

INTRASPECIFIC VARIATION IN NEUROTOXIC AND MYOTOXIC ACTIVITIES OF *Bothrops neuwiedii* VENOMS

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ABSTRACT: Snake venoms frequently vary in composition. In this work, we compared the neurotoxic and myotoxic activities of 16 lots of *Bothrops neuwiedii* venoms from different regions of Brazil, using chick biventer cervicis preparations. The neuromuscular blockade varied from 2% to 100% after 120 min incubation with venoms (50 µg/ml). In all cases, this blockade was irreversible and concentration-dependent; at low concentrations (10-20 µg/ml), 15 of the 16 venom lots failed to abolish responses to acetylcholine (110 µM), but blocked responses to KCl (13.4 µM), and induced contracture. At 5-20 µg/ml, the most active venom totally blocked twitch-tension without affecting responses to acetylcholine and KCl. Polyacrylamide gel electrophoresis for basic proteins showed that the most active samples contained a band that was absent in the less active venoms. These results indicate that there may be considerable intraspecific variation in the neurotoxic activity of *B. neuwiedii* venoms, whereas myotoxic activity is less variable.

KEY WORDS: *Bothrops neuwiedii* venom, intraspecific variation, myotoxicity, neurotoxicity, neuromuscular blockade.

INTRODUCTION

Snake venoms are complex mixtures of proteins that can vary in composition and toxicity according to geographic origin (1). In Brazil, *Bothrops* snakes are responsible for 90% of snakebites. *Bothrops* venoms are rich in proteolytic, coagulant, and hemorrhagic activities. In recent years, venoms of several *Bothrops* species, including *B. jararacussu* (16), *B. insularis* (2,3), *B. neuwiedii* (23), and *B. pirajai* (4) have been shown to affect neuromuscular transmission in amphibian, avian and mammal nerve-muscle preparations. *B. neuwiedii* venom induces complete and irreversible blockade of twitch-tension response in chick biventer cervicis neuromuscular preparations, without affecting responses to acetylcholine or reducing contracture to KCl (23). However, nothing is known about the extent to which this and other activities present in the venom of this species may vary. For this reason, we investigated the intraspecific variation in neurotoxic and myotoxic activities of *B. neuwiedii* venoms from the Brazilian states of Minas Gerais, Rio Grande do Sul, and São Paulo.

MATERIALS AND METHODS

Animals

Male HY-LINE W36 chicks (4-8 days old) were supplied by Granja Ito S/A (Sumaré, SP, Brazil). The animals were housed at 24°C with access to food and water *ad libitum*.

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Venoms

B. neuwiedii venoms obtained from 1-5 individuals from different regions were provided by the Instituto Butantan (São Paulo, SP, Brazil), Instituto Eva (Paulínia, SP, Brazil) and Centro de Estudos de Venenos e Animais Peçonhentos -CEVAP (Botucatu, SP, Brazil). Samples BnSP1-BnSP7 were from specimens collected in São Paulo State; BnRS1-BnRS8 Rio, Grande do Sul; and BnMG, Minas Gerais. All venom concentrations used in this study refer to venom dry weight.

Chick biventer cervicis preparation

The biventer cervicis was removed as described by Ginsborg and Warriner (9) and mounted under a tension of 1 g in a 5 ml organ bath containing aerated (95% O₂, 5% CO₂) Krebs solution (pH 7.5, 37°C) of the following composition (mM): NaCl 118.6, KCl 4.69, CaCl₂ 1.88, KH₂PO₄ 1.17, MgSO₄ 1.17, NaHCO₃ 25.0, and glucose 11.65. Stimuli (4 x threshold, 0.1 Hz, 0.2 ms) were delivered to the preparations with a Grass S4 stimulator to the tendon via bipolar electrodes. The resulting muscle tension was recorded isometrically using a force displacement transducer (BG 25 GM Kulite) coupled to a Gould RS 3400 recorder. The preparation was allowed to stabilize for at least 15 min before venom addition (1, 5, 10, 20, 50, or 100 µg/ml). Contractures to exogenously applied submaximal concentrations of acetylcholine (110 µM for 60 s) and KCl (13.4 µM for 120-180 s) were obtained in the absence of nerve stimulation prior to venom addition and at the end of the experiment, as an assay for the presence of neurotoxic and myotoxic activities (10).

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was performed in 10% polyacrylamide gels as described by Reisfeld *et al.* (14), using 0.03 M acid acetic buffer, pH 4.5. The samples were dissolved in 50% glycerol electrode buffer.

Data analysis

The extent of blockade levels induced by exogenous acetylcholine and KCl were expressed as a percentage of the control response obtained before venom addition. Each experiment was repeated at least three times and the results expressed as mean \pm S.E.M. of *n* experiments. Students *t*-test was used for data statistical comparison. Values of *P* < 0.05 were considered significant.

RESULTS

Effects of *B. neuwiedii* venoms in chick biventer cervicis preparations

B. neuwiedii venoms produced different degrees of neuromuscular blockade in chick biventer cervicis preparations, as shown by the residual contractile activity after 120 min incubation with venom (50 µg/ml) (Figure 1).

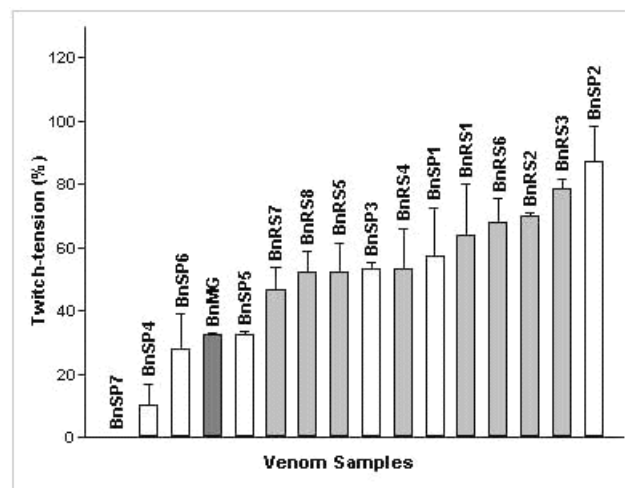


Figure 1. Comparison of neuromuscular activity of *B. neuwiedii* venoms in indirectly stimulated chick biventer cervicis preparations. Columns represent the residual contractile activity in indirectly stimulated preparations after 120 min incubation with venom at 50 µg/ml (mean \pm S.E.M. of 3-6 experiments/venom). Neuromuscular blockade decreases from left to right in the figure: white bars - venoms from São Paulo State; dark gray bar - venom from Minas Gerais; and light gray bars - venoms from Rio Grande do Sul. In control experiments, (preparations incubated with Krebs solution alone) no effect on twitch-tension was observed after 120 min.

In all cases, the extent of neuromuscular blockade was concentration-dependent and could not be reversed by washing.

Sample BnSP7 was the most active. At 5 µg/ml, BnSP7 and BnSP4 produced 50% blockade in 45.0 ± 2.0 min (*P*<0.05; *n*=3) and 64.5 ± 4.3 min (*P*<0.05; *n*=4), whereas concentrations above 20 µg/ml or 50 µg/ml were necessary to inhibit the contractile responses within 120 min for other venoms.

At concentrations of 10-20 µg/ml, all venoms, except BnSP7, abolished responses to KCl and produced contracture, but showed varying effects on responses to exogenous acetylcholine. At higher concentrations (above 50 µg/ml), all venoms abolished responses to both acetylcholine and KCl and induced pronounced contractures. BnSP7 at 5-20 µg/ml blocked twitch-tension responses, without inhibiting responses to KCl and acetylcholine or inducing contracture; BnSP4 produced the same effect only at 5 µg/ml. At 10-20 µg/ml, BnSP4 did not abolish responses to acetylcholine, but blocked the responses to KCl.

Tables 1, 2, and 3 show the effect of the various venoms on responses to KCl and acetylcholine.

Table 1. Effect of *B. neuwiedii* venoms from São Paulo on responses to exogenous acetylcholine and KCl in indirectly stimulated chick biventer cervicis preparations. The preparations were mounted as described in Methods, and responses to exogenous acetylcholine (110 µM) and KCl (13.4 µM) were obtained before and after venom addition. The extent of blockade was expressed as a percentage of the control response obtained before venom addition. The values are the mean ± S.E.M. of the number of experiments indicated.

Venom Sample	Concentration (µg/ml)	Acetylcholine (% blockade)	KCl (% blockade)	N
BnSP1	20	25.0 ± 20.1	90.8 ± 8.0	3
	50	85.0 ± 15.0	94.9 ± 5.1	3
BnSP2	20	11.1 ± 11.1	100.0	3
	50	51.8 ± 9.2	100.0	3
BnSP3	20	41.0 ± 20.5	100.0	3
	50	76.0 ± 5.7	100.0	3
BnSP4	20	22.2 ± 12.1	98.2 ± 1.8	3
	50	90.2 ± 5.5	94.7 ± 5.3	3
BnSP5	20	38.9 ± 20.0	94.6 ± 5.4	3
	50	63.3 ± 31.8	100.0	3
BnSP6	20	60.4 ± 21.3	100.0	4
	50	100.0	97.7 ± 2.3	4

Sample BnSP7 did not affect response to acetylcholine or KCl (data not shown).

Table 2. Effect of *B. neuwiedii* venoms from Rio Grande do Sul on responses to exogenous acetylcholine and KCl in indirectly stimulated chick biventer cervicis preparations. The preparations were mounted as described in Methods, and responses to exogenous acetylcholine (110 µM) and KCl (13.4 µM) were obtained before and after venom addition. The extent of blockade was expressed as a percentage of the control response obtained before venom addition. The values are the mean ± S.E.M. of the number of experiments indicated.

Venom Sample	Concentration (µg/ml)	Acetylcholine (% blockade)	KCl (% blockade)	N
BnRS1	20	46.1 ± 8.4	100.0	3
	50	100.0	100.0	3
BnRS2	20	53.3 ± 3.3	100.0	3
	50	88.6 ± 5.9	94.4 ± 2.9	3
BnRS3	20	22.2 ± 22.0	100.0	3
	50	100.0	100.0	3
BnRS4	20	58.6 ± 17.9	100.0	3
	50	91.7 ± 8.3	100.0	3
BnRS5	20	0	100.0	3
	50	54.7 ± 16.0	100.0	3
BnRS6	20	37.9 ± 14.0	100.0	3
	50	70.9 ± 5.8	100.0	3
BnRS7	20	60.0 ± 20.5	100.0	3
	50	86.1 ± 10.0	100.0	3
BnRS8	20	78.3 ± 11.7	100.0	3
	50	100.0	100.0	3

Table 3. Effect of *B. neuwiedii* venoms from Minas Gerais on responses to exogenous acetylcholine and KCl in indirectly stimulated chick biventer cervicis preparations. The preparations were mounted as described in Methods, and responses to exogenous acetylcholine (110 µM) and KCl (13.4 µM) were obtained before and after venom addition. The extent of blockade was expressed as a percentage of the control response obtained

before venom addition. The values are the mean \pm S.E.M. of the number of experiments indicated.

Venom Sample	Concentration ($\mu\text{g/ml}$)	Acetylcholine (% blockade)	KCl (% blockade)	N
BnMG	20	58.0 ± 4.2	100.0	3
	50	51.7 ± 5.7	100.0	3

Electrophoretic profiles

Figure 2 shows that the electrophoretic profiles of the venom samples were very similar, but BnMG and BnSP4-BnSP7 contained an additional band not found in the other venoms.

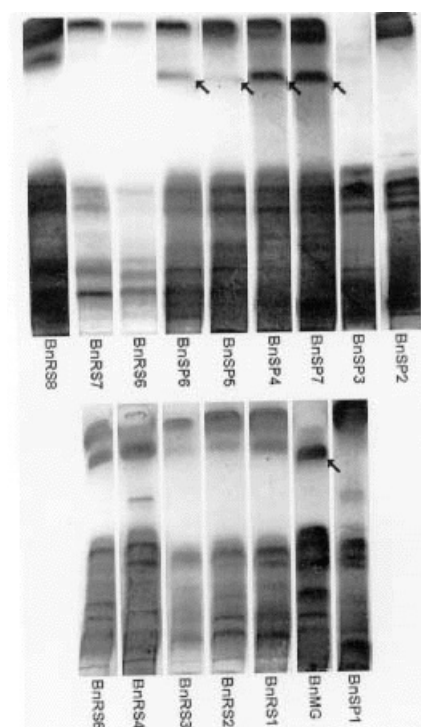


Figure 2. PAGE electrophoretic profiles of *B. neuwiedii* venoms. Venom samples (5-50 μg) were run as described by Reisfeld *et al.* (14) and stained with Coomassie brilliant blue R250. The arrows indicate the extra band in samples BnMG and BnSP4 - BnSP7.

DISCUSSION

Many factors such as age, sex, diet, geographic origin, and venom milking and storage conditions can affect the biological activity of venoms (1). We have shown significant variations in the neurotoxic potencies of *B. neuwiedii* venoms from different regions. At concentrations $\geq 20 \mu\text{g/ml}$, most venoms reduced twitch-tension without completely abolishing contracture to exogenous acetylcholine, suggesting a presynaptic action. There was no clear correlation between geographic origin and venom ability to reduce electrically stimulated twitches.

Myotoxicity was less variable, based on venom ability to abolish slow contractures caused by KCl (10). Although our results confirmed myotoxicity in all *B. neuwiedii* venoms tested, not all samples abolished responses to KCl at low concentrations (5-20 $\mu\text{g/ml}$). Gel electrophoresis has revealed intraspecific variation in *B. neuwiedii* venom myotoxin content from different regions (15,18). Ontogenic regulation of myotoxin expression could contribute to this variation, as suggested for *B. asper* (12).

As reported by Harvey *et al.* (10), low venom concentrations frequently reveal the presence of neurotoxins, while high venom concentrations are required to demonstrate the presence of myotoxic components. Thus, at 5 $\mu\text{g/ml}$, BnSP4 blocked twitch-tension response of chick muscle without inhibiting response to KCl or inducing contracture; the latter two effects were seen at 10 $\mu\text{g/ml}$.

B. neuwiedii venom contains phospholipases (5,6,8,19,22), which may be involved in venom neuromuscular action. Intraspecific variation in phospholipase activity, such as reported in *B. asper* (13), *C. durissus* (17), *C. ruber* (20), and *Daboia russelli* (21) could account for variation in *B. neuwiedii* neuromuscular activity. Although samples BnMG1 and BnSP4-BnSP7, which had the highest neuromuscular potency, also had an additional electrophoretic

band in relation to the other venoms, our results do not permit correlation between these two findings.

Although neuromuscular action has been demonstrated *in vitro* for various *Bothrops* venoms (2-4,16,23), there is no conclusive evidence for such an effect *in vivo*, including human envenomations. In the case of *B. neuwiedii*, the main envenomation effects are very similar to those of other *Bothrops* and include edema, hemorrhage, necrosis, and coagulation disorders (7,11). Thus, the clinical relevance of our findings is difficult to judge. However, the fact that we observed extensive variations suggests that further investigations into the above venom actions are required for a more direct bearing on the consequences of envenomation.

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