclei. 30 μg/mL of *C. atrox* induced 19.2 ± 4.3% of muscle

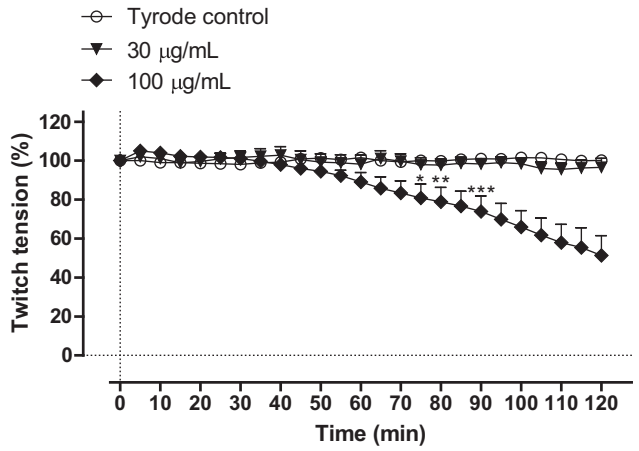

Fig. 1. Twitch tension responses (%) of PND preparations under indirect stimuli and 120 min incubation with Tyrode control or *Crotalus atrox* venom (30 and 100 μg/mL), at 37 °C. Each point represents the mean ± SEM (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to Tyrode control).

damage (Fig. S2), being significantly different to the Tyrode control (1.0 ± 0.8% of cell damage). Higher venom concentrations (100 μg/mL) were able to produce 36.3 ± 3.2% of cell damage.

3.2. Activity in chick biventer cervicis preparation (BC)

Avian preparations were more sensitive to whole venom than mammalian models. *C. atrox* venom caused a concentration and time-dependent blockade of the twitch response of BC preparations (Fig. 4). After 120 min of incubation with 3 μg/mL of venom, there was no significant difference in the twitch heights when compared to the Tyrode control. Higher venom concentrations showed twitch heights of 63.7 ± 3.0, 41.2 ± 10.2 and 7.2 ± 1.5% of control after 120 min of incubation with 10, 30 and 100 μg/mL of venom, respectively. The times of 50% blockade (t50) were 90.8 ± 9.7 and 39.5 ± 10.1 min with 30 and 100 μg/mL, respectively, while the time of 90% blockade (t90) was reached only with 100 μg/mL, being 107.3 ± 2.0 min. Facilitation of the twitch responses was not observed at any concentration (Fig. 4). There was no reversal of the neuromuscular blockade by washing, but a slight continuation of the developing paralysis was observed (Fig. S3).

The contractures in response to ACh, CCh and KCl were also investigated (Fig. 5). Comparison of the contractures before and after a 120 min incubation with venom revealed a concentration-dependent reduction in the respective responses. The contractures in response to ACh were reduced to 88.3 ± 7.5, 70.2 ± 5.4, 43.2 ± 11.9 and 5.6 ± 1.3% of control after incubation with 3, 10, 30 and 100 μg/mL of venom, respectively (Fig. 5A). The contractures in response to CCh were also reduced, being 88.6 ± 4.3, 57.6 ± 3.3, 21.1 ± 5.6 and 1.5 ± 0.7% of control with 3, 10, 30 and 100 μg/mL of venom, respectively (Fig. 5B). Lastly, the contractures in response to KCl were 86.9 ± 9.6, 47.1 ± 4.1, 25.3 ± 7.1 and 3.7 ± 1.8% of control in the incubations with 3, 10, 30 and 100 μg/mL of venom, respectively (Fig. 5C).

Histological analyses evidenced that *C. atrox* venom was able to produce concentration-dependent muscle damage, with the presence of edematous cells, delta lesions, vacuolated and hypercontracted fibers and myonecrosis (Figs. 6 and 7). It was observed 1.7 ± 0.2, 25.5 ± 2.0, 43.3 ± 1.4 and 67.7 ± 3.3% of cell damage with 3, 10, 30 and 100 μg/mL of venom, respectively. Only 3 μg/mL was not significantly different from the Krebs control; 2.5 ± 0.8% of cell damage (Fig. S4).

The venom phenotype of *C. atrox* is notably different from South America snake venoms; which are truly neurotoxic, more potent and fast acting. The weak effects caused by *C. atrox* venom are markedly delayed and it only has neuromuscular blocking activity at very high concentrations of venom used (Table 1), which corroborates with role of myotoxicity in this bioactivity.

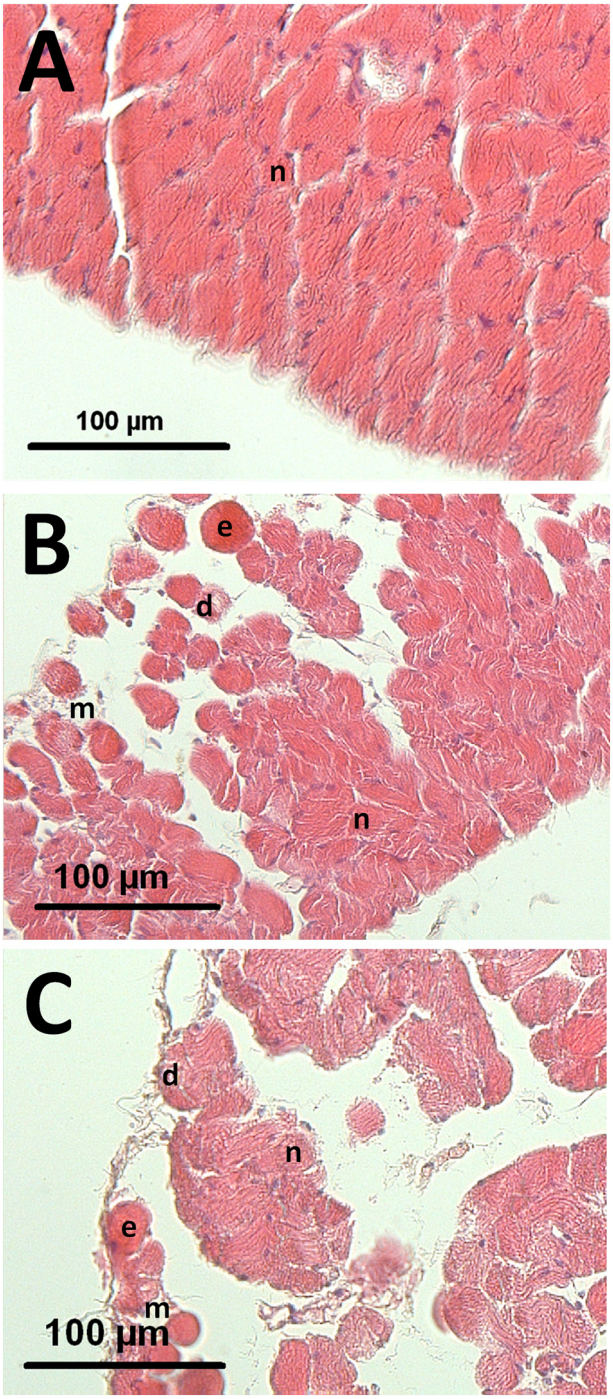

Fig. 2. Histological analysis of transversal PND muscles sections after a 120 min incubation with Tyrode solution (A); 30 (B) and 100 μg/mL (C) of *C. atrox* venom. The sections are representative of six preparations per treatment. Note the presence of normal fibers (n), edematous (e), delta lesions (d) and myonecrosis (m). Bar = 100 μm.

4. Discussion

Research into *Crotalus* venoms from North American snakes has mainly focused on the proteomic composition and their hemotoxic and

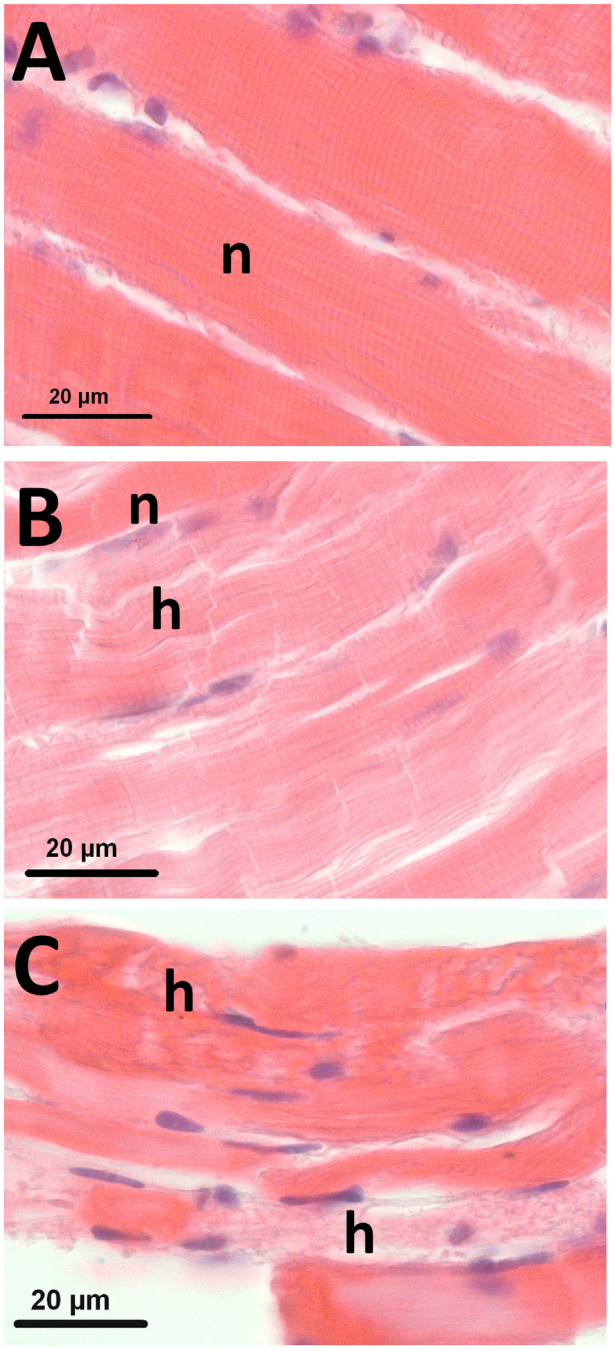

Fig. 3. Histological analysis of longitudinal PND muscles sections after a 120 min incubation with Tyrode solution (A); 30 µg/mL (B) and 100 µg/mL (C) of *C. atrox* venom. The sections are representative of 6 preparations per treatment. Note the presence of normal fibers (n) and hypercontracted myofilaments (h). Bar = 20 µm.

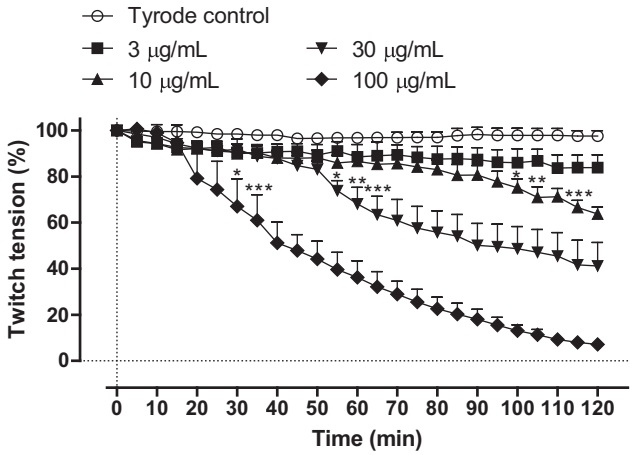

Fig. 4. Twitch tension responses (%) of the BC preparations under campus stimuli and 120 min incubation with Krebs control or *Crotalus atrox* venom (3–100 µg/mL), at 37 °C. Each point represents the mean ± SEM (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to Krebs control).

local toxicity (Calvete et al., 2009; Bosak et al., 2014; Almeida et al., 2016b). The neurotoxic action of North American venoms has not been fully explored, which although little pronounced has previously been reported in some snakebites by *C. horridus*, *C. oreganus helleri* and *C. atrox* (Richardson et al., 2007; Vohra et al., 2008; Bosak et al., 2014).

Crotalus atrox is a large deadly and medically-important rattlesnake that possesses a venom with properties similar to other American pit vipers, such as *Crotalus oreganus abyssus*; characterized predominantly by local biological effects (Calvete et al., 2009; Almeida et al., 2016a). Recent works have suggested interesting biotechnological and clinical uses of *C. atrox* venom in new and efficient therapies for reducing peripherative hemorrhage stemming from the venom's capacity to produce fibrin cleavage products (Kim et al., 2017; Nielsen, 2017). However, despite the high biomedical potential, detailed investigation into the toxic effects triggered by western diamondback is also essential to evaluate these promising therapeutic applications and their safety.

The biological examination showed that higher *C. atrox* venom concentrations (100 µg/mL) induced neuromuscular blocking activity in mouse (partial; twitch heights 51.4 ± 10.1% of control) and chicken nerve-muscle preparations (total); while lower venom concentrations (10 and 30 µg/mL) only partially affect neuromuscular responses in chick preparations. However, using both animal models the incubation with 3 µg/mL of venom, there was no significant difference in the twitch heights when compared to the control solution. The mechanism of action for such neuromuscular effect may result from an integration of the drastic myonecrotic effect characterized in histological analyses and a significant reduction in responses to acetylcholine, carbachol and KCl. Briefly, the non-specific myotoxic effect seems responsible for the failure of twiches in both nerve-muscle preparations and non-selective inhibition of responses to agonists. The venoms of *Bothrops alcatraz* and *Bothrops fonsecai* act by a similar mechanisms, which is believed to be dependent on damage to skeletal muscle (de Moraes et al., 2012; Fernandes et al., 2014). The mode of action of *C. atrox* is more similar to bothropic venoms (myotoxic) than those of crotalic venoms from South America; which act predominantly in presynaptic mode, abolishing nerve-evoked twiches without affecting cholinoreceptor agonist response (Beghini et al., 2004). This neurotoxic data is consistent with experimental results obtained by Harvey et al. (1994), who first described the very low neuromuscular blocking activity of *C. atrox*. However, in the present work, we extend the understanding of the molecular underpinnings of this biological activity; describing the time-dependent neuromuscular blockade in both models; the absence of reversal after washing; the contracture in response to agonists and KCl and principally, the role of myotoxic effect in this pharmacological action.

The contractures in response to ACh, CCh and KCl were reduced after a 120 min incubation with venom when compared to control. The reduction in the responses to ACh and KCl is in line with histological analyses, which showed tissue damage characterized mainly by the presence of edematous cells, delta lesions, hypercontracted fibers and myonecrosis. Experimental studies of the morphological effects triggered by snake venoms in vertebrate and invertebrate preparations, such as *Lachesis muta muta* (Damico et al., 2005) and *Bothrops fonsecai* (Fernandes et al., 2014) have reported morphological changes similar to tissue damage induced by *C. atrox*. At higher concentrations, it was

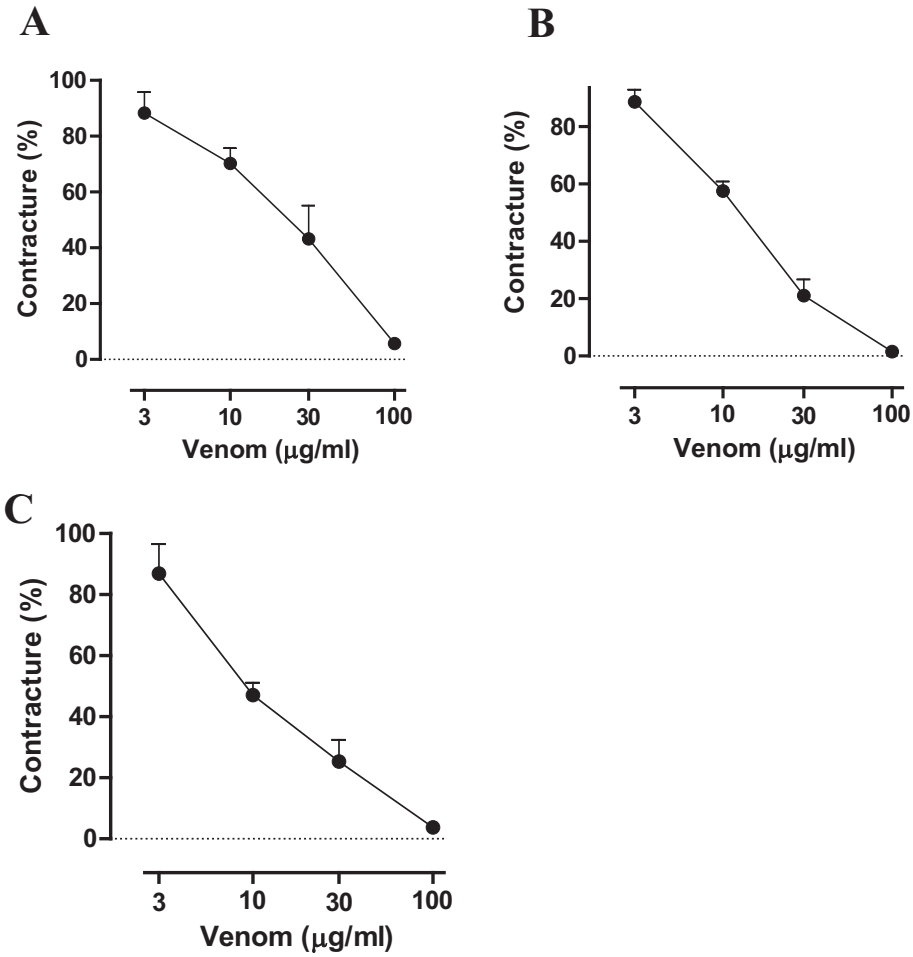

Fig. 5. Contracture of BC muscle incubated with *Crotalus atrox* venom (3–100 μg/ml) in response to 1 mM acetylcholine (ACh) (A), 20 μM carbachol (CCh) (B) and 40 mM KCl (C). Each point represents mean ± SEM of the contrature after incubation with venom compared to the contrature before incubation.

noted 36.3 ± 3.2% of cell damage in avian preparations and 67.7 ± 3.3% of cell damage in mice models. Neurotoxic effects were not observed in the absence of myotoxic effect, and higher neuromuscular blockade seems associated with higher toxicity to muscle cells. Thus, these pharmacological findings suggested that muscle fiber damage plays a significant role in the low neuromuscular activity of *C. atrox* venom. This non-specific effect may be the result of the synergistic action of the functionally diverse catalog of proteins and peptides present in *C. atrox* venom or may derive from a highly specific protein acting alone. Presumably, the myotoxicity evidenced in our histological assessments could be provoked by abundant proteolytic enzymes, including metalloproteinases and serine proteases, and some phospholipases A_2 (PLA_2), which can act together or alone. Previous studies have shown that a Lys49 PLA_2 homologue from *C. atrox* promotes damage muscle in animal models (Tsai et al., 2001). The importance and role of myotoxicity in neuromuscular blockade varies from venom to venom, reflecting distinct mechanisms of action and proteomes. The myotoxic effects contribute to the neuromuscular action of some venoms (Damico et al., 2005; Carreiro da Costa et al., 2008), whereas some snake venoms, such as *C. durissus cascavella* (Beghini et al., 2004) and *C. durissus ruruima* (Cavalcante et al., 2015) presented a high neurotoxic activity at low doses even in the absence of any damage to muscle tissue.

Crotalus atrox snakes are important elements of the food chain, as they feed primarily on small rodents, lizards, and birds (Beavers, 1976; McCue, 2007). Beavers (1976) analyzed the digestive tracts of 205 *Crotalus atrox* snakes from the United States and reported that mammals, composed 94.8% of this species´ diet by weight. Interestingly, the same study revealed seasonal fluctuations in food ingestion. The diamondbacks collected and examined during the spring had consumed greater amounts of food, relative to their body weight, when compared to specimens collected in the winter or summer.

Our experimental results showed a prey-specific variation in *C. atrox* venom neurotoxicity and myotoxicity. The chick *biventer cervicis* nerve-muscle preparations were more sensitive to *C. atrox* venom than the mouse diaphragm preparations. Higher venom concentration showed twitch heights 51.4 ± 10.1% and 7.2 ± 1.5% of control within 120 min of incubation in mouse and chick nerve-muscle preparations, respectively. Other toxicological studies have reported the specific sensitivity of neuromuscular preparations to whole snake venoms or isolated toxins. For example, the venom of *Philodryas olfersii* induces potent effects in avian preparations with very low effect on neurotransmission in mammalian preparations (Prado-Franceschi et al., 1996), similar to the toxic effects induced by *Bothrops insularis* (Cogo et al., 1993). Resende et al. (2017) isolated and characterized an acidic PLA_2, AplTx-I, from *Agkistrodon piscivorus leucostoma*, that triggered a selective and highly neuromuscular effect in chick *biventer cervicis* preparations. No effect was noted in mice models using the same PLA_2 toxin. In the same way, crotoxin and β-bungarotoxin has been found to induce a more pronounced effect on chick neuromuscular preparations (Chang, 1985) when compared to PND preparations.

Other investigations have also described the correlation between variances in diet and ontogenetic changes in venom proteomes, which are related to prey preference of North American snakes (Mackessy, 1988; Saviola et al., 2015). The intriguing relationship between prey

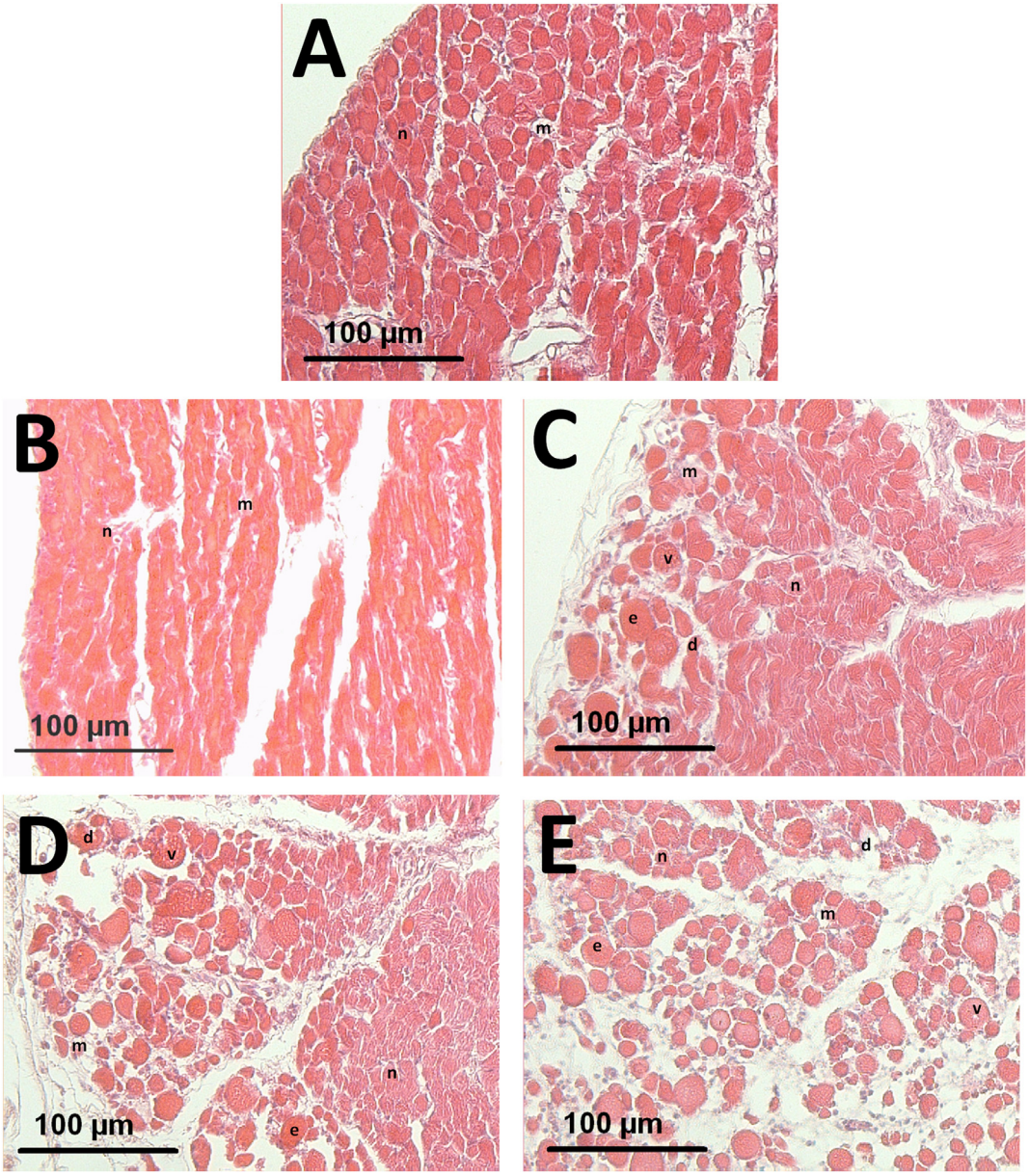

Fig. 6. Histological analysis of transversal BC muscles sections after a 120 min incubation with Tyrode solution (A); 3 (B), 10 (C), 30 (D) and 100 µg/mL (E) of *C. atrox* venom. The sections are representative of 6 preparations per treatment. Note the presence of normal fibers (n), edematous (e), delta lesions (d), vacuolated fibers (v) and myonecrosis (m). Bar = 100 µm.

type (animal models) and sensitivity to snake venoms represents a great challenge in toxicological research that reveals the need for more efforts to better understanding. The more potent myotoxic and neuromuscular effect of *C. atrox* on BC in relation to PND preparations can be from the result of distinct anatomical and physiological features of the muscle fibers and the kind of innervation of the nerve-muscle preparations used in the experiments, as discussed by Cavalcante et al. (2011) and Fernandes et al. (2014). Other valuable hypothesis to be considered when analyzing the prey-specific variation in diamondback neurotoxic activity is a mammalian adaptation, that confers resistance to the venom and its components. Beavers (1976), Lagler and Salyer (1945) and Cottam et al. (1959) suggested in their studies that western diamondback rattlesnakes feed mainly on small mammals owing to their greater availability, which reflects the ease of capturing the prey and results from the integration of several factors, such as homoiothermic condition, the nocturnal activity of rodents and rattlesnake habits. Usually, *C. atrox* is an animal that hunts and feeds at night, using heat sensitive pits to detect its prey (Beavers, 1976; Calvete et al., 2009). Birds on the other hand are diurnal, limiting their exposure and prey potential to *C. atrox* (Beavers, 1976). With this in mind, it is suggested that the greater potency of this whole venom in avian preparations could confer an evolutionary advantage for possible predation of an animal that is difficult to capture and not so abundant during nocturnal activity of the snake. Typically, snake venoms evolve with highly complex and integrated systems of proteins and peptides, that show biochemical arsenal and biological activities directed towards to immobilization and digestion of their prey (Mackessy, 1988; Hargreaves et al., 2014; Aird et al., 2015). However, more research is essential to unravel this puzzle.

Proteomic and genomic approaches have revealed the evolutionary biology of the genus *Crotalus* and provided invaluable insights into ecology, biochemistry, phylogenetic aspects and protein compositions (Calvete et al., 2009; Calvete et al., 2010; Dowell et al., 2016; Zancolli et al., 2016). Venomic analyses have demonstrated that North

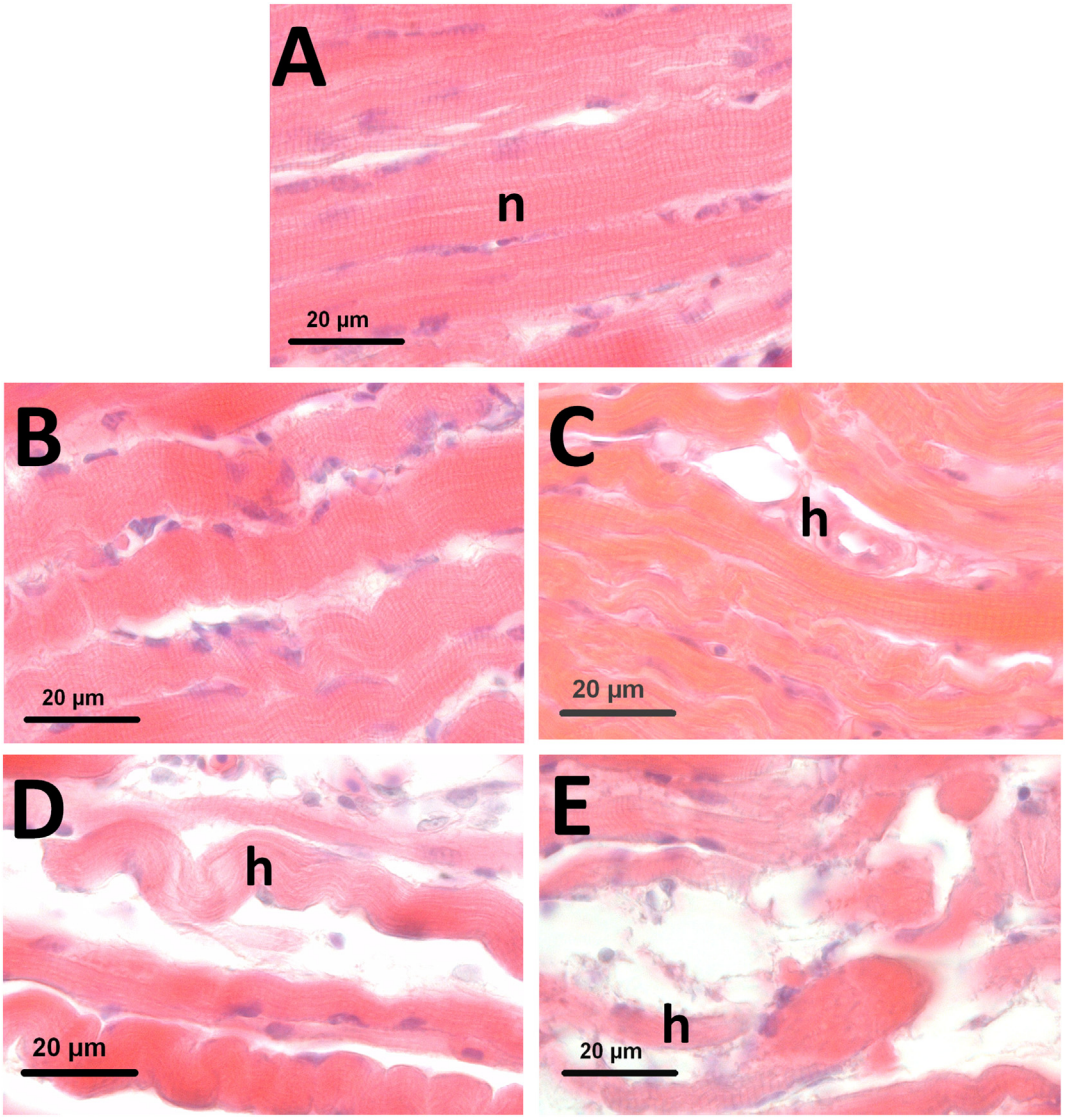

Fig. 7. Histological analysis of longitudinal BC muscles sections after 120 min incubation with Tyrode solution (A); 3 (B), 10 (C), 30 (D) and 100 µg/mL (E) of *C. atrox* venom. The sections are representative of 6 preparations per treatment. Note the presence of normal fibers (n) and hypercontracted myofilaments (h). Bar = 20 µm.

American snakes, such as *C. atrox* have a proteomic profile very different from snakes from South America. The two predominant *C. atrox* venom toxins are metalloproteinases and serine proteases, which compose approximately the 70% of the venom proteome. Present in smaller quantities are: phospholipases, disintegrins, L-amino acids, vasoactive peptides, C-type lectin and other proteins (Calvete et al., 2009). In marked contrast, *Crotalus* snakes from South America produce a venom rich in crotoxin, a heterodimeric protein, responsible for blockade neuromuscular and muscle paralysis (Calvete et al., 2010; Cavalcante et al., 2015). The venomic analysis of *C. durissus terrificus* demonstrated the presence of a high number and diversity of crotoxin isoforms (Georgieva et al., 2010). The experimental values of the time taken to cause 50% or 90% inhibition of nerve mediated twitches were compared with the literature on crotalic venom from South America and they are according to these genomic and venomic analyses. The times of 50% blockade (t50) were 90.8 ± 9.7 and 39.5 ± 10.1 min with 30 and 100 µg/mL of *C. atrox venom*, respectively, while the time of 90% blockade (t90) was reached only with 100 µg/mL, being 107.3 ± 2.0 min. It is noteworthy that based on these values, *C. atrox* presented a very low ability to cause neuromuscular blockade in mouse and chick preparations when compared to *C. durissus terrificus*, *C. durissus cascavella*, *C. durissus ruruima* and *C. durissus cumanensis* venoms. Some studies have explained the underlying mechanisms for the loss of venom neurotoxic genes in snakes from North American on the basis of dietary shift and the resulting expression of other protein classes (Calvete et al., 2010; Dowell et al., 2016). The increased production of neurotoxic molecules in South American snakes, such as crotoxin in *C. durissus* venoms, constitutes a key evolutionary event for the neurotoxicity and lethal activity of snake venoms, that facilitated the colonization of new territory (Calvete et al., 2010).

In conclusion, our results showed that *C. atrox* is a venom with myotoxic properties that play a significant role in weak neuromuscular blockade in isolated nerve-muscle preparations. A prey-specific variation in *C. atrox* was observed; with the venom being more myotoxic and neurotoxic in BC preparations. This characterization of the molecular patterns of bioactivity from North American snake venom reveals crucial clues, which integrated with genomic and proteomic data from literature and further transcriptomic investigations, will prove useful in gaining a better understanding of the evolutionary biology of rattlesnakes, of paradigms in origins of multifunctional toxins and of differences in the phenotypes of their venoms.

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Table 1
Comparison of the potency of *Crotalus atrox* and other *Crotalus* snake venoms determined as the times required to induce neuromuscular blockade in BC and PND preparations. *C. atrox* venom is weakly neurotoxic. This bioactivity is due to its myotoxic effect. The t_{50} and t_{90} values were estimated from graphs provided in the cited publications.

Snake species	Venom concentration (μg/mL)	Neuromuscular preparations				References
		BC		PND		
		t_{50} (min)	t_{90} (min)	t_{50} (min)	t_{90} (min)	
Crotalus atrox	3	NE	NE	NE	NE	Present work
	10	NE	NE	NE	NE	
	30	≈91	NE	NE	NE	
	100	≈40	≈107	NE	NE	
Crotalus durissus terrificus	5	–	–	≈70	≈100	de Jesus et al. (2010)
	10	≈30	≈49	≈64	≈100	
	20	–	–	≈40	≈64	
Crotalus durissus cascavella	1	≈35	≈93	–	–	Beghini et al. (2004)
	5	≈26	≈37	–	–	
	25	≈20	≈30	–	–	
Crotalus durissus ruruima	1	–	–	–	NE	Cavalcante et al. (2015)
	5	–	–	NE	NE	
	10	–	–	≈48	NE	
Crotalus durissus cumanensis	1	≈70	NE	–	–	Cavalcante et al. (2015)
	5	≈35	NE	≈42	≈60	
	10	≈30	≈48	≈36	≈60	
Crotalus scutulatus salvini	3	≈22	≈32	–	–	Dobson et al. (2017)
Crotalus scutulatus scutulatus Cochise	3	≈19	≈28	–	–	Dobson et al. (2017)

NE The venom did not induce 50 or 90% neuromuscular blockade at the indicated concentration during the frame of the experiment.
– Data not reported.

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Conflict of interest

The authors declare no relevant conflicts of interest concerning the present manuscript. All authors have read and agreed with the final version of the article.

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