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Coagulant effects of black snake (*Pseudechis* spp.) venoms and *in vitro* efficacy of commercial antivenom

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ABSTRACT

The coagulant effects of Australasian black snakes (*Pseudechis* spp.) are poorly understood and differ to the procoagulant venoms of most dangerous snakes in Australia. This study aimed to investigate *in vitro* coagulant effects of *Pseudechis* venoms and the efficacy of commercial black snake antivenom (BlSAV), tiger snake antivenom (TSAV) and specific rabbit anti-snake IgG to neutralise these effects. Using a turbidimetric assay, all six *Pseudechis* venoms had anticoagulant activity, as well as phospholipase A_2 (PLA_2) activity. Inhibition of PLA_2 activity removed anticoagulant effects of the venoms. *Pseudechis porphyriacus* was unique and had procoagulant activity independent of PLA2 activity. Both BlSAV and TSAV completely inhibited the coagulant and PLA2 activity of all Pseudechis venoms. PLA2 activity was also inhibited completely by p-Bromophenacyl bromide (pBPB) and partially by specific anti-*N. scutatus* IgG antibodies. Anti-*N. scutatus* IgG also completely inhibited anticoagulant activity of *Pseudechis* venom. All *Pseudechis* venoms showed immunological cross reactivity with specific anti-snake IgG antibodies to *P. porphyriacus*, *Pseudechis australis* and *Notechis scutatus*. *Pseudechis* venoms have *in vitro* anticoagulant activity that appears to be attributable to PLA_2 activity. Both antivenoms inhibited anticoagulant and PLA_2 activity at concentrations below those occurring in patients treated with one vial of antivenom. There was cross-neutralisation of *Pseudechis* venoms and *N. scutatus* antibodies that might be attributable to immunological similarities between the venoms.

1. Introduction

The *Pseudechis* genus (black snakes) is one of five groups of clinically important venomous snakes in Australia (White, 1998). *Pseudechis* spp. are widespread across Australia. The two most common and clinically significant black snakes, *Pseudechis* porphyriacus (Red-bellied black snake) and *Pseudechis australis* (Mulga snake) are found along the East Coast of Australia and throughout mainland Australia respectively (Cogger, 2000). Other members of the genus include *Pseudechis butleri* (Butler's snake), *Pseudechis colletti* (Collett's snake), *Pseudechis guttatus* (Spotted or blue-bellied black snake) and *Pseudechis papuanus* (Papuan black snake) which are less widespread in Australasia and cause far fewer bites (Isbister et al., 2006; Jansen et al., 2007).

The majority of medically important venomous Australian snakes cause a venom induced consumptive coagulopathy (VICC) that results in clotting factor deficiencies and the risk of bleeding (Isbister, 2006; Isbister et al., 2010a, b). This is not the case with *Pseudechis* spp.

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which cause a reversible anticoagulant coagulopathy (Campbell, 1984; Isbister et al., 2006; Churchman et al., 2010). However, despite extensive investigation of procoagulant toxins in Australasian elapids (Isbister, 2009) there has been limited study of the anticoagulant toxins (Madaras et al., 1983). Anticoagulant toxins have been identified in other snake venoms and many of these have been identified as phospholipase A_2 (PLA_2) enzymes (Kini, 2006). PLA_2 enzymes are present in many animals including humans and serve many biological functions. Snake venoms PLA_2 have a toxic activity (e.g. myotoxic, anticoagulant, neurotoxic) in addition to their enzymatic activity and these functions may or may not be independent (Lomonte et al., 2003; Kini, 2006). PLA_2 toxins including myotoxins, platelet inhibitors and anticoagulant toxins have been identified in some *Pseudechis* spp. but a comprehensive study of anticoagulant activity of *Pseudechis* venoms and whether this is associated with PLA_2 activity has not been undertaken (Kamiguti et al., 1994; Laing et al., 1995; Ramasamy et al., 2004; Kini, 2006).

The current clinical knowledge regarding envenoming by black snakes comes mainly from case reports and anecdotes. Effects of envenoming include systemic symptoms, myotoxicity, anticoagulant coagulopathy, and localised effects at the bite site which rarely result in necrosis (Vines, 1978; Campbell, 1984; Lalloo et al., 1994; Pearn et al., 2000; Currie, 2004; Isbister et al., 2006; Jansen et al., 2007; Churchman et al., 2010). Myotoxicity appears to be the most important clinical effect and may result in acute renal failure in some cases. The presence and importance of an anticoagulant coagulopathy is more contentious with mixed reports of its presence. From a recent large series of *Pseudechis porphyriacus* envenoming anticoagulant coagulopathy appears to be common, occurring in 61% of patients, and was characterised by a raised activated partial thromboplastin time (aPTT). This anticoagulant coagulopathy was rapidly reversed with administration of one vial of antivenom (Churchman et al., 2010). It is therefore important to characterise the coagulant effects of *Pseudechis* venoms, both anticoagulant and procoagulant, including the efficacy of antivenoms in neutralising these coagulant effects.

This study aimed to investigate the anticoagulant and procoagulant effects of *Pseudechis* venoms for a range of venom concentrations including those found in patients with envenoming. In addition it investigated the effects of black snake antivenom (BlSAV) and tiger snake antivenom (TSAV) on the anticoagulant effects.

2. Materials and methods

2.1. Venom and antivenom preparation

P. porphyriacus, *P. australis*, *Notechis scutatus* (Tiger snake) and *Oxyuranus scutellatus* (Taipan) venoms were obtained from Venom Supplies. *P. butleri*, *P. colletti*, *P. guttatus* and *P. papuanus* venoms were gifted by Monash Venom Group. Venom solutions were made to 1.0 mg/ml in 50% glycerol and stored at −20 °C until required.

CSL tiger snake antivenom (TSAV; batch 10402, expiry October 2008) and black snake antivenom (BlSAV; batch 07301; expiry July 2007) were obtained from John Hunter Hospital pharmacy. CSL antivenoms are horse derived $F(ab')_2$ immunoglobulins raised against each of the five clinically important terrestrial snake groups in Australia – *Pseudonaja* (Brown snake), *Notechis* (Tiger snake), *Oxyuranus* (Taipan), *Pseudechis* (Black snake) and *Acanthophis* (Death Adder). One unit (1U) of antivenom is defined as the amount that binds or neutralises 0.01 mg of venom from the respective snake species. Expired CSL antivenoms have been shown to remain active past their stated expiry dates and are suitable for use in *in vitro* experiments (O'Leary et al., 2009).

2.2. Chemicals and other reagents

Fresh frozen plasma was purchased from the Australian Red Cross and aliquots of 10 ml were thawed at 37 °C. Calcium (50 μL 0.4 M $CaCl_2$) was added immediately prior to the assay. Tris Buffered Saline (TBS) is tris(hydroxymethyl)methylamine 25 mM, NaCl (137 mM), KCl (3.4 mM) adjusted to pH 7.4 with HCl. Innovin® (recombinant tissue factor) was obtained from Dade. Solutions (76 mg/ml) were prepared according to the manufacturer's instructions, then diluted to 10% in TBS.

Anti-*P. australis* venom IgY was purchased from Gen-Way Biotech Inc (San Diego USA). Rabbit anti-*N. scutatus* venom IgG and rabbit anti-*P. porphyriacus* venom IgG were purchased from the Western Australian Institute of Medical Research and their preparation has been previously described in detail (Isbister et al., 2010a, b). For ELISA experiments, Greiner high-binding 96 well plates were used (#655061). Carbonate buffer used for coating is 50 mM, pH 9.5. Antibodies were biotinylated using the Pierce EZ-Link Sulfo-NHS-LC-Biotin reagent. Washing solution is 0.02% Tween 20 in phosphate buffered saline (PBS). Blocking solution is 0.5% Bovine Serum Albumin in PBS. Streptavidin-horseradish peroxidase (HRP) was purchased from Millipore/Chemicon and tetramethylbenzidine (TMB) from Sigma. p-Bromophenacyl bromide (pBPB) was obtained from Sigma. All other chemicals used were of analytical grade.

2.3. Clotting studies

Clotting studies were performed using a turbidimetric assay described elsewhere (O'Leary and Isbister, 2010). In brief, procoagulant activities were tested by adding 1:1 serial dilutions of *Pseudechis* venom (starting at 1 μg) in 100 μl TBS to wells of a 96-well microtitre plate. Clotting was triggered by adding 100 μL of recalcified fresh frozen plasma, incubated at 37 °C. Wells were mixed for 5 s and optical density at 340 nm at 37 °C was recorded immediately and at 30 s intervals in a BioTek ELx808 plate reader. Clotting time was calculated based on previous definitions as the lag time before a 0.02 increase in optical density (OD) at 340 nm from the mean of the first two values (O'Leary and Isbister, 2010). Procoagulant activity of *Pseudechis* venoms was determined as a shortening of the clotting time compared to no venom addition.

Anticoagulant activity was measured in a similar way but recombinant tissue factor (Innovin; including synthetic