

# IN VITRO NEUROTOXIC AND MYOTOXIC EFFECTS OF THE VENOM FROM THE BLACK WHIP SNAKE (*DEMANSIA PAPUENSIS*)

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## SUMMARY

**1. Black whip snakes belong to the family elapidae and are found throughout the northern coastal region of Australia. The black whip snake (*Demansia papuensis*) is considered to be potentially dangerous due to its size and phylogenetic distinctiveness. Previous liquid chromatography–mass spectrometry analysis of *D. papuensis* venom indicated a number of components within the molecular mass ranges compatible with neurotoxins. For the first time, this study examines the *in vitro* neurotoxic and myotoxic effects of the venom from *D. papuensis*.**

**2. Venom (10 µg/mL) caused significant inhibition of twitches elicited by stimulation (0.2 ms, 0.1 Hz, supramaximal V) of motor nerves in the chick biventer cervicis nerve–muscle preparation. This neurotoxic effect, which was postsynaptic in origin, was weak in comparison to that of most other Australian elapids. Prior addition (10 min) of polyvalent (PSAV) or tiger snake (TSAV) antivenom (5 units/mL) prevented venom-induced twitch inhibition. Addition of PSAV (5 units/mL) at t<sub>50</sub> failed to reverse the inhibitory effect but prevented further inhibition of nerve-mediated twitches.**

**3. The venom (20–50 µg/mL) is also myotoxic as indicated by a slowly developing contracture and inhibition of twitches elicited by direct stimulation (2 ms, 0.1 Hz, supramaximal V, in the presence of tubocurarine 10 µmol/L) of the chick biventer muscle. This activity was confirmed by histological examination of the muscle.**

**4. Fractionation and characterization of venom components is required to further investigate the reasons for the weak neurotoxic activity of *D. papuensis* venom.**

**Key words:** antivenom, myotoxicity, neurotoxicity, phospholipase A<sub>2</sub>, snake, venom.

## INTRODUCTION

Australian elapids have a reputation as being among the most venomous snakes in the world.<sup>1</sup> Many of these venoms contain highly potent neurotoxins (both presynaptic and postsynaptic), myotoxins and haemotoxins.<sup>2</sup> However, there are still many species

of Australian snakes whose venoms have not been pharmacologically characterized. Whip snakes (*D. papuensis* and *D. atra*, also called *D. vestigata*) belong to the family elapidae and are found throughout the northern coastal region of Australia.<sup>3</sup> The black whip snake (*D. papuensis*) is considered to be potentially dangerous due to its size (up to 2 m) and phylogenetic distinctiveness which may suggest the presence of unique venom components.<sup>2</sup> LC-MS analysis of *D. papuensis* venom indicated a number of components within the molecular mass ranges of 6–7 and 13–14 kDa.<sup>4</sup> This may indicate the presence of short and long chain α-neurotoxins or PLA<sub>2</sub> components, respectively. Liquid chromatography–mass spectrometry (LC-MS) analysis also indicated the presence of components not found in other Australian elapid venoms highlighting the possibility that the commercially available antivenoms may not neutralize the toxic effects of this venom.<sup>4</sup> A prospective study in the Northern Territory (Australia), over the period from September 1989 to March 2003, identified 17 bites by *D. papuensis* and *D. atra* and a further five by the marble-headed whip snake, *D. olivacea*.<sup>5</sup> *D. papuensis* was not implicated in any serious envenoming<sup>5</sup> which may indicate that the venom contains only small quantities of neurotoxins or myotoxins, or toxins with weak activity. Alternatively, an inefficient biting mechanism or low venom production may result in only low quantities of venom being injected into victims.

Apart from studies on the *in vivo* toxic effects of *D. olivacea* venom,<sup>6</sup> no recently published studies on the venom from any whip snake have been located in the literature. Therefore, we examined the *in vitro* effects of the venom from *D. papuensis* at the skeletal neuromuscular junction and their neutralization by antivenoms.

## METHODS

### Venom preparation and storage

Venom was collected from a specimen of *D. papuensis* (Darwin, Northern Territory, Australia) by sliding a pipette tip over the fang and wiggling to stimulate venom delivery. The venom was filtered (20 µm) to remove any mucosal contaminants. Polyethylene materials (e.g. pipette tips, Eppendorf tubes, specimen bottles) were used to handle and contain the milking due to the strong affinity some peptides possess for glass and polystyrene. Freeze-dried venom and stock solutions of venom prepared in 0.1% bovine serum albumin (BSA) in 0.9% saline were stored at –20°C until required.

### Chick biventer cervicis nerve–muscle (CBCNM) preparation

Chickens (4–10-day-old male) were killed with CO<sub>2</sub> and both biventer cervicis nerve–muscle preparations were dissected. These were mounted under

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Received 30 June 2005; revision 2 October 2005; accepted 20 November 2005.

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1 g resting tension in 5 mL organ baths containing physiological salt solution of the following composition (in mmol/L): NaCl, 118.4; KCl, 4.7; MgSO<sub>4</sub>, 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.5; NaHCO<sub>3</sub>, 25 and glucose, 11.1. The solution was maintained at 34°C bubbled with carbogen (95% O<sub>2</sub> and 5% CO<sub>2</sub>). Motor nerves were stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator.<sup>7</sup> d-Tubocurarine (dTC) was added (10 µmol/L) and the subsequent abolition of twitches confirmed the selective stimulation of nerves. Responses to nerve stimulation were re-established by thorough washing. Contractile responses to acetylcholine (ACh; 1 mmol/L for 30 s), carbachol (CCh; 20 µmol/L for 60 s) and potassium chloride (KCl; 40 mmol/L for 30 s) were obtained in the absence of stimulation.<sup>7</sup> The preparations were then equilibrated for at least 30 min with electrical stimulation (as above) before addition of venom. In all experiments, venom was left in contact with the preparations until responses to nerve stimulation were abolished, or for a maximum of 3 h if total twitch blockade did not occur. At the conclusion of the experiment, responses to ACh, CCh and KCl were obtained as previously described. Time taken to reduce the amplitude of the nerve-mediated twitches by 50% (i.e., t<sub>50</sub>) was calculated in order to provide a quantitative measure of neurotoxicity.<sup>8</sup>

Where indicated CSL Ltd PSAV (batch number: 055513801) or TSAV (batch number: 055010201) antivenom (5–30 units/mL) was added 10 min prior<sup>9</sup> to the addition of venom (10–50 µg/mL). Reversibility of the inhibitory effects of venom was examined in additional experiments in which antivenom (5 units/mL) was added at t<sub>50</sub>. Twitch height was then monitored for a further 2 h. In experiments examining the myotoxic effects of venom, the biventer cervicis muscle was directly stimulated every 10 s with pulses of 2 ms duration at supramaximal voltage. In these experiments the electrode was placed around the belly of the muscle and dTC (10 µmol/L) 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-hour period (as above). Venom was considered to be myotoxic if it inhibited twitches elicited by the direct stimulation or caused a contracture of the skeletal muscle.<sup>7</sup>

### Determination of PLA<sub>2</sub> activity

PLA<sub>2</sub> activity of the venom was determined using a colourimetric assay kit (Cayman Chemical, Ann Arbor, MI) designed to test the activity of secretory PLA<sub>2</sub>s. This assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine which serves as a substrate for PLA<sub>2</sub> enzymes. Free thiols generated following the hydrolysis of the thio ester bond at the sn-2 position by PLA<sub>2</sub> are detected using DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid)). Colour changes were monitored using a CERES900C microplate reader (Bio-Tek Instruments, Winooski, VT) at 405 nm, sampling every minute for a 5-min period. PLA<sub>2</sub> activity was expressed as micromoles of phosphatidylcholine hydrolysed per minute per milligram of enzyme.

### Analysis of results and statistics

For isolated tissue experiments, responses were measured via a Grass force displacement transducer (FTO3) and recorded on a MacLab System. In both neurotoxicity and myotoxicity studies, twitch height was expressed as a percentage of the twitch height prior to the addition of venom or antivenom. A one way ANOVA was performed for multiple comparisons at the time point indicated, in order to determine statistical significance. Data are expressed as mean±SE with statistical significance whenever  $P < 0.05$ .

### Morphological studies

At the conclusion of the myotoxicity studies, tissues were removed from the organ bath, immediately placed in Tissue Tek, frozen in liquid nitrogen and stored at -80°C until required. Tissues were cut into transverse sections (14 µm) using a Leica Cryocut 1 800 cryostat (Leica, Wetzlar, Germany) and placed onto gelatin-coated slides. Tissue sections were post fixed for 15 min in 4% formaldehyde, stained with haematoxylin and eosin and examined under a light microscope (Olympus BH-2, Olympus Optical Co. Tokyo, Japan). Areas exhibiting pathological changes typical of myotoxicity were photographed using an Olympus C-35AD camera (Olympus Optical).

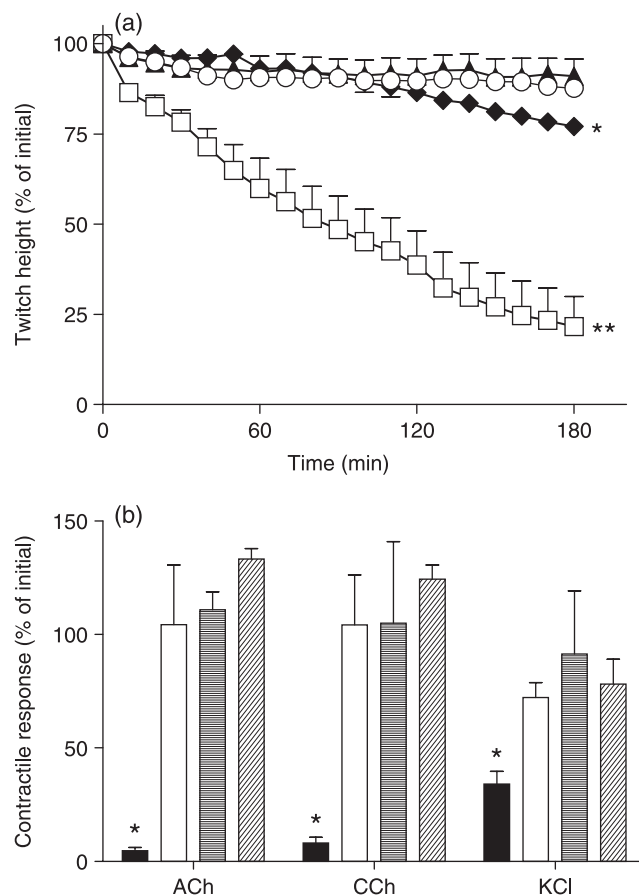
## RESULTS

### Neurotoxicity studies

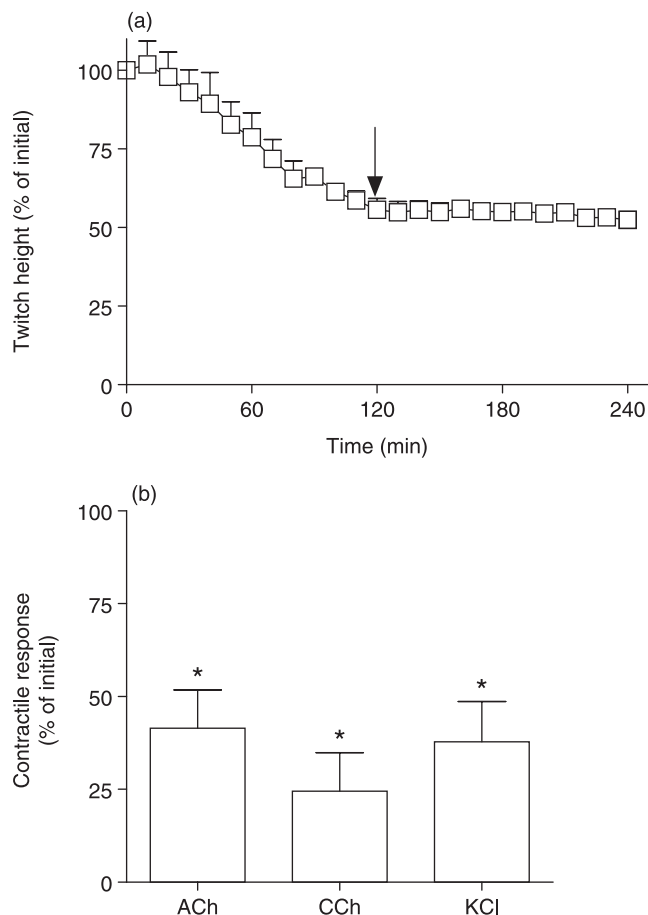
*D. papuensis* venom (10 µg/mL) caused significant inhibition of nerve-mediated twitches in the CBCNM preparation over a 3-hour period (Fig. 1a;  $P < 0.05$ ; one-way ANOVA; t<sub>50</sub> 83.5 ± 8.2 min;  $n = 4-6$ ). Venom also significantly inhibited contractile responses to exogenous ACh (1 mmol/L), CCh (20 µmol/L) and KCl (40 mmol/L) (Fig. 1b;  $P < 0.05$ ; one-way ANOVA;  $n = 4-6$ ).

Prior addition of either PSAV (5 units/mL) or TSAV (5 units/mL) prevented the venom-induced inhibition of twitches (Fig. 1a;  $P < 0.05$ ; one-way ANOVA;  $n = 4-6$ ). Although, in the presence of PSAV, the twitch height was still significantly less than the vehicle control. Prior addition of PSAV or TSAV (5 units/mL) prevented the venom-induced inhibition of the contractile responses to exogenous agonists (Fig. 1b;  $P < 0.05$ ; one-way ANOVA;  $n = 4-6$ ).

Addition of PSAV (5 units/mL), at t<sub>50</sub>, failed to reverse the venom-induced inhibition of twitch height, but prevented further inhibition of twitches. In addition, the addition of PSAV at t<sub>50</sub> did not fully restore the responses to exogenous agonists (Fig. 2a,b;  $P < 0.05$ ; paired *t*-test;  $n = 5$ ).



**Fig. 1** Effect of vehicle (i.e. BSA), venom (10 µg/mL) alone or venom in the presence of antivenom (5 units/mL) on (a) nerve-mediated twitches of the CBCNM preparation (▲, Vehicle control; ○, TSAV + venom; ◆, PSAV + venom; □, venom) or (b) contractile response to exogenous agonists. \* $P < 0.05$ , significantly different from vehicle control; one-way ANOVA. \*\* $P < 0.05$ , significantly different from venom in the presence of antivenom (5 units/mL) one-way ANOVA;  $n = 4-6$ . For (b): (■), venom; (□), PSAV + venom; (▨), TSAV + venom; (▩), vehicle control.



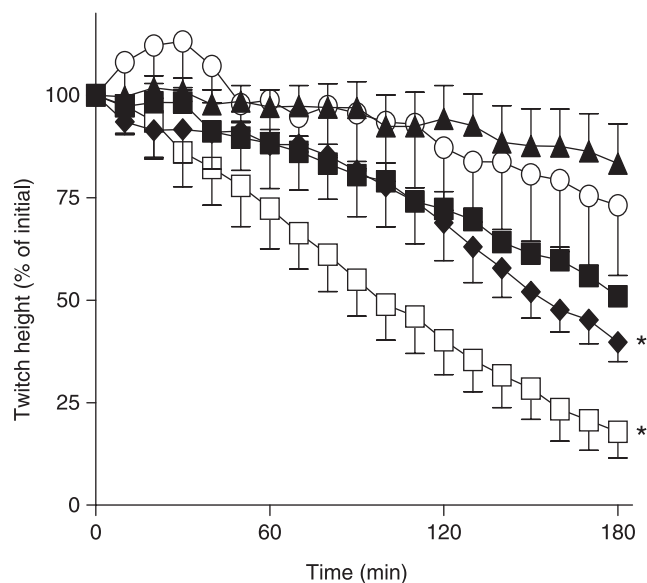
**Fig. 2** Effect of PSAV (5 units/mL) added at  $t_{50}$  (indicated by arrow) on (a) nerve-mediated twitches and (b) response to exogenous agonists of the CBCNM preparation. \* $P < 0.05$ , significantly different from the vehicle control; one-way ANOVA;  $n = 5$ .

### Myotoxicity studies

Venom caused concentration-dependent inhibition of direct twitches in the CBCNM preparation (Fig. 3;  $P < 0.05$ ; one-way ANOVA;  $n = 4-5$ ) and a transient increase in the baseline tension (data not shown). Prior addition of antivenom (30 units/mL; PSAV or TSAV) failed to inhibit the increase in baseline tension in response to venom (50  $\mu\text{g/mL}$ ; data not shown). However, prior addition of antivenom prevented the venom-induced inhibition of the twitches elicited by direct muscle stimulation, although twitch height was still significantly different compared to the vehicle control ( $n = 4-6$ ;  $P < 0.05$ , one-way ANOVA).

### Morphological studies

Light microscopy studies of the tissues exposed to venom showed concentration-dependent myotoxic effects (Fig. 4a,b). These changes included swollen cells, oedema, vacuolation and breakdown of myofibres. Prior addition of PSAV (30 units/mL) markedly reduced these morphological changes. However, slight oedema and swollen cells were still evident (Fig. 4c). Similar effects were observed in tissues pretreated with TSAV (30 units/mL; data not shown). Tissues exposed to vehicle (i.e. BSA; Fig. 4d) had no detectable morphological changes.



**Fig. 3** Effect of venom (20–50  $\mu\text{g/mL}$ ), venom (50  $\mu\text{g/mL}$ ) in the presence of PSAV or TSAV antivenoms (30 units/mL) or vehicle (i.e. BSA) on direct twitches of the CBCNM preparation. \* $P < 0.05$ , significantly different from the vehicle control; one-way ANOVA;  $n = 4-6$ . ( $\blacktriangle$ ), Vehicle; ( $\circ$ ), venom (20  $\mu\text{g/mL}$ ); ( $\square$ ), venom (50  $\mu\text{g/mL}$ ); ( $\blacksquare$ ), TSAV + venom; ( $\blacklozenge$ ), PSAV + venom.

### Phospholipase A<sub>2</sub> activity

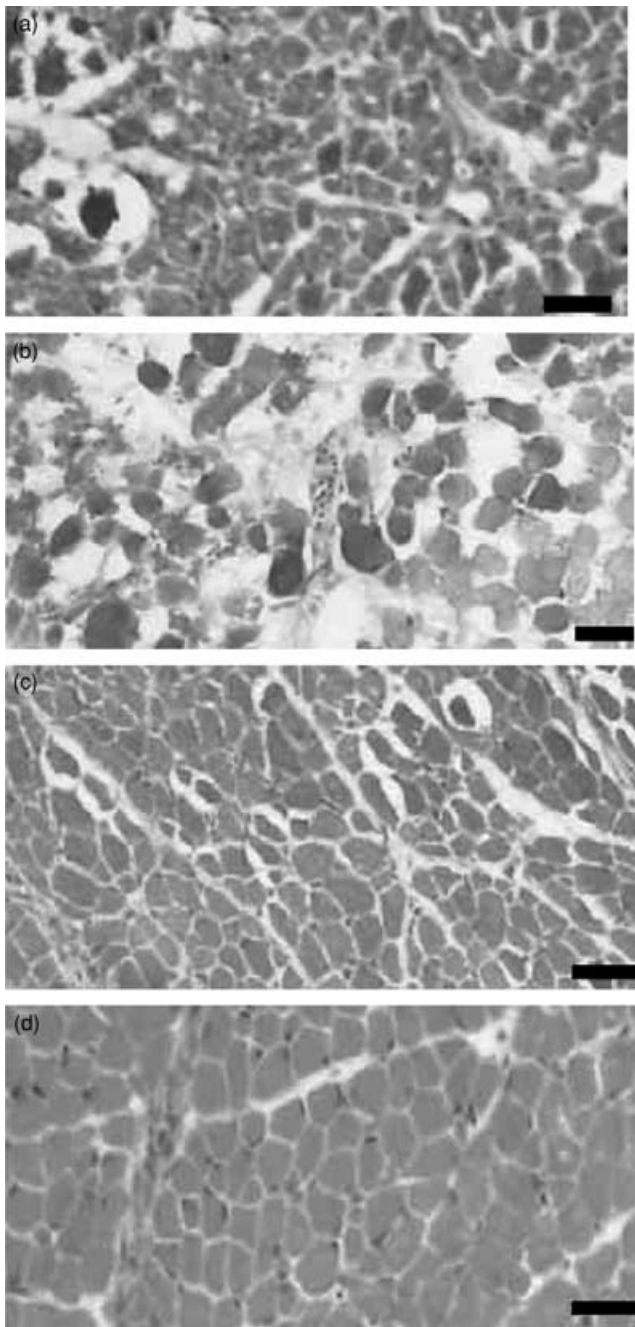
Specific activity of the whole venom was  $17.5 \pm 0.2$   $\mu\text{mol/min per mg}$  ( $n = 3$ ) while that of the positive control (i.e. bee venom) was  $312.5 \pm 26.4$   $\mu\text{mol/min per mg}$  ( $n = 3$ ).

## DISCUSSION

In the CBCNM (i.e. a skeletal muscle preparation), *D. papuensis* venom caused inhibition of nerve-mediated twitches and inhibited contractile responses to exogenous ACh and CCh indicating the presence of postsynaptic neurotoxins. However, responses to exogenous KCl were also inhibited indicating that myotoxic activity may contribute to the observed decrease in twitch height. Postsynaptic neurotoxins bind to nicotinic acetylcholine receptors with a high affinity.<sup>10</sup> Normally the presence of postsynaptic neurotoxins, which have a much quicker onset of action than presynaptic neurotoxins, mask the effects of presynaptic neurotoxins making it difficult to confirm their presence in whole venoms. The neurotoxic effect of *D. papuensis* venom appears to be fairly weak as evidenced by only 80% inhibition of twitches over 3 h and a  $t_{50}$  of  $83.5 \pm 8.2$  min. In contrast, the venom of the Papuan taipan (10  $\mu\text{g/mL}$ ) caused 100% inhibition of nerve-mediated twitches within 1 h and has a  $t_{50}$  value of  $27 \pm 3$  min.<sup>8</sup>

As venom inhibited responses to KCl, the presence of myotoxic activity in the venom was also examined. Venom caused a concentration-dependent inhibition of twitches elicited by direct stimulation of the preparation, as well as an increase in baseline tension, indicative of myotoxicity.<sup>7</sup> Myotoxic effects of the venom were further proven in light microscopy studies, which showed morphological changes (i.e. the development of areas of oedema) in the biventer muscle exposed to venom.

PLA<sub>2</sub> enzymes are found in the venom and oral secretions of snakes of all families.<sup>11</sup> Previous LCMS analysis of *D. papuensis*



**Fig. 4** Transverse sections of CBCNM preparations exposed to (a) venom (20  $\mu\text{g/mL}$ ) (b) venom (50  $\mu\text{g/mL}$ ), (c) venom (50  $\mu\text{g/mL}$ ) in the presence of PSAV (30 units/mL) or (d) vehicle (i.e. 0.1% BSA). Scale bars represent 50  $\mu\text{m}$  in all micrographs.

venom has indicated the presence of compounds of a molecular weight consistent with  $\text{PLA}_2$ . Hence, *D. papuensis* venom was assayed for  $\text{PLA}_2$  activity.<sup>4</sup> Although  $\text{PLA}_2$  activity was detected, this activity was much less than that of other Australasian elapid venoms such as death adders (ranging from  $119.8 \pm 6.2$ – $476.4 \pm 12.4$   $\mu\text{mol/min}$  per mg depending on the species).<sup>12,13</sup> This is surprising considering that myotoxic activity usually correlates with high  $\text{PLA}_2$  activity.<sup>14</sup>

The treatment of envenoming by whip snakes is normally supportive, mainly due to the lack of life-threatening symptoms

following envenoming which may be related to the low venom yields of these snakes.<sup>2</sup> However, our study suggests the presence of myotoxic and neurotoxic components in this venom. There is no specific antivenom raised against the venom of *D. papuensis*. Therefore, the efficacy of polyvalent snake antivenom and tiger snake antivenom was examined against these activities. CSL polyvalent snake antivenom is raised against the venom of five species. The minimum amount of each antivenom contained in an ampoule of polyvalent antivenom is: Brown snake (*Pseudonaja textiles*; 1 000 units), death adder (*Acanthophis antarcticus*; 6 000 units), king brown (*Pseudechis australis*; 18 000 units), taipan (*Oxyuramus scutellatus*; 12 000 units) and tiger snake (*Notechis scutatus*; 3 000 units). Tiger snake antivenom was chosen due to its recommended use in treating envenoming by a range of Australian snakes.<sup>15</sup> In addition, it is a cheaper alternative to polyvalent snake antivenom. Prior incubation of tissues with either antivenom prevented venom-induced twitch inhibition. Although the inhibitory effects were marginally better for tiger snake antivenom, the batch used in this study contained antibodies against the venoms from king Brown, taipan, death adder and Brown snakes (Keiran Ragas, CSL; personnel communication). This indicates the likelihood that the antivenoms were raised in horses that had been hyper-immunized against a range of venoms. Therefore, it was not a monovalent antivenom. However, as tiger snake antivenom was marginally more effective than polyvalent antivenom it may indicate that it is the tiger snake antibodies which are neutralizing the effects of *D. papuensis* venom.

In order to simulate a more clinically relevant situation polyvalent snake antivenom was added after the venom (i.e. at  $t_{50}$ ). Although this resulted in the prevention of further inhibition of nerve-mediated twitches, it failed to reverse the decrease in twitch height. This result may indicate a high affinity (i.e. pseudo irreversible) of the postsynaptic neurotoxins in this venom to the skeletal muscle nicotinic acetylcholine receptors. Clinicians in Australia and Papua New Guinea have used anticholinesterases in order to reduce the amount of antivenom given to patients after envenoming by elapids such as death adders.<sup>16</sup> The use of anticholinesterases may be useful under circumstances where the efficacy of the antivenom is incomplete. However, in the event of high affinity binding of venom neurotoxins to the nicotinic acetylcholine receptors, this therapy may not be efficacious.

Prior addition of either antivenom prevented venom-induced inhibition of twitches elicited by direct stimulation of the CBCNM preparation. The efficacy of both antivenoms in neutralizing the myotoxic effects was also evident upon histological examination of tissues, with the addition of antivenom prior to venom resulting in far less detectable morphological changes than venom alone.

In conclusion, *D. papuensis* venom causes *in vitro* myotoxic and neurotoxic effects. This supports the earlier LCMS analysis of this venom but is in contract to the clinical effects observed. While this study presents for the first time a basic pharmacological profile of this venom there is still scope for further investigation. In particular, examination of the potential haemotoxic effects of this venom as previously suggested,<sup>6</sup> fractionation of the venom to identify the components responsible for the above mentioned effects as well as an examination of other *Demansia* venoms for similar activity.

## ACKNOWLEDGEMENTS

SK was supported by a Monash University Postgraduate Publication Award during the writing of this manuscript.

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