

NEUROTOXIC EFFECTS OF VENOMS FROM SEVEN SPECIES OF AUSTRALASIAN BLACK SNAKES (*PSEUDECHIS*): EFFICACY OF BLACK AND TIGER SNAKE ANTIVENOMS

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SUMMARY

1. *Pseudechis* species (black snakes) are among the most widespread venomous snakes in Australia. Despite this, very little is known about the potency of their venoms or the efficacy of the antivenoms used to treat systemic envenomation by these snakes. The present study investigated the *in vitro* neurotoxicity of venoms from seven Australasian *Pseudechis* species and determined the efficacy of black and tiger snake antivenoms against this activity.

2. All venoms (10 µg/mL) significantly inhibited indirect twitches of the chick biventer cervicis nerve–muscle preparation and responses to exogenous acetylcholine (ACh; 1 mmol/L), but not to KCl (40 mmol/L), indicating activity at post-synaptic nicotinic receptors on the skeletal muscle.

3. Prior administration of either black or tiger snake antivenom (5 U/mL) prevented the inhibitory effects of all *Pseudechis* venoms.

4. Black snake antivenom (5 U/mL) added at t_{90} (i.e. the time-point at which the original twitch height was reduced by 90%) significantly reversed the effects of *P. butleri* ($28 \pm 5\%$), *P. guttatus* ($25 \pm 8\%$) and *P. porphyriacus* ($28 \pm 10\%$) venoms. Tiger snake antivenom (5 U/mL) added at the t_{90} time-point significantly reversed the neurotoxic effects of *P. guttatus* ($51 \pm 4\%$), *P. papuanus* ($47 \pm 5\%$) and *P. porphyriacus* ($20 \pm 7\%$) venoms.

5. We show, for the first time, the presence of neurotoxins in the venom of these related snake species and that this activity is differentially affected by either black snake or tiger snake antivenoms.

Key words: antivenom, elapid, king brown, mulga, neurotoxicity, neurotoxin, *Pseudechis*, venom.

INTRODUCTION

Elapid snakes form a conspicuous component of the herpetofauna of Australasia and represent the majority of snakes found in Australia. In addition, these snakes are of special significance as

the sole clad of venomous snakes capable of inflicting bites of medical importance in the region.^{1–3} The *Pseudechis* genus (black snakes) is one of the most widespread, occupying temperate, desert and tropical habitats and ranging in size from 1 to 3 m. *Pseudechis australis* is one of the largest venomous snakes found in Australia and is responsible for the vast majority of black snake envenomations. As such, the venom of *P. australis* has been the most extensively studied and is used in the production of black snake antivenom. It has been documented that a number of other *Pseudechis* from the Australasian region can cause lethal envenomation.⁴

The envenomation syndrome produced by *Pseudechis* species varies across the genus and is difficult to characterize because the offending snake is often not identified.^{3,5} However, symptoms of envenomation may include local pain, generalized itchiness, sweating, faintness, nausea, vomiting, prostration and headache.⁵ Phospholipase A₂ (PLA₂) toxins have been isolated from *P. australis*,^{6,7} *P. colletti*,⁸ *P. porphyriacus*⁹ and *P. papuanus*.¹⁰ In addition, pre-^{9,11} and post-synaptic^{6,7} neurotoxins, platelet inhibitors¹² and myotoxic,^{13–15} pro-¹⁶ and anticoagulating,¹⁷ antibacterial,¹⁸ haemorrhagic¹⁶ and necrotic⁵ activities have been demonstrated for *Pseudechis* venoms.

Recommended treatment following black snake envenomation includes application of a pressure immobilization bandage immediately after the bite and, depending on the severity of the envenomation, black snake antivenom.^{19,20} Although, tiger snake antivenom is recommended for systemic *P. porphyriacus* envenomation, it is not indicated for use in the case of *P. australis* or *P. colletti* envenomation.²¹ The pharmacological basis for the apparent cross-reactivity of tiger snake antivenom against venoms from snakes of different genera is unknown. To date, there has been no examination of the efficacy of black or tiger snake antivenom to neutralize the activity of *Pseudechis* venoms. Therefore, in the present study, we investigated the *in vitro* neurotoxicity of venoms from seven *Pseudechis* species and the ability of black or tiger snake antivenom to neutralize these effects.

METHODS

Snake collection and venom preparation

The following venoms were purchased from Venom Supplies (Tanunda, SA, Australia): *P. australis* (mulga snake) from Eyre Peninsula, *P. colletti* (Collett's snake) from central Queensland; *P. guttatus* (spotted black snake) from south-east Queensland; *P. papuanus* (Papuan black snake) from Merauke, West Papua; and *P. porphyriacus* (red-bellied black snake) from

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Received 3 June 2004; revision 3 August 2004; accepted 1 October 2004.

the Barossa Valley, South Australia. *Pseudechis butleri* (Butler's snake) from Yalgoo, Western Australia, and *P. pailsii* (eastern pygmy mulga) from Mt Isa, Queensland, were milked by BGF. Freeze-dried venoms and stock solutions of venoms were prepared in 0.1% bovine serum albumin (BSA) in 0.9% saline and stored at -20°C until required. Owing to the lack of availability of *P. pailsii* venom, this was not included in the antivenom reversal studies.

Chick isolated biventer cervicis nerve–muscle preparation

Male chicks (4–10 days old) were killed by CO_2 inhalation and exsanguination and both biventer cervicis preparations were removed.²² Tissues were mounted under 1 g resting tension in organ baths containing a physiological solution of the following composition (in mmol/L): NaCl 118.4; KCl 4.7; NaHCO_3 2.5; KH_2PO_4 1.2; MgSO_4 1.2; CaCl_2 2.5; glucose 11.1. The

physiological solution was bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 34°C . Indirect twitches were evoked by stimulating the motor nerve every 10 s with pulses of 0.2 msec duration at a supramaximal voltage⁷ using a Grass S88 stimulator (Grass Instrument Co., Quincy, MA, USA). After a 30 min equilibration period, the nicotinic receptor antagonist D-tubocurarine ($10\ \mu\text{mol/L}$) was added and the subsequent abolition of twitches confirmed the selective stimulation of the somatic nerve. Tissues were then washed thoroughly to re-establish twitches. Contractile responses to acetylcholine (ACh; $1\ \text{mmol/L}$ for 30 s) and potassium chloride (KCl; $40\ \text{mmol/L}$ for 30 s) were obtained in the absence of stimulation.²³ Electrical stimulation was then recommenced and the preparations were allowed to equilibrate for a further 30 min period before commencement of the experiment. Venom was left in contact with the preparations until complete twitch blockade occurred or for a 60 min period. At the conclusion of the experiment, contractile responses to ACh and KCl were then obtained, as described previously.

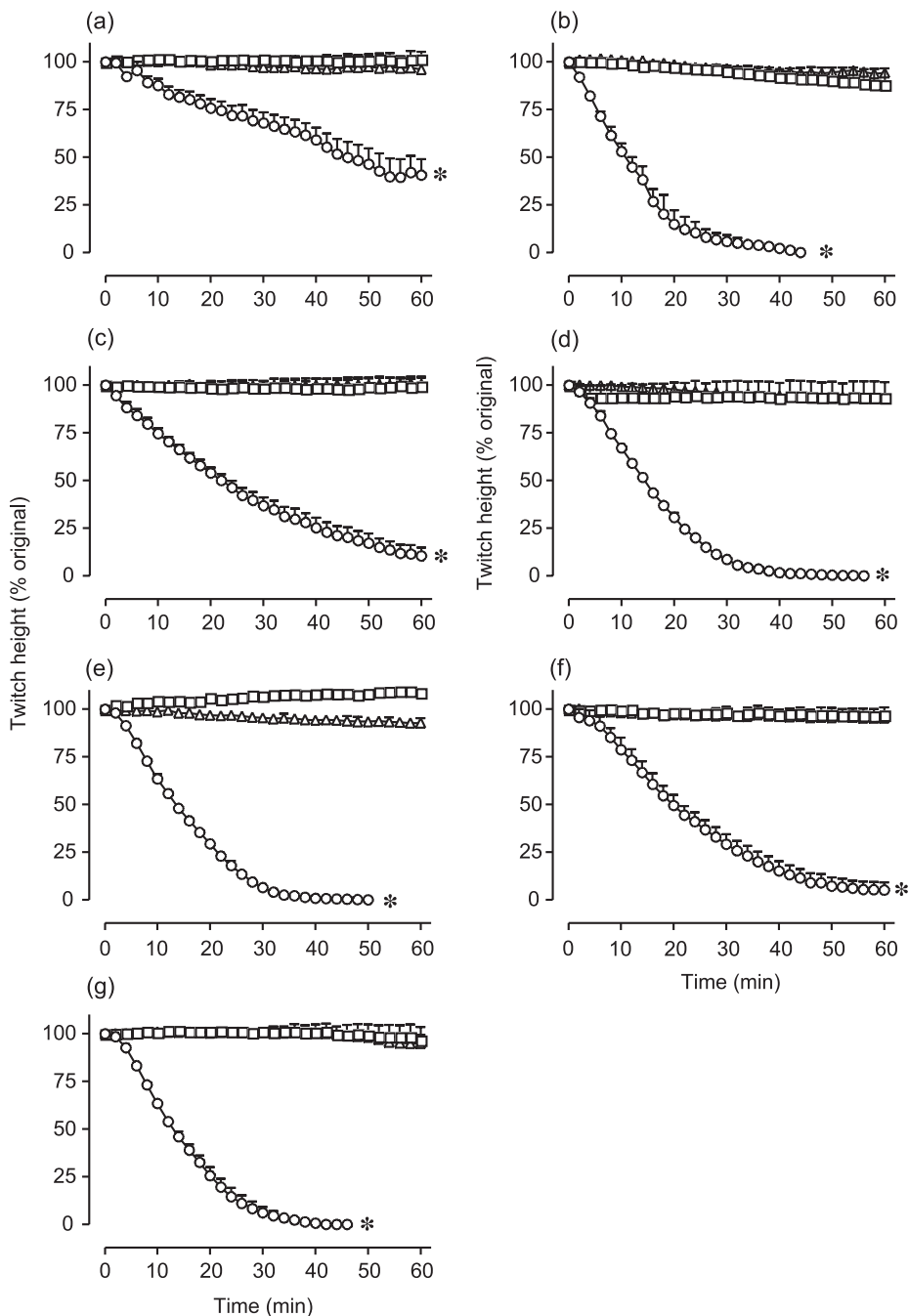


Fig. 1 Effect of prior administration of black snake antivenom (5 U/mL; $n = 3-5$; \square) or tiger snake antivenom (5 U/mL; $n = 3-4$; \triangle) on *Pseudechis* venom ($10\ \mu\text{g/mL}$; $n = 4-7$)-induced inhibition of indirect twitches in the chick biventer cervicis nerve–muscle preparation. (\circ), *Pseudechis* venom alone. (a) *Pseudechis australis*; (b) *Pseudechis butleri*; (c) *Pseudechis colletti*; (d) *Pseudechis guttatus*; (e) *Pseudechis pailsii*; (f) *Pseudechis papuanus*; (g) *Pseudechis porphyriacus*. * $P < 0.05$ compared with vehicle control (one-way ANOVA followed by a Bonferroni post hoc test).

Black or tiger snake antivenom (5 U/mL) was added 10 min prior to venom or at t_{90} (i.e. the time-point at which the original twitch height was reduced by 90%) of individual tissues and kept in contact with the tissue for the remainder of the experiment.

Ethical approval for all animal experiments was obtained from the Monash University Animal Ethics Committee.

Drugs

Acetylcholine chloride and D-tubocurarine chloride were purchased from Sigma (St Louis, MO, USA). Stock solutions were made up in distilled water. Black snake antivenom, raised against *P. australis* venom, and tiger snake antivenom, raised against *Notechis scutatus* venom, were purchased from CSL Ltd (Melbourne, Victoria, Australia).

Statistics

All statistics were performed using Sigmasat v.2.0 (Systat Software, Richmond, CA, USA). Responses are expressed as the mean \pm SEM. Data were analysed using one-way ANOVA, followed by a post hoc test (Bonferroni). In all cases, statistical significance is indicated by $P < 0.05$.

RESULTS

Effect of *Pseudechis* venoms on indirect twitches

All *Pseudechis* venoms (10 μ g/mL) produced a significant reduction of indirect twitches of the chick biventer cervicis nerve–muscle preparation ($n = 4-7$; $P < 0.05$, one-way ANOVA; Fig. 1a–g) with the following rank order: *P. butleri* \approx

Table 1 Time taken for *Pseudechis* venoms (10 μ g/mL) to produce 90% inhibition (t_{90}) of indirect twitches in the chick biventer cervicis nerve–muscle preparation

Species	n	t_{90} (min)
<i>Pseudechis australis</i>	5	ND (> 60)
<i>Pseudechis butleri</i>	5	22 \pm 3
<i>Pseudechis colletti</i>	5	56 \pm 5
<i>Pseudechis guttatus</i>	4	30 \pm 1
<i>Pseudechis pailsii</i>	6	28 \pm 1
<i>Pseudechis papuanus</i>	4	40 \pm 5
<i>Pseudechis porphyriacus</i>	4	25 \pm 2

ND, not determined.

Table 2 Effect of *Pseudechis* venoms, in the absence or presence of black snake (5 U/mL) or tiger snake (5 U/mL) antivenom, on the contractile response of the chick biventer cervicis nerve–muscle preparation to acetylcholine (1 mmol/L)

	Venom alone	Acetylcholine response (% of original)			
		BSAV (prior)	TSAV (prior)	BSAV (after)	TSAV (after)
<i>Pseudechis australis</i>	2 \pm 1*	102 \pm 5 [†]	110 \pm 7 [†]	ND	ND
<i>Pseudechis butleri</i>	0 \pm 0*	88 \pm 1* [†]	102 \pm 1 [†]	70 \pm 5* [†]	53 \pm 13*
<i>Pseudechis colletti</i>	0 \pm 0*	116 \pm 3 [†]	103 \pm 1 [†]	45 \pm 18* [†]	59 \pm 2* [†]
<i>Pseudechis guttatus</i>	1 \pm 0*	89 \pm 11 [†]	97 \pm 3 [†]	98 \pm 4 [†]	75 \pm 7* [†]
<i>Pseudechis papuanus</i>	0 \pm 0*	94 \pm 6* [†]	98 \pm 3 [†]	13 \pm 8*	66 \pm 6*
<i>Pseudechis porphyriacus</i>	0 \pm 0*	91 \pm 7* [†]	112 \pm 9 [†]	72 \pm 5* [†]	59 \pm 12* [†]
<i>Pseudechis pailsii</i>	1 \pm 0*	81 \pm 9* [†]	110 \pm 2 [†]	ND	ND
Vehicle control	102 \pm 6	–	–	–	–
BSAV alone	120 \pm 8	–	–	–	–
TSAV alone	103 \pm 5	–	–	–	–

Data are presented as the mean \pm SEM percentage of the original response. * $P < 0.05$ compared with vehicle or antivenom alone (one-way ANOVA); [†] $P < 0.05$ compared with venom alone (one-way ANOVA).

BSAV, black snake antivenom; TSAV, tiger snake antivenom. ND, not determined.

P. porphyriacus \approx *P. pailsii* \approx *P. guttatus* > *P. papuanus* \geq *P. colletti* > *P. australis* (t_{90} values are given in Table 1). All venoms produced a significant decrease in the contractile response of the chick biventer nerve–muscle preparation to ACh (1 mmol/L; $n = 4-7$; $P < 0.05$, one-way ANOVA; Table 2), with venoms from some species significantly affecting contractile responses to KCl (40 mmol/L; $n = 4-7$; $P < 0.05$, one-way ANOVA; Table 3).

Black snake antivenom (5 U/mL), tiger snake antivenom (5 U/mL) or vehicle (BSA) alone had no significant effect on either the indirect twitches (data not shown, $n = 4$) or the contractile responses of the chick biventer cervicis nerve–muscle preparation to exogenous ACh (1 mmol/L; Table 2) or KCl (40 mmol/L; Table 3).

Prior administration of the antivenom

Black snake antivenom (5 U/mL) prevented the inhibition of indirect twitches induced by all *Pseudechis* venoms (10 μ g/mL; Fig. 1a–g) and at least partially restored contractile responses to exogenous ACh (1 mmol/L) to an extent that was indistinguishable from that of the antivenom control ($n = 3-5$; $P < 0.05$, one-way ANOVA; Table 2). With the exception of *P. porphyriacus* venom, pretreatment with black snake antivenom prevented the venom-induced reduction of contractile responses to KCl (40 mmol/L; $n = 3-5$; $P < 0.05$, one-way ANOVA; Table 3). Tiger snake antivenom (5 U/mL) also prevented the inhibition of indirect twitches induced by all *Pseudechis* venoms (10 μ g/mL; Fig. 1a–g) and prevented the venom-induced attenuation of contractile responses to exogenous ACh (1 mmol/L) to an extent that was indistinguishable from that of the antivenom control ($n = 3-4$; $P < 0.05$, one-way ANOVA; Table 2). Pretreatment with tiger snake antivenom prevented the *P. papuanus* and *P. guttatus* venom-induced reduction of contractile responses to KCl, but had no significant effect on the inhibition produced by *P. butleri*, *P. colletti* or *P. pailsii* venoms ($n = 3-4$; $P < 0.05$, one-way ANOVA; Table 3).

Antivenom reversal study

Black snake antivenom (5 U/mL), added at t_{90} , partially reversed the neurotoxic effects of *P. butleri* (28 \pm 5%), *P. guttatus* (25 \pm 8%) and *P. porphyriacus* (28 \pm 10%) venoms (10 μ g/mL;

Fig. 2a) on indirect twitches compared with tissues incubated with venom alone (Fig. 1b,d,g). However, with the exception of *P. guttatus*, venom-induced attenuation of tissue responses to ACh (1 mmol/L; $n = 4$; $P < 0.05$, one-way ANOVA; Table 2) were unaffected by the addition of black snake antivenom (5 U/mL).

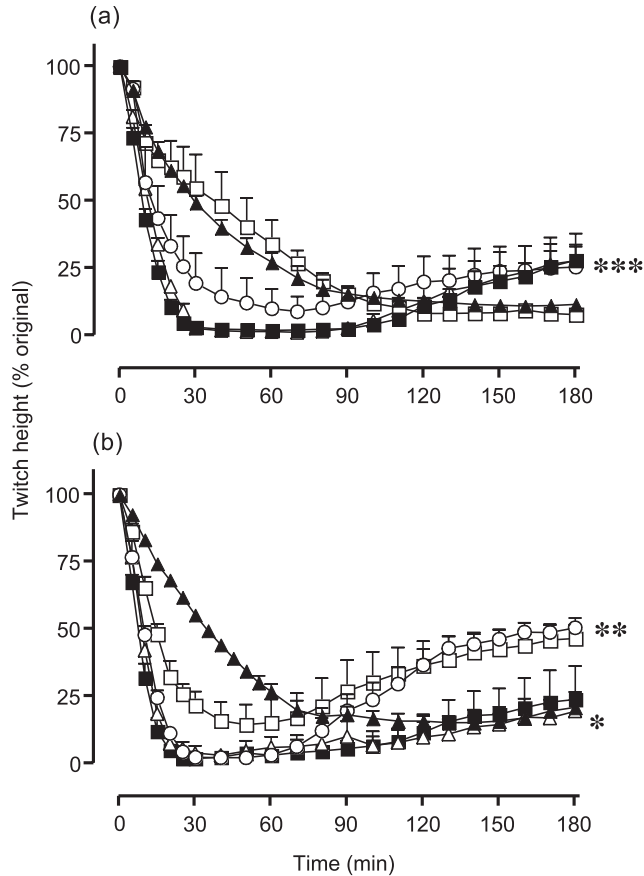


Fig. 2 Effect of (a) black snake antivenom (5 U/mL; $n = 4$) or (b) tiger snake antivenom (5 U/mL; $n = 4$), added at the t_{90} time-point, on indirect twitch inhibition produced by *Pseudechis* venoms (10 μ g/mL) in the chick biventer cervicis nerve–muscle preparation. * $P < 0.05$ compared with vehicle control (one-way ANOVA followed by a Bonferroni post hoc test). (■), *Pseudechis butleri*; (▲), *Pseudechis colletti*; (○), *Pseudechis guttatus*; (□), *Pseudechis papuanus*; (△), *Pseudechis porphyriacus*.

Reductions in contractile responses to KCl (40 mmol/L; $n = 4$; $P < 0.05$, one-way ANOVA; Table 3) remained unchanged in the presence of black snake antivenom.

Tiger snake antivenom (5 U/mL), added at t_{90} , partially reversed the neurotoxic effects of *P. guttatus* (51 \pm 4%), *P. papuanus* (47 \pm 5%) and *P. porphyriacus* (20 \pm 7%) venoms (10 μ g/mL; Fig. 2b) compared with tissues incubated with venom alone (Fig. 1d,f,g). Furthermore, tiger snake antivenom had no significant effect on the *P. butleri* or *P. papuanus* venom-induced reductions of contractile responses to ACh (1 mmol/L) and only partially restored the inhibition produced by *P. colletti*, *P. guttatus* and *P. porphyriacus* venoms ($n = 4$; $P < 0.05$, one-way ANOVA; Table 2). However, with the exception of *P. guttatus* and *P. porphyriacus*, tiger snake antivenom restored the venom-induced inhibition of contractile responses to KCl (40 mmol/L; $n = 4$; $P < 0.05$, one-way ANOVA; Table 3).

Pseudechis australis venom was not included in the antivenom reversal study because a t_{90} value was unobtainable owing to the low neurotoxicity of this venom.

DISCUSSION

The variation in the neurotoxic activity, across the range of *Pseudechis* venoms examined in the present study, suggests that Australasian black snake venoms contain neurotoxin(s) of differing potency or quantity, which may be acting to block post-synaptic nicotinic receptors. Systemic symptoms of neurotoxicity are not frequently present following Australian *Pseudechis* envenomation. This is most likely because a large number of envenomations are inflicted by *P. australis* (mulga snake), which, as indicated by the present study, has the lowest level of neurotoxic activity. Previous *in vitro* studies have suggested that phospholipase components isolated from the venom of *P. australis* have neurotoxic effects.^{6,7,13} These components are likely to contribute to the weak activity observed in the present study.

In contrast with *P. australis*, *P. papuanus* is capable of producing neurotoxicity and life-threatening paralytic symptoms in humans,¹⁶ as well as displaying neurotoxic activities in rodents.¹² Interestingly, in the present study, the venoms of *P. butleri*, *P. guttatus*, *P. pailsii* and *P. porphyriacus* were all more neurotoxic than *P. papuanus* in the chick biventer

Table 3 Effect of *Pseudechis* venoms, in the absence or presence of black snake (5 U/mL) or tiger snake (5 U/mL) antivenom, on the contractile response of the chick biventer cervicis nerve–muscle preparation to KCl (40 mmol/L)

	Venom alone	KCl response (% of original)			
		BSAV (prior)	TSAV (prior)	BSAV (after)	TSAV (after)
<i>Pseudechis australis</i>	81 \pm 10	87 \pm 8	72 \pm 12	ND	ND
<i>Pseudechis butleri</i>	86 \pm 14	77 \pm 9	65 \pm 10*	47 \pm 7*	84 \pm 11
<i>Pseudechis colletti</i>	70 \pm 12	87 \pm 15	46 \pm 5*	31 \pm 4*	51 \pm 11
<i>Pseudechis guttatus</i>	47 \pm 9*	89 \pm 8	100 \pm 6 [†]	50 \pm 8*	74 \pm 7*
<i>Pseudechis papuanus</i>	61 \pm 6*	79 \pm 11	95 \pm 5 [†]	42 \pm 14*	70 \pm 16
<i>Pseudechis porphyriacus</i>	67 \pm 11*	71 \pm 8*	96 \pm 7	79 \pm 21	40 \pm 20*
<i>Pseudechis pailsii</i>	53 \pm 17*	92 \pm 8	61 \pm 11*	ND	ND
Vehicle control	100 \pm 6	–	–	–	–
BSAV alone	97 \pm 10	–	–	–	–
TSAV alone	99 \pm 6	–	–	–	–

Data are represented as the mean \pm SEM percentage of the original response. * $P < 0.05$ compared with vehicle or antivenom alone (one-way ANOVA); [†] $P < 0.05$ compared with venom alone (one-way ANOVA).

BSAV, black snake antivenom; TSAV, tiger snake antivenom. ND, not determined.

muscle. This indicates the potential for these species to produce neurotoxicity in humans.

Previous clinical studies have indicated that tiger snake antivenom is as effective as black snake antivenom in the case of severe black snake envenomation.^{24–27} In addition, the use of tiger snake antivenom has been recommended following *P. porphyriacus* envenomation because it is cheaper and has a lower mass of equine protein per ampoule compared with that of black snake antivenom.⁵ In Papua New Guinea, snake bite requiring antivenom is best treated with CSL polyvalent antivenom, which has been shown to be clinically effective in treating the symptoms of *P. papuanus* envenomation,¹⁶ in contrast with the poor neutralization produced by tiger snake antivenom. However, the *in vitro* efficacy of tiger snake antivenom to neutralize *Pseudechis* venom has not been investigated previously.

Although the administration of antivenom prior to the addition of venom does not mimic the clinical situation, such experiments provide vital information regarding dosage and efficacy of antivenoms and are therefore still important. For example, if an antivenom given prophylactically cannot neutralize a venom at a given dose, then it is highly unlikely to be effective if administered after the addition of venom. In the present study, both black and tiger snake antivenom (5 U/mL) prevented the venom-induced reduction in indirect twitches when administered prophylactically. Preliminary studies using a lower dose of black or tiger antivenom (i.e. 1 U/mL) yielded variable neutralization of the neurotoxic effects of the *Pseudechis* venoms investigated (data not shown), indicating a dose-dependent effect. In addition, the variable neutralizing ability of the two antivenoms at the lower dose may also suggest that there are differences in the venom composition and/or potency of these related snake species.

The results from the present study show that tiger snake antivenom (5 U/mL) is as equally effective as black snake antivenom in neutralizing *Pseudechis* venom-induced neurotoxicity *in vitro* and may support its preferred clinical use in black snake envenomation,^{23–27} even though symptoms of neurotoxicity are not common following *Pseudechis* envenomation. Black or tiger snake antivenom added at t_{90} partially reversed the neurotoxic effects of some *Pseudechis* venoms. In addition, the inability of the antivenoms to partially or completely reverse the effects of the *Pseudechis* venoms may also indicate toxins that can produce irreversible damage to tissues, such as myotoxins, whose presence has been demonstrated by previous studies.^{13–15}

Results from the present study suggest that *Pseudechis* venoms contain neurotoxic activity despite the infrequency of patients presenting to hospitals with signs of neurotoxicity following envenomation. Prophylactic administration of tiger snake antivenom (5 U/mL) was as equipotent as black snake antivenom in neutralizing the neurotoxic effects of all *Pseudechis* venoms. These results support current clinical practice whereby tiger snake antivenom is the recommended treatment for severe *P. porphyriacus* envenomation considering its wider availability and the reduction in the incidence of side-effects associated with this antivenom.⁵ Alternatively, administration of either snake antivenom after the administration of venom gave varying results, suggesting that there may be other toxins (e.g. myotoxins) contained within the venom of *Pseudechis* species that cause irreversible damage to the isolated chick biventer cervicis.

ACKNOWLEDGEMENTS

We are grateful to the Commonwealth of Australia (Department of Health and Ageing) for on-going funding of the Australian Venom Research Unit and for financial assistance from the Australia and Pacific Science Foundation, Australia Geographic Society and the University of Melbourne.

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