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The *in vitro* Neurotoxic and Myotoxic Effects of the Venom from the *Suta* Genus (Curl Snakes) of Elapid Snakes

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Abstract: Australia has a tremendous diversity of elapid snakes, including many unique smaller sized species of this venomous snake family. However, little if anything is known about the majority of the venoms of these lesser studied snakes. In the current study, the venoms of *Suta suta* (curl snake) and *Suta punctata* (spotted-curl snake) were examined for *in vitro* activity using a skeletal muscle preparation (i.e. chick biventer cervicis nerve-muscle preparation). Both venoms caused concentration-dependent (3–10 μg/ml) inhibition of nerve-mediated twitches, and inhibited responses to exogenous acetylcholine and carbachol, indicating the presence of postsynaptic neurotoxins. These effects were prevented by prior addition of CSL Ltd. polyvalent snake antivenom (5 units/ml) but only partially reversed by the addition of antivenom (5 units/ml) at the t_{90} time-point (i.e. time at which twitches were inhibited by 90%). *Suta punctata* venom (10 μg/ml) was also myotoxic as indicated by the inhibition of direct twitches of the chick biventer cervicis nerve-muscle preparation. This effect was not reversed by antivenom (5 units/ml). This study highlights the danger of underestimating the potential severe clinical effects posed by these small but highly venomous snakes.

The vast majority of venomous Australian snakes, in particular those of medical importance, are elapids (i.e. snakes with small, fixed, front fangs). Australia has a tremendous biodiversity of small elapids, with these lesser studied species comprising a greater biodiversity than their larger, and more well studied, cousins (e.g. tiger snakes, taipans, death adders, black snakes). We have previously described the presynaptic neurotoxic effects of *Furina tristis* (brown-headed snake) venom [1], a study undertaken following a report of a severe envenoming on Lizard Island (northern Queensland, Australia). The current study focuses on the bioactivity of *Suta suta* (curl snake) and *Suta punctata* (spotted-curl snake) venoms. This investigation is a follow-up to a report of a severe envenoming by *S. suta*. The clinical symptoms included progressive neurotoxicity over a 4-hr period that resulted in flaccid paralysis. The patient was also in extreme pain that started in the lower back. Polyvalent antivenom was administered and recovery from the neurotoxicity was slow (Fry, unpublished observations).

A previous study that analysed the molecular mass of snake venom constituents by liquid chromatography with mass spectrometry (LC/MS) suggests the presence of three finger toxins, or at least components with corresponding molecular weights (e.g. 6420 Da, 6739 Da, 6702 Da) in *S. suta* venom [2]. However, conclusive identification of these components would require structural studies such as N-terminal sequencing and *in vitro* analysis utilizing skeletal muscle preparations. Three finger toxins are characterised by the presence of three finger-like β strands emerging from the globular core, stabilised by four disulfide bridges [3,4]. They are some of the most abundant and highly recognised protein families in snake venoms, and can affect targets ranging from nicotinic acetylcholine receptors [5] to proteins involved in blood coagulation [6].

The curl snake (*S. suta*) is found in all states of mainland Australia but not Tasmania. The common name is due to the snake's characteristic defensive behaviour of curling up in a knot with its head out of sight [7]. Initial studies on the venom of *S. suta* indicated that it is likely to have neurotoxic effects [8] supporting the more recent LC/MS examination [2]. While a study examining the lethality of *S. suta* venom in mice reported a subcutaneous LD_{50} of 1 mg/kg [9]. *S. punctata*, or the spotted curl snake, is found in Western Australia, Northern Territory and Queensland. To the best of our knowledge, there have been no previously published studies on the bioactivities of either venom.

This study provides the first report of the *in vitro* neuromuscular activity of *S. suta* and *S. punctata* venoms. The ability of commercially available antivenom to neutralise this activity was also examined.

Materials and Methods

Venom or toxin preparation and storage. Suta suta and *Suta punctata* were collected by Bryan G. Fry from the Barkly Tableland region of the Northern Territory. *S. suta* and *S. punctata* venoms were prepared in Milli-Q water and stored at –20°C until required.

Chick biventer cervicis nerve muscle preparation. Chicks (4–10 days old) were killed by CO_2 and exsanguination. The biventer cervicis

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nerve muscle preparations were dissected and mounted under 1 g tension in 5 ml organ baths containing physiological salt solution (NaCl 118.4 mM; KCl 4.7 mM; $MgSO_4$ 1.2 mM; KH_2PO_4 1.2 mM; $CaCl_2$ 2.5 mM; $NaHCO_3$ 25 mM and glucose 11.1 mM) at 34°C, bubbled with carbogen (95% oxygen; 5% CO_2). Indirect twitches (i.e. nerve-mediated) of the preparation were evoked by electrical stimulation of the motor nerves using a Grass S88 stimulator (0.2 msec., 0.1 Hz, supramaximal V; Grass Instruments Co., Quincy, Medfield, MA, USA). Blockade of twitches by the addition of d-tubocurarine (10 µM) confirmed the selective stimulation of the motor nerves. The preparation was then washed repeatedly until twitches were re-established. In the absence of electrical stimulation, responses to acetylcholine (ACh, 1 mM for 30 sec.), carbachol (CCh, 20 µM for 60 sec.) and KCl (40 mM for 30 sec.) were obtained. The preparation was washed thoroughly, electrical stimulation recommenced and the preparation allowed to equilibrate for 30 min. Venom was added to the organ bath and twitch height was recorded for 3 hr or until twitches were abolished. Where indicated, CSL Ltd. polyvalent snake antivenom (5 units/ml; CSL Ltd., Melbourne, Australia) was added 10 min. prior to the addition of venom. Contractile responses to ACh, CCh and KCl were obtained (as described above) at the conclusion of the experiment.

In additional experiments, polyvalent snake antivenom (5 units/ml) was added at the t_{90} time-point (i.e. time at which twitches were inhibited by 90%), and twitch height was recorded for 3 hr or until no further changes in twitch height were observed.

In experiments examining the myotoxic effects of venom, the biventer cervicis muscle was directly stimulated every 10 sec. with pulses of 2 msec. duration at supramaximal voltage. In these experiments the electrodes were placed around the belly of the muscle and d-tubocurarine (10 µM) remained in the organ bath for the duration of the experiment. Venom was left in contact with the preparation until twitch blockade occurred, or for a 3-hr period (as above). Where indicated, polyvalent snake antivenom was added 1 hr after the addition of venom. Venom was considered to be myotoxic if it inhibited direct twitches or caused a contracture of the skeletal muscle.

Drugs. The following drugs were used: carbamylcholine chloride (carbachol); acetylcholine chloride (Sigma Chemical Co., St. Louis, MO, USA); polyvalent snake antivenom; tubocurarine chloride (Calbiochem, San Diego, CA, USA). All stock solutions were made up in distilled water.

Results

Neurotoxicity studies.

Suta suta (fig. 1A) and *Suta punctata* (fig. 1B) venoms caused concentration-dependent (3–10 µg/ml) inhibition of nerve-mediated twitches in the chick biventer preparation (n = 4). In addition, both venoms significantly inhibited responses to exogenous ACh and CCh (fig. 2; n = 4), while having no significant effect on the response to KCl. This indicates a postsynaptic mode of action. Prior addition of antivenom (5 units/ml) prevented the venom-induced inhibition of twitches (fig. 1A and B; n = 4–8; one-way ANOVA) and responses to exogenous nicotinic receptor agonists (data not shown, n = 4–8). The addition of antivenom (5 units/ml) at the t_{90} time-point partially reversed the inhibition induced by *S. suta* venom (3 µg/ml; 35 ± 9% recovery) and *S. punctata* venom (3 µg/ml; 18 ± 4% recovery; fig. 3; n = 6) over a period of 3–4 hr.

Mytoxicity studies.

Suta suta venom (10 µg/ml) had no significant effect on the direct twitches of the chick biventer preparation compared to vehicle (i.e. 0.1% bovine serum albumin; data not shown). In contrast, *S. punctata* venom (10 µg/ml) caused significant inhibition of direct twitches (fig. 4; n = 4–5; one-way ANOVA), indicating myotoxic activity of the venom. This was further supported by the significant increase in baseline tension of the muscle induced by *S. punctata* (1.1 ± 0.1 g) venom (10 µg/ml) compared to *S. suta* (0.0 ± 0.2 g) venom (n = 4–5; one-way ANOVA; 10 µg/ml).

The addition of antivenom (5 units/ml) 1 hr after the addition of *S. punctata* venom (10 µg/ml) failed to reverse, nor prevent further decrease in direct twitches (data not shown). In addition, antivenom (5 units/ml) had no significant effect on the *S. punctata* (10 µg/ml) venom-induced increase in

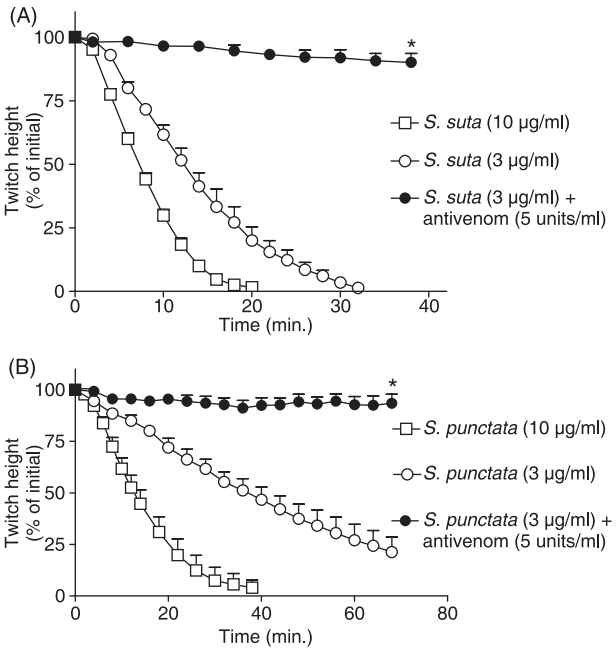

Fig. 1. The effect of (A) *S. suta* (3–10 µg/ml) or (B) *S. punctata* (3–10 µg/ml) venom alone or in the presence of antivenom on indirect twitches of the chick biventer cervicis nerve-muscle preparation. *P < 0.05; one-way ANOVA compared to venom (3 µg/ml) alone; n = 4–8.

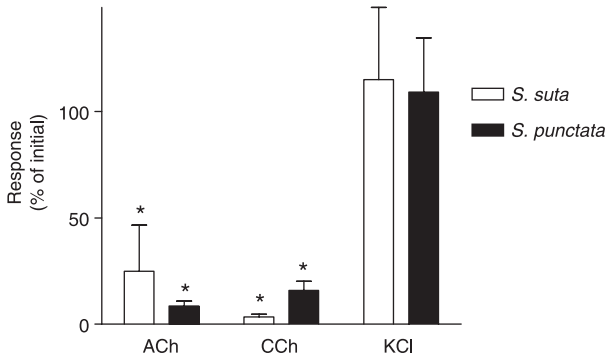

Fig. 2. The effect of *S. suta* and *S. punctata* (3 µg/ml) venom responses of the chick biventer cervicis nerve-muscle preparation to exogenous ACh, CCh or KCl. *P < 0.05; paired t-test compared to initial response; n = 4.

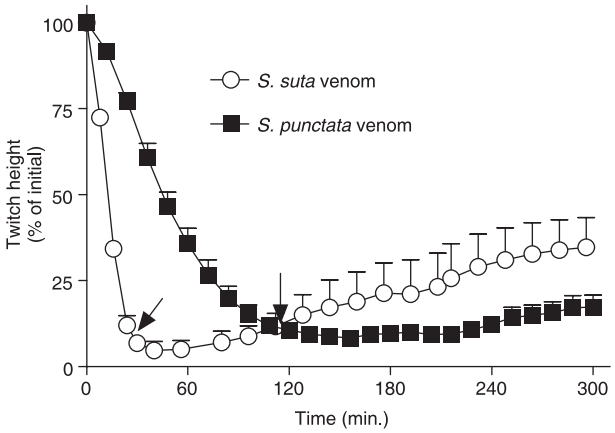

Fig. 3. The effect of antivenom (5 units/ml), added at t_{90} (indicated by arrow), following the addition of *S. suta* (3 μg/ml) or *S. punctata* (3 μg/ml) venom on indirect twitches of the chick biventer cervicis nerve-muscle preparation; n = 6.

baseline tension (1.0 ± 0.3 g) compared to venom alone (1.1 ± 0.1 g; n = 4–5; one-way ANOVA).

Discussion

The *in vitro* neurotoxicity of the venoms was examined using the chick biventer cervicis nerve-muscle preparation (i.e. a skeletal muscle preparation). Both venoms caused a concentration-dependent decrease in indirect twitches that was postsynaptic in origin, as evidenced by the subsequent inhibition of responses to the exogenous nicotinic receptor agonists ACh and CCh, while having no effect on the response to KCl. However, the presence of presynaptic neurotoxins in these venoms cannot be ruled out since postsynaptic neurotoxins, which have a more rapid onset of action, would mask the action of the slower acting presynaptic neurotoxins, making it difficult to confirm the presence of the latter. *S. suta* venom appears to be more neurotoxic than *S. punctata* venom as indicated by a faster onset of action, at the same concentrations and the markedly lower t_{90} value. The neurotoxic effects of *S. suta* venom seem to be comparable to that of *Acanthophis wellsi* (black head death adder) as evidenced by the similar t_{90} values (table 1).

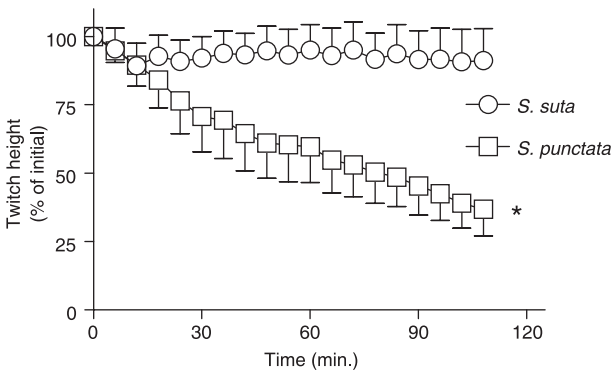

Fig. 4. The effect of *S. suta* and *S. punctata* venom (10 μg/ml) on direct twitches of the chick biventer cervicis nerve-muscle preparation. *P < 0.05; one-way ANOVA compared to vehicle (i.e. 0.1% bovine serum albumin); n = 5.

Although bites by *S. suta* and *S. punctata* are rare, a detailed study of the effects of these venoms is necessary given the widespread distribution of *S. suta* and the increase in number of visitors to the areas inhabited by *S. punctata*. Due to the lack of a specific monovalent antivenom against these venoms, the efficacy of polyvalent snake antivenom was examined. Interestingly, it has been previously shown that the CSL venom detection kit does not recognise *S. punctata* venom [10]. This would appear to indicate a lack of cross reactivity between *S. punctata* venom and the monovalent antivenoms raised against the five most clinically relevant genus in Australia (NB. *S. suta* venom was not included in the study). The polyvalent antivenom contains the following (with the minimum amount of each antivenom in an ampoule indicated): brown snake (*Pseudonaja textilis*; 1000 U), death adder (*Acanthophis antarcticus*; 6000 U), king brown (*Pseudechis australis*; 18,000 U), taipan (*Oxyuranus scutellatus*; 12,000 U) and tiger snake (*Notechis scutatus*; 3000 U). Prior addition (10 min.) of polyvalent snake antivenom prevented the inhibition of twitches and agonist responses induced by each of the venoms. In order to further test the efficacy of the antivenom, and to better approximate a clinical situation, antivenom was added at the t_{90} time-point. Antivenom partially reversed the twitch inhibition induced by *S. suta* venom but had less of an effect on *S. punctata* venom. The relative lack of effect of the antivenom added after the addition of *S. punctata* venom is likely to be due to the damage to the skeletal muscle by myotoxins. In support of this suggestion, *S. punctata* venom caused an increase in baseline tension and a decrease in direct twitches, both indicative of myotoxic activity [11], in the chick biventer cervicis preparation. To confirm and characterise the myotoxic effects of *S. punctata* venom histological examination of skeletal muscle tissue samples is required. However, the apparent *in vitro* myotoxic effects of *S. punctata* venom are comparable to that of *Acanthophis* sp. Seram venom, which we have previously shown to be the most myotoxic of the death adder venoms [12]. In contrast, the venom of *S. suta* appears to be devoid of myotoxic effects.

In the current study, the efficacy of polyvalent snake antivenom against the myotoxic effects of the *S. punctata* venom was investigated. Addition of antivenom 1 hr after the addition of venom failed to inhibit the myotoxic activity. This is likely to be due to the irreversible damage done to the myofibres by myotoxins in the venom.

In conclusion, the venoms of *S. suta* and *S. punctata* cause neurotoxic effects *in vitro*, which appear to be postsynaptic in origin. Although neurotoxicity is prevented by polyvalent snake antivenom, it only causes the partial reversal of the venom-induced twitch inhibition. *S. punctata* also causes significant myotoxic effects *in vitro* in comparison to *S. suta* venom, effects not reversible by the polyvalent snake antivenom. While this study examines for the first time the *in vitro* neuromuscular effect of these venoms, there is still

Table 1.

A comparison of neurotoxicity (as indicated by t_{90} values), obtained in the chick biventer cervicis nerve-muscle preparation, for a range of Asian-Pacific snake venoms.

Common name	Scientific name	t_{90} (min.) at 3 μg/ml	t_{90} (min.) at 10 μg/ml
Common death adder (New South Wales)	*Acanthophis antarcticus*	16 ± 1 [13]	10 ± 1 [13]
Black-head death adder	*Acanthophis wellsi*	24 ± 5 [13]	13 ± 2 [13]
Curl snake	*Suta suta*	23 ± 1	13 ± 1
Tiger snake	*Notechis scutatus*	38 ± 6 [14]	22 ± 2 [14]
Yellow-banded snake	*Hoplocephalus stephensi*	47 ± 7 [14]	20 ± 2 [14]
Spotted-curl snake	*Suta punctata*	86 ± 12	25 ± 2
Australian copperhead	*Austrelaps superbus*	89 ± 10 [14]	26 ± 3 [14]

scope for further investigation. Fractionating the venoms would help to identify and characterize the toxins responsible for the above mentioned effects.

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References

1 Kuruppu S, Fry BG, Hodgson WC. Presynaptic neuromuscular activity of venom from the Brown-Headed Snake (*Furina tristis*). Toxicon 2005;**45**:383–8.

2 Fry BG, Wuster W, Ryan Ramjan SF, Jackson T, Martelli P, Kini RM. Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxinological implications. Rapid Commun Mass Spectrom 2003;**17**:2047–62.

3 Endo T, Tamiya N. Current view on the structure-function relationship of postsynaptic neurotoxins from snake venoms. Pharmacol Ther 1987;**34**:403–51.

4 Kini RM. Molecular moulds with multiple missions: functional sites in three-finger toxins. Clin Exp Pharmacol Physiol 2002;**29**:815–22.

5 Tsetlin V. Snake venom alpha-neurotoxins and other 'three-finger' proteins. Eur J Biochem 1999;**264**:281–6.

6 Banerjee Y, Mizuguchi J, Iwanaga S, Kini RM. Hemextin AB complex, a unique anticoagulant protein complex from *Hemachatus haemachatus* (African Ringhals cobra) venom that inhibits clot initiation and factor VIIa activity. J Biol Chem 2005;**280**:42601–11.

7 Worrell E. Reptiles of Australia. Angus and Robertson, Sydney, Australia, 1970.

8 Kellaway CH. The venoms of some of the small and rare Australian venomous snakes. Med J Aust 1934;**2**:249–55.

9 Gillam MW, Williams OJ. The curl snake *Denisonia suta* (Peters). A dangerous elapid in Northern Territory. North Terr Nat 1984;**7**:12–7.

10 Jelinek GA, Tweed C, Lynch D, Celenza T, Bush B, Michalopoulos N. Cross reactivity between venomous, mildly venomous, and non-venomous snake venoms with the Commonwealth Serum Laboratories venom detection kit. Emerg Med Australas 2004;**16**:459–64.

11 Harvey AL, Barfaraz A, Thomson E, Faiz A, Preston S, Harris JB. Screening of snake venoms for neurotoxic and myotoxic effects using simple *in vitro* preparations from rodents and chicks. Toxicon 1994;**32**:257–65.

12 Wickramaratna JC, Fry BG, Hodgson WC. Species-dependent variations in the *in vitro* myotoxicity of death adder (*Acanthophis*) venoms. Toxicol Sci 2003;**74**:352–60.

13 Fry BG, Wickramaratna JC, Jones A, Alewood PF, Hodgson WC. Species and regional variations in the effectiveness of antivenom against the *in vitro* neurotoxicity of death adder (*Acanthophis*) venoms. Toxicol Appl Pharmacol 2001;**175**:140–8.

14 Hodgson WC, Eriksson CO, Alewood PF, Fry BG. A comparison of the *in vitro* neuromuscular activity of venom from three Australian snakes, *Hoplocephalus stephensi*, *Austrelaps superbus* and *Notechis scutatus*: efficacy of tiger snake antivenom. Clin Exp Pharmacol Physiol 2003;**30**:127–32.

