Investigation of the neuromuscular effects of snake envenoming and the role of antivenom as treatment.

Nanayakkarawasam Karijjawattage Anjana Silva

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DECLARATION

Thesis including published works

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes five original papers and a review article published in peer reviewed journals and one manuscript ready for submission. The core theme of the thesis is the neuromuscular effects in snake envenoming and the role of antivenom as treatment. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Pharmacology, School of Biomedical Sciences, Monash University under the supervision of Prof. Wayne C. Hodgson, Prof. Geoffrey K. Isbister, Prof. Sisira H. Siribaddana and Dr. D.M. Sanjaya Kuruppu.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 2, 3, 4, 5 and 6, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-author(s), Monash student Y/N*
2	Antivenom for neuromuscular paralysis resulting from snake envenoming.	Published	70%: Literature search, analysis and writing of the first draft of the manuscript.	Isbister GK (C, S, W) 15%; Hodgson WC (C, S, W) 15%	No No
3	Neuromuscular Effects of Common Krait (*Bungarus caeruleus*) Envenoming in Sri Lanka.	Published	60%: Design of the study, clinical and neurophysiological data collection, data analysis, writing the first version of the manuscript.	Maduwage K (L) 2.5% Segdwick M (DC) 2.5% Pilapitiya S (DC) 2.5% Weerawansa P (DC) 2.5% Dahanayake NJ (DC) 2.5% Buckley NA (A,C) 2.5% Johnston C (L) 2.5% Siribaddana S (DC, S) 7.5% Isbister GK (C,S,A,W) 15%	No No No No No No No No No
3	Neurotoxicity in Russell's viper (*Daboia russelii*) envenoming in Sri Lanka: a clinical and neurophysiological study.	Published	60%: Design of the study, clinical and neurophysiological data collection, data analysis, writing the first version of the manuscript	Maduwage K (L) 5% Segdwick M (A) 2.5% Pilapitiya S (DC) 2.5% Weerawansa P (DC) 2.5% Dahanayake NJ (DC) 2.5% Buckley NA (A) 2.5% Siribaddana S (DC, S) 7.5% Isbister GK (C, S, A, W) 15%	No No No No No No No No
4	Neurotoxicity in Sri Lankan	Published	70%: Design of the study,	Kuruppu S (L, S) 5% Othman I (L) 2.5%	No No

	Russell's Viper (*Daboia russelii*) Envenoming is Primarily due to U1-viperitoxin-Dr1a, a Pre-Synaptic Neurotoxin.		Laboratory Experiments, Data analysis, writing the first version of the manuscript	Goode RJA (L) 2.5% Hodgson WC (C, S,A,W) 10% Isbister GK (C, S, A, W) 10%	No No No
4	Clinical and Pharmacological Investigation of Myotoxicity in Sri Lankan Russell's Viper (*Daboia russelii*) Envenoming.	Published	60%: Design of the study, Clinical data collection, Laboratory Experiments, data analysis, writing the first version of the manuscript	Johnston C (L) 2.5% Kuruppu S (S, L) 2.5% Kneisz D (L) 2.5% Maduwage K (L) 2.5% Kleifeild O (L) 2.5% Smith I (L) 2.5% Siribaddana S (DC, S) 2.5% Buckley NA (A) 2.5% Hodgson WC (C,S,A, W) 10% Isbister GK (C, S, A, W) 10%	No No No No No No No No No No
5	Cross-Neutralisation of In Vitro Neurotoxicity of Asian and Australian Snake Neurotoxins and Venoms by Different Antivenoms.	Published	80%: Design of the study, Laboratory Experiments, Data analysis, writing the first version of the manuscript	Isbister GK (C, S, W) 10%; Hodgson WC (C, S, W) 10%	No No
6	Defining the role of snake alpha neurotoxins in the neuromuscular paralysis in snake envenoming in humans.	Manuscript in preparation for submission	60%: Design of the study, laboratory experiments, data analysis, writing the first version of the manuscript.	Christofu-Armstrong BC (L) 10% Rash LD (L) 10% Hodgson WC (C, S) 10% Isbister GK (C, S) 10%	No No No No

C, concept; S, supervision; W, writing of the manuscript; L, laboratory work; DC, clinical data collection; A, data analysis

I have renumbered sections of published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] Date: 20.07.2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] Date: 21.07.2017

DEDICATION

I dedicate this thesis to

my beloved mother

and

my loving wife

for the constant encouragement,

countless sacrifices

and

for being always there with me

in good times and hard times.

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LIST OF PUBLICATIONS INCLUDED AS PART OF THIS THESIS

1. **Silva A**, Maduwage K, Sedgwick M, Pilapitiya S, Weerawansa P, Dahanayaka NJ, Buckley NA, Johnston C, Siribaddana S, Isbister GK. Neuromuscular Effects of Common Krait (*Bungarus caeruleus*) Envenoming in Sri Lanka. *PLoS Negl Trop Dis.* 2016;10 (2): e0004368.

2. **Silva A**, Maduwage K, Sedgwick M, Pilapitiya S, Weerawansa P, Dahanayaka NJ, Buckley NA, Siribaddana S, Isbister GK. Neurotoxicity in Russell's viper (*Daboia russelii*) envenoming in Sri Lanka: a clinical and neurophysiological study. *Clin Toxicol (Phila).* 2016;54 (5):411-9.

3. **Silva A**, Kuruppu S, Othman I, Goode RJ, Hodgson WC, Isbister GK. Neurotoxicity in Sri Lankan Russell's Viper (*Daboia*) Envenoming is Primarily due to U1-viperitoxin-Dr1a, a Pre-Synaptic Neurotoxin. *Neurotox Res*. 2016; 31 (1): 11-9.

4. **Silva A**, Johnston CJ, Kuruppu S, Kniesz D, Maduwage K, Kleifield, O, Smith I, Siribaddana S, Buckley N, Hodgson WC, Isbister GK. Clinical and Pharmacological Investigation of Myotoxicity in Sri Lankan Russell's Viper (*Daboia russelii*) Envenoming. *PLoS Negl Trop Dis*. 2016; 10 (12): e0005172.

5. **Silva A**, Hodgson WC, Isbister GK. Cross-neutralisation of in vitro neurotoxicity of Asian and Australian Snake neurotoxins and venoms by different antivenoms. *Toxins (Basel)*. 2016;8(10). pii: E302.

6. **Silva A**, Hodgson WC, Isbister GK. Antivenom for neuromuscular paralysis resulting from snake envenoming. *Toxins (Basel)*. 2017; 9,143. doi:10.3390/toxins9040143

7. **Silva A**, Christofu-Armstrong B, Rash LD, Hodgson WC, Isbister GK. Defining the role of post-synaptic α-neurotoxins in neuromuscular paralysis due to snake envenoming in humans. Manuscript in preparation for submission to *Nature Communications*

CONFERENCE PRESENTATIONS DURING PhD CANDIDATURE

1. **Silva A.** Biomarkers of snake envenoming: local data prompts broad applications. XXXVII Annual conference of the European Association of Poison Centres and Clinical Toxicologists, Basel, Switzerland 19th May 2017. **(Key-note address)**

2. **Silva A**., Hodgson WC, Isbister GKI. Universal antivenoms? Ability of Australian and Asian antivenoms to cross neutralise different neurotoxins. Toxicology and Poisons Network Australasia conference (TAPNA), Brisbane, Australia. 4th - 6th May, 2016 [oral presentation]

3. **Silva, A.** Investigating the neuromuscular dysfunction in Sri Lankan Russell's viper envenoming. Australian Society for Medical Research – 3rd Satellite meeting – 29th April 2016 at Hunter Medical Research Institute, Newcastle, Australia. Topic: **(Invited Early Career Speaker)**

4. **Silva A**., Kneisz D, Kuruppu S, Hodgson WC, Isbister GKI. Pharmacological characterization of the major neurotoxin and myotoxin from Sri Lankan Russell's viper (*Daboia russelii*) venom. [Oral presentation at the 14th International Scientific Conference of the Asia Pacific Association of Medical Toxicology, Perth, Australia, 2-4 December 2015] **Won the best oral presentation award**

5. **Silva A**, Maduwage K, Siribaddana S, Isbister GK. Adult snakebite patients admitted to a tertiary care centre: A cohort study. [Poster presentation at the 14th International Scientific Conference of the Asia Pacific Association of Medical Toxicology, Perth, Australia, 2-4 December 2015]

6. **Silva A**., Siribaddana S, Sedgwick ME, Maduwage K, Kuruppu, S, Buckley N, Hodgson WC, Isbister GKI. Neurotoxicity due to Sri Lankan Russell's viper envenomation is caused by a weak presynaptic neurotoxin. Oral presentation at the 2015 Annual meeting of the North American congress of Clinical Toxicology, San Francisco, USA, 9-12 October 2015. **Won the Outstanding Oral Presentation award**

7. **Silva A**., Siribaddana S, Sedgwick ME, Maduwage K, Buckley N, Isbister GKI. Indian Krait (*Bungarus caeruleus*) envenoming: A clinical and neurophysiological investigation of neuromuscular dysfunction. Clin Toxicology 2015; 53 (4): 352-353. [oral presentation at the XXXVth annual congress of the European association of poison centers and clinical toxicology, St. Julian's, Malta, 26-29 May, 2015] **Won the young investigator of the year award**

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SUMMARY

Snake envenoming is a major public health hazard in the tropics. Paralysis is a major clinical effect of snake envenoming neurotoxicity which does not appear to respond well to antivenom. Neuromuscular dysfunction in snake envenoming results from the actions of toxins that either irreversibly damage motor nerve terminals (pre-synaptic) or reversibly inhibit nicotinic acetylcholine receptors (nAChR; post-synaptic). The clinical importance of pre-synaptic versus post-synaptic neurotoxins, and whether antivenom can reverse or neutralise the toxin effects, requires further clarification.

The broad aim of the present doctoral study was to investigate the pathophysiology of neuromuscular dysfunction in human snake envenoming and the role of antivenom in treating venom-induced neuromuscular dysfunction.

To investigate the current clinical evidence for antivenom therapy for neurotoxic snake envenoming, a systematic review was conducted. A one-year cohort study of authenticated Indian Krait and Russell's viper bites in Sri Lanka was conducted with serial recording of clinical effects and single fibre electromyography. Neurotoxins were isolated from snake venoms using liquid chromatography. In vitro neurotoxicity of toxins/venoms was studied using the chick biventer cervicis nerve muscle preparation. *Xenopus* oocytes with expressed human and rat nAChR were subjected to two electrode voltage clamping to study binding of long and short chain post-synaptic (α) toxins. All toxin/venom studies included studies with antivenom.

The systematic review yielded no randomised placebo-controlled trials for 70 years indicating lack of quality clinical evidence. In the cohort study, severe paralysis occurred in 17/33 krait bite patients, requiring mechanical ventilation for up to 7 days, despite early antivenom administration binding circulating venom. Mild neurotoxicity occurred in 130/245 Russell's

viper bite patients which also did not improve with antivenom. A 13.6kDa weak pre-synaptic toxin with phospholipase A_2 activity (U1-viperitoxin Dr-1a), was identified as the major neurotoxin in Sri Lankan Russell's viper venom that accounted for the mild non-life-threatening effects in humans. The myotoxicity caused by Sri Lankan Russell's viper envenoming was mild and the causative toxins were two weak phospholipase A_2 toxins (U1-viperitoxin Dr-1a and b). Thai neuro-polyvalent, Australian polyvalent antivenom and Indian antivenom neutralised both *in-vitro* pre and post synaptic neurotoxic effects of Asian krait venoms. In further experiments there was significant cross-neutralisation by Asian and Australian antivenoms of post-synaptic and pre-synaptic neurotoxins. Short chain α-toxins were markedly less potent and more reversible on human nAChR compared to rat nAChR. Long chain toxins bound to both species nAChR with similar affinity. A review of venomic proteome and clinical data indicates that paralysis in humans following snakebite is likely to be due to Long chain α-toxins, but not Short chain α-toxins.

The ineffectiveness of antivenom is due to the irreversible pathophysiology of pre-synaptic neurotoxins, despite antivenom binding circulating venom. Post-synaptic α-neurotoxins are less likely to cause reversible neurotoxicity in humans, except with massive envenoming. All neurotoxins share common antigenic regions enabling them to be cross-neutralised.

This study demonstrated that clinical ineffectiveness of snake antivenom is not due to poor efficacy, but related to the pathophysiology of neurotoxicity. The ability of antivenoms to cross neutralise diverse neurotoxins indicates the potential for a universal neurotoxic antivenom. The study challenges the long-established idea of the clinical importance of post-synaptic toxins.

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
ARF	Acute Renal Failure
CBVNM	Chick biventer cervicis nerve-muscle preparation
CMAP	Compound Muscle Action Potential
DIC	Disseminated Intravascular Coagulation
HPLC	High Performance Liquid Chromatography
nAChR	Nicotinic acetylcholine receptor
NMJ	Neuromuscular junction
PLA_2	Phospholipase A_2
PT	Prothrombin time
RPNHD	Rat phrenic nerve hemi-diaphragm preparation
RP-HPLC	Reverse-phase high performance liquid chromatography
sfEMG	Single Fiber Electromyography
TFTx	Three-finger Toxin
VICC	Venom-induced consumption coagulopathy
WBCT20	20 Minutes Whole Blood Clotting Test
WHO	World Health Organization

CHAPTER ONE: LITERATURE REVIEW

1. Snakebite: Global picture

Envenoming due to snakebite is a common and important health issue in the tropics. There is currently no accurate estimate of the global burden of snakebite, mainly due to poor reporting (Chippaux, 2008; Warrell, 2010). Based on reported snakebites in some countries, an estimated 421,000-1,841,000 envenomings and 20,000-92,000 deaths are believed to occur annually across the globe (Kasturiratne *et al.*, 2008). The highest incidence of snakebite and mortality occurs in South Asia, South-East Asia, Sub-Saharan Africa and Latin America (Kasturiratne *et al.*, 2008).

Snakebite mostly affects rural farming communities in resource poor countries in the above regions and, for these communities, snakebite is an occupational and environmental health hazard (Williams *et al.*, 2010). Further, snakebite is considered as a disease of poverty (Harrison *et al.*, 2009). Hospital statistics from the most affected areas do not appear to represent the true burden of snakebite as many snakebite victims seek treatment from traditional practitioners rather than present to hospitals (Fox *et al.*, 2006). These factors are highly likely to collectively give less recognition to snakebite as a globally important health issue, despite its high burden. Hence, snakebite has been recognized as a Neglected Tropical Disease by the World Health Organization (World Health Organisation, 2009, 2017).

2. Venomous snakes

Of the approximately 3150 known snake species (Vidal *et al.*, 2007), nearly 2700 species belong to a relatively recently diverged group Caenophidia, or advanced snakes, who possess a complete venom delivery system or part of such system (Vonk *et al.*, 2008). These snakes belong to four families, i.e. Viperidae, Elapidae, Atractaspididae and Colubridae. Of these only about 600 species, almost exclusively belonging to the three monophyletic families Viperidae, Elapidae and Atractaspididae, are known to be potentially dangerous to humans (Vonk *et al.*, 2011). The remaining caenophidians have classically been treated within the

paraphyletic ‘catch-all’ family, Colubridae, which includes snakes largely posing no danger to humans (Vonk *et al.*, 2011). However, recent molecular studies have suggested promotion of several snake groups to the family level, which have long been classified under Colubridae (Vidal *et al.*, 2007; Vonk *et al.*, 2008). Venomous snakes inhabit a range of arboreal, aquatic, terrestrial and fossorial habitats throughout the world, except Antarctica.

The heterogenous snake family, Elapidae, contains 60 genera and approximately 300 species which includes snake groups of high medical importance such as Kraits, Cobras, Mambas, Sea snakes, Coral snakes and Australian snakes (Chippaux, 2006). This family is distributed across Asia, Africa, America and Australia, including tropical waters. Elapids possess non-movable front fangs which are usually tubular to facilitate the delivery of venom during the bite (Jackson, 2007). Further, because of the remarkable ability of these snakes to control venom delivery, sometimes no venom is delivered during a bite resulting in ‘dry bites’ (Jackson, 2003). Typically, envenoming by elapids leads to rapid and progressive neuromuscular paralysis, resulting in asphyxia due to paralysis of the respiratory muscles.

Viperids (family: Viperidae) are highly adaptable having colonized many environments including deserts. These snakes represent 33 genera and more than 230 species. Vipers have a highly effective venom apparatus with long movable/rotatable, tubular front fangs which deliver the venom at relatively high pressure from their large venom glands. The family is subdivided into two main groups; the pit vipers (subfamily: Crotalinae) are found in Asia and America and the true vipers (subfamily: Viperinae) are found only in the Old World (Africa, Europe and Asia) (Chippaux, 2006). Pit vipers possess infrared sensitive loreal pits which help the snake to track warm-blooded prey in the dark.

Although colubrids are the largest snake family in the world (family: Colubridae), and widespread across the globe, only a few species possess glands (Duvernoy’s glands) producing venom-like secretions. When present, these glands may be associated with a low

pressure delivery system that includes grooved or ungrooved, posteriorly placed, enlarged maxillary teeth (Weinstein *et al.*, 2011). This system requires the wide opening of the snake's mouth for an adequate duration in order to transfer the venom through a shallow channel during a bite. Therefore, effective venom delivery to a human during a bite is less likely in the usual brief and shallow bite by these snakes (Valenta, 2010). In general, apart from a few taxa, venomous colubrids pose little danger to humans.

The snake family Atractaspididae contains 10 genera and 70 species of primitive and burrowing snakes, which are restricted to Africa and the Middle East. Some of these species possess movable, even to the side of the mouth, front fangs enabling them to strike side-ways. Some atractaspidids have non-movable rear fangs (Valenta, 2010). Only a few species are considered dangerous to humans, with systemic envenoming potentially leading to cardiovascular and neurological disturbances, and may be fatal.

3. Snake venom

The primary purpose of venom is to enable venomous snakes to quickly neutralize or subdue prey animals (Calvete *et al.*, 2009; Casewell *et al.*, 2013). The venom components play diverse roles related to foraging such as immobilizing, paralysing, liquefying prey as well as deterring competitors (Juárez *et al.*, 2008). It is believed that the venom systems of reptiles originated from a single ancestor 200 million years ago (Fry *et al.*, 2006) and the front-fanged venom systems evolved in elapids, viperids and atractaspidids separately at different times (Jackson, 2003). Since diet plays a major role in venom evolution (Daltry, Wuster and Thorpe, 1996), subsequent diversification lead to a high variation of venom components not only among closely related taxa, but also led to variation within the same species by factors such as geographical distribution (Suzuki *et al.*, 2010), ontogeny (Andrade and Abe, 1999) and sex (Menezes *et al.*, 2006).

Snake venom is a highly complex natural secretion, a mixture of a large number of toxins and enzymes with a wide range of biological actions (Calvete *et al.*, 2009; Casewell *et al.*, 2013). The venom glands, usually located in the head of the snake, produce venom. Sophisticated, diverse and efficient delivery systems have been developed to deliver the venom from the venom glands to the prey or the victim (Jackson, 2003). Of the dry weight of venom, 90 to 95% consists of proteins and peptides, which have enzymatic activity and/or ligand binding ability in other organisms (Markland, 1998; Mackessy, 2009), and are referred to as toxins. The collective effects of these toxins result in the varying clinical effects of envenoming in animals and humans.

Structurally and functionally diverse snake venom proteins with high molecular weight show enzymatic activity on diverse substrates in target sites including components of the coagulation cascade, red blood cell membrane, neuromuscular junction, skeletal and cardiac muscles, extracellular space and vascular endothelium (Chippaux, 2006). Some snake venom enzyme groups, such as metalloproteases, depend on metal ions such as calcium or zinc for their enzymatic activity. Although enzymatic activity is related to the toxic activity of venom enzymes in most cases, the toxicity of some venom enzymes, such as certain phospholipases, may not be related to their enzymatic activity (Kini, 2003). Snake venom phospholipase A_2 (PLA_2) are present in all snake venoms to varying amounts and possess diverse properties including neurotoxic, myotoxic, cardiotoxic, haemolytic, anticoagulant, hypotensive and local tissue necrotic activity (Kini, 2003; Gutiérrez and Lomonte, 2013). Enzymes such as snake venom metalloproteinases and thrombin-like snake venom serine proteinases, which are associated with coagulopathy, haemorrhage and fibrinolysis, are more abundant in Viperid venoms (Chippaux, 2006; Mackessy, 2009; Markland and Swenson, 2013).

Snake venoms are also rich in low molecular weight polypeptide toxins without enzymatic action. These bind with high affinity to a wide range of receptors, ion channels and plasma

proteins, exerting diverse toxic effects and can lead to the immobilization of prey (McCleary and Kini, 2013). Three finger toxins, proteinase inhibitors, bradykinin-potentiating peptides, c-type lectins and related proteins, and vascular endothelial growth factors are among the better studied toxin families. Although members within each toxin family share remarkably similar primary, secondary and tertiary structures, the pharmacological properties of these differ greatly (Vonk *et al.*, 2011; Casewell *et al.*, 2013; McCleary and Kini, 2013).

Table 1: Major groups of enzymatic and non-enzymatic toxins in snake venoms.

(Kini, 2002, 2011; Yamazaki and Morita, 2004; Kang *et al.*, 2011; Arlinghaus and Eble, 2012; Bruserud, 2013; Junqueira-de-Aazevedo *et al.*, 2016; Lodovicho *et al.*, 2017; Pla *et al.*, 2017)

Toxin group	**Enzymatic activity**	**Origin**	**Major toxicities**
Phospholipase A_2	Yes	All species	Neurotoxicity, myotoxicity, cardiotoxicity, haemolysis, anticoagulation, hypotension, local tissue necrosis
L-amino acid oxidase	Yes	Crotalinae, Elapinae	Haemorrhagic, anticoagulant, platelet aggregation
Snake Venom Metalloproteinases	Yes	Crotalinae, Colubridae	Haemorrhagic
Snake venom Serine Proteinases	Yes	Crotalinae	Fibrinogenolytic

Hyaluronidase	Yes	Crotalidae, Elapidae	Local tissue necrosis, procoagulant
Three-finger toxins	No	Elapids, Viperids, Colubrids	Neurotoxicity, cytotoxicity, cardiotoxicity
Cysteine-rich secretory proteins (CRISP)	No	Elapids, Colubrids, Viperids	Relaxation of smooth muscles, Myotoxicity
C-type lectins	No	Viperids, Colubrids	Anti-coagulant, antithrombotic

4. Clinical effects of snake envenoming

A venomous snakebite results in venom being injected into a prey or victim's body through a bite wound created by the fangs of the snake (or absorption through mucous membranes during an attack by a 'spitting' snake). For an animal to be effectively envenomed, the venom must be delivered in sufficient amounts to cause local or systemic toxic features. Several factors such as the amount of injected venom, venom composition, route of venom absorption, and the body mass of the animal, determine the degree of envenoming (Chippaux, 2006; Mackessy, 2009; Valenta, 2010). In the case of humans there are further factors that influence the severity of envenoming, including first aid measures, time to antivenom administration and other therapeutic interventions. This is the reason that there is a wide range in the clinical severity of clinical envenoming in patients, from non-envenoming to severe and life-threatening envenoming. Snake envenoming also manifests in varying combinations of clinical features in humans, including local tissue damage, neurotoxicity, coagulopathy and

haemorrhage, acute kidney injury, myotoxicity, primarily depending on the composition of the venom that is being injected during the bite.

4.1 Local envenoming

Direct tissue damage and the alteration of normal physiological processes by the venom, and the fangs, at the bite site and/or the surrounding area result in local envenoming. Local envenoming is more severe in most viper and cobra bites, and at times requires surgical intervention that may cause permanent disability, such as limb amputation. Usually local envenoming initially manifests with fang marks (usually two or more), pain, erythema, oedema and bluish discolouration. With more severe local envenoming this may progress with haemorrhagic blistering, local tissue necrosis and gangrene (Chippaux, 2006; Kularatne *et al.*, 2009; Valenta, 2010). In many pit-viper bites, local envenoming can result in profound and rapidly worsening oedema that leads to the rapid rise in tissue pressure within muscle compartments and may require decompression by surgical intervention (Bucaretchi *et al.*, 2010). In addition, the oedema and fluid sequestration may cause vascular depletion and hypotension, particularly for lower limb bites.

Treatment of local envenoming and minimizing the tissue damage that occurs has always been a great challenge, despite many therapeutic approaches have been tested (Sellahewa, Gunawardena and Kumararatne, 1995; Rojnuckarin *et al.*, 2006; Chotenimitkhun and Rojnuckarin, 2008). Many survivors of snakebite can end up with varying degrees of permanent disability due to local envenoming (Gutiérrez, Theakston and Warrell, 2006).

4.2 Coagulopathy

Abnormalities in blood coagulation are probably the most common feature of systemic envenoming due to snakebite in humans, because all venomous snake families can cause disturbances in coagulation to varying degrees (Chippaux, 2006; Isbister, 2009; Warrell,

2010). In most cases snake toxins cause a consumptive coagulopathy that leads to defective blood clotting in snakebite victims and potentially life-threatening haemorrhage, particularly when vascular damage also co-exists. Although previously referred to as disseminated intravascular coagulation (DIC), or defibrination syndrome in various publications, a more appropriate term for this clinico-pathological entity is venom induced consumption coagulopathy (VICC) (Isbister, 2010). Many snake venoms, particularly those of viperids and Australasian elapids, contain potent procoagulant toxins that activate the clotting pathway at different parts of the pathway (Isbister, 2009; Maduwage and Isbister, 2014). The procoagulant toxins that cleave fibrinogen (e.g. thrombin-like enzymes and fibrinogenases) usually result in milder coagulopathy, whereas those activating clotting factors higher up the clotting pathway, such as factor X and prothrombin, cause more severe coagulopathy, as seen in envenoming by Russell's vipers, Saw Scaled vipers and Australasian elapids (Maduwage and Isbister, 2014).

Activation of the clotting pathway and rapid consumption of clotting factors, most importantly fibrinogen, leads to low concentrations or complete depletion of fibrinogen. This results in prolonged clotting times, including prothrombin time (or international normalised ratio [INR], activated partial thromboplastin time, thrombin time and decreased fibrinogen concentrations (Isbister, 2009; Maduwage and Isbister, 2014). In most cases of severe coagulopathy, the clinical features are only bleeding from the bite site. cannula sites, or any open wound, but often no detectable bleeding (Jacob, 2006). Severe gastrointestinal bleeding and intracranial bleeding will occur in some cases (Phillips *et al.*, 1988; Kularatne, 2000; Allen *et al.*, 2012). The effectiveness of antivenom therapy in treating VICC is controversial and appears to differ for different snakes/toxins (Maduwage and Isbister, 2014), whilst the effectiveness of fresh frozen plasma for treating VICC has recently been the subject of much interest (Isbister *et al.*, 2013).

4.3 Acute kidney injury

Although acute kidney injury is being recognised as a major envenoming syndrome in snake envenoming, its pathophysiology remains unclear. The kidney, being a highly vascularized organ, is highly susceptible to cytotoxic agents (i.e. direct toxic injury) and many breakdown products generated from pathological processes induced by the venom toxins at distant sites (i.e. indirect injury). On theoretical grounds, and in experimental animal models, direct toxic injury to the kidneys has been previously discussed and studied (Willinger *et al.*, 1995; Sitprija, 2006, 2008; Kanjanabuch and Sitprija, 2008). However, more recent insights into the pathophysiology have emphasized the role of thrombotic microangiopathy as a plausible cause of acute kidney injury in snake envenoming in humans (Isbister *et al.*, 2007; Malbranque *et al.*, 2008; Isbister, 2010; Isbister *et al.*, 2012) The most important clinical event related to the kidneys in a snakebite patient is acute renal failure (ARF). Clinically, this is characterized by reduced urinary output, at times leading to complete anuria, elevation of blood urea nitrogen and electrolyte imbalances often with high serum K^+ levels (Sitprija, 2006, 2008). Many viperine species, such as the Russell's viper and Saw-scaled viper, are known for their ability to cause life-threatening ARF (Myint-Lwin *et al.*, 1985; Ali *et al.*, 2004; Inho, Anetta and Urdmann, 2005). Further, development of chronic renal failure in patients who developed ARF has also been reported (Wanigasuriya, 2011; Herath *et al.*, 2012).

4.4 Neurotoxicity

Neurotoxicity is a common toxic manifestation of snake envenoming, particularly following envenoming by members of the family Elapidae and some members of the family Viperidae. Many elapids including cobras, kraits, mambas, taipans, coral snakes, death adders, tiger snakes and some sea snakes are known to cause neurotoxicity. Envenoming by several true

viper species and pit vipers species can result in neurotoxicity in humans (Chippaux, 2006; Mackessy, 2009; Ranawaka, Lalloo and de Silva, 2013).

Snakebite primarily leads to toxic effects in the peripheral nervous system, particularly the neuromuscular junction of skeletal muscle, resulting in flaccid paralysis. The severity of the flaccid paralysis can vary among envenomed patients ranging from isolated mild weakness of the eyelid muscles (clinically evident as ptosis) to life-threatening total neuromuscular paralysis that involves the respiratory muscles. Although the time scale and the severity of the neurotoxicity vary depending on the type of snake species and the degree of envenoming, the involvement of the muscle groups in descending order is described in most cases (Warrell *et al.*, 1983; Watt *et al.*, 1986; Connolly *et al.*, 1995; Kularatne, 2002; Ariaratnam *et al.*, 2008; Faiz *et al.*, 2010; Isbister *et al.*, 2012).

5. Neurotoxic snake envenoming

5.1 Clinical impact

Compared to other toxic effects of snake envenoming, neurotoxicity becomes rapidly life-threatening due to the progressive paralysis of the bulbar and respiratory muscles within a short time period following the bite (Warrell *et al.*, 1983; Kularatne, 2002; Warrell, 2010; Ranawaka, Lalloo and de Silva, 2013). Many patients complain about double vision or diplopia and paralysis of eye lid muscles (leading to ptosis) after neurotoxic snake bite. Paralysis of the extraocular muscles or external ophthalmoplegia, sometimes resulting in a prominent strabismus, is also commonly observed in patients with neurotoxic snake envenoming. Facial muscle weakness, and difficulty in swallowing, due to weakness in bulbar muscles are seen in severely envenomed patients. Paralysis of the intercostal muscles and diaphragm contribute to respiratory paralysis in patients with severe neuro-muscular paralysis (Warrell *et al.*, 1983; Theakston *et al.*, 1990; Kularatne, 2000; Kularatne, 2002; Westerström, Petrov and Tzankov, 2010; Johnston *et al.*, 2012). Paralysis decreases the ability of the lungs

to ventilate, which manifests as decreased tidal volume and variable decrease in respiratory rate, until complete paralysis results in the inability to ventilate. Clinically this is seen as hypoventilation with a rising PCO_2 and eventually a decreasing PO_2, and is referred to as type II respiratory failure. This can only be treated by ventilatory support. Paralysis of the bulbar muscles results in the inability to protect the airway, which leads to airway obstruction, apnoea, and death. Therefore, patients with neurotoxic snake envenoming need medical care that is not only capable of delivering the specific antidote, e.g. antivenom, but also with adequate facilities to secure the airway and perform mechanical ventilation, within a limited time frame. Unfortunately, this is a major challenge globally, because there is limited access to modern intensive care and mechanical ventilation, apart from a few regions such as Oceania, North America and Europe. In regions such as South Asia, South-East Asia, Sub-Saharan Africa and Latin America (i.e. regions accounting for more than 80% of the global snakebite burden) many factors prevent snakebite victims receiving adequate healthcare, reducing their chances of survival (Kasturiratne *et al.*, 2008; Alirol *et al.*, 2010). Poor farming communities in developing countries of these regions are most affected by snakebite (Harrison *et al.*, 2009; Williams *et al.*, 2010). Therefore, the collective unavailability of many critical factors such as healthcare facilities in rural areas, transport facilities and road access, capable and qualified medical staff, facilities for mechanical ventilation, and safe and effective antivenoms often prevent the snakebite victim receiving the necessary care before respiratory paralysis develops (Gutiérrez *et al.*, 2010; Warrell, 2010; Williams *et al.*, 2010). Even when the victims reach a medical care facility, a considerable number of victims require prolonged ventilator assistance, in some cases for several weeks (Kularatne, 2002). Therefore, neurotoxic snake envenoming is a serious clinical and public health challenge globally.

5.2 Neuromuscular paralysis seen in snake bite victims: clinical issues

5.2.1 Clinical sequelae of neuromuscular paralysis due to neurotoxic snake envenoming

Envenoming by Kraits (*Bungarus* species), Cobras (*Naja* species), Coral snakes (e.g. species of *Micrurus, Micruroides, Leptomicrurus*), some Australasian elapids (*Oxyuranus*, *Acanthophis* and *Notechis* species), some viperids (*Crotalus*, *Daboia*, *Vipera* species) and some sea snakes (*Laticauda, Hydrophis, Enhydrina, Lapemis* species) are generally known to cause neuromuscular paralysis in humans(Harris, 2009; Kularatne and Senanayake, 2014). Although different types/subtypes of neurotoxins are found in the venoms of different snake species, the clinical effects are remarkably similar, with most variation occurring in the degree of severity (Warrell *et al.*, 1983; Connolly *et al.*, 1995; Kularatne, 2002; Kularatne *et al.*, 2009; Faiz *et al.*, 2010; Malina *et al.*, 2011; Isbister *et al.*, 2012; Johnston *et al.*, 2012). The pattern of the involvement of different muscle groups in neurotoxic snake envenomed patients is generally in descending order. The paralysis usually starts from eyelid and external eye muscles, then proceeds to facial muscle paralysis, followed by bulbar muscle involvement, then involves the diaphragm and intercostal muscles, and finally the peripheral muscles including limb muscles. The recovery usually occurs in reverse order (Theakston *et al.*, 1990; Connolly *et al.*, 1995; Warrell, 2010). The neuromuscular paralytic effects of snake envenoming are commonly clinically evident at least a few hours after the bite and may last for 3-4 weeks in patients who develop severe neuromuscular paralysis (Kularatne, 2002; John *et al.*, 2008; Prakash, Mathew and Bhagat, 2008; Hung, Du Thi and Hojer, 2009; Isbister *et al.*, 2012; Dayal *et al.*, 2014). Clinically evident long-term neurological features have not been observed. However, nerve conduction abnormalities in patients one year after the snakebite have been reported. These patients had no residual effects in neuromuscular junction transmission (Bell *et al.*, 2010).

The reported case fatality rate of neurotoxicity ranges from 4 to 37%, depending on the facilities available for managing patients with neurotoxicity (Ranawaka, Lalloo and de Silva, 2013). Although there are many clinical studies describing neurotoxicity due to snake

envenoming, precise descriptions of the time scale course of the appearance and the recovery of the clinical features of neurotoxic envenoming are extremely rare.

5.2.2 Treatment of neuromuscular paralysis in snake bite victims: the role of antivenom and AChE inhibitors.

Despite the fact that antivenom has been recommended as the mainstay for treating snakebite patients globally (Warrell, 2010), the effectiveness of antivenom in reversing neuromuscular paralysis in the clinical setting is inconclusive (Ranawaka, Lalloo and de Silva, 2013). In studies favouring the usefulness of antivenom, the heterogeneity and subjectivity of the clinical parameters used and the confounding effect of natural recovery questions the generalizability of the results. A controlled clinical trial of a new antivenom raised against the many banded krait (*Bungarus multicinctus*) in Vietnam revealed the antivenom-treated group had significantly lower durations of ptosis, limb paralysis and respiratory paralysis, over the control group. However, the control used in this study was a historical control group, i.e. from a previous study, limiting the applicability of the results.

There are some reports of recovery from neuromuscular paralysis due to snakebite, without antivenom treatment (Pochanugool, Wilde and Jitapunkul, 1997; Hung, Du Thi and Hojer, 2009). Of 54 patients who had severe neuromuscular paralysis, including respiratory paralysis, due to many banded krait (*Bungarus multicinctus*) bites, and who were not treated with antivenom, 50 recovered with mechanical ventilation and supportive therapy (Hung, Du Thi and Hojer, 2009). Further, antivenom has reportedly been ineffective in reversing the neurotoxic effects due to envenoming by death adder (Johnston *et al.*, 2012), kraits (R. Theakston *et al.*, 1990; Kularatne, 2002; Pillai *et al.*, 2012), Papuan taipan (Connolly *et al.*, 1995) and rattlesnake species (Richardson *et al.*, 2007). Therefore, it is difficult to conclude whether antivenom is useful in reversing neurotoxic effects due to snake envenoming. A

randomized trial showed that antivenom was ineffective in treating the paralysis from Philippine cobra envenoming (Watt *et al.*, 1989)

AChE inhibitors (AChEI), such as neostigmine, have been tested as supportive therapy in treating snakebite induced neuromuscular paralysis. Several studies have documented the potential benefits of AChEI, mainly for bites caused by Philippine cobra (Warrell *et al.*, 1983; Watt *et al.*, 1986, 1989; Lee *et al.*, 2004). However, neostigmine has been shown to be ineffective in treating the neuromuscular paralysis in krait bite victims (Anil *et al.*, 2010; Pillai *et al.*, 2012). Before administration of neostigmine, initial testing with a short-acting AChEI, such as Tensilon, to predict the effectiveness of neostigmine has also been suggested (R. Theakston *et al.*, 1990).

5.2.3 Tools used to study the neuromuscular paralysis in clinical setting.

Clinical neurological features

Clinical descriptions of neuromuscular paralysis due to snakebite are largely dependent on clinical observation. A major weakness of most of the available clinical studies on neurotoxic envenoming is the reliance on subjective clinical features making it difficult for comparisons between studies. Many studies have described the presence or absence of clinical features in neurotoxic snake envenoming, rather than the grading of clinical features (Watt *et al.*, 1986; Phillips *et al.*, 1988; R. Theakston *et al.*, 1990; Kularatne *et al.*, 2009; Johnston *et al.*, 2012). Careful and frequent documentation of the clinical features in an objective manner would be useful in understanding the time course of the weakness of different muscle groups.

Neurophysiological studies

Clinical features of neuromuscular paralysis are evident in patients, when the defects in neuromuscular junction transmission are severe enough. However, many events occur in neuromuscular junctions at the subclinical stages of the neuromuscular paralysis.

Neurophysiological studies are useful in better defining the abnormalities of neuromuscular junction transmission with a high sensitivity, and facilitate a better understanding of the pathophysiology.

Repetitive nerve stimulation in patients bitten by Papuan taipans showed initial high compound muscle action potential (CMAP) soon after tetanic stimulation (i.e. compared to resting state), followed by rapid decremental responses compared to the rest. These deficits did not respond to intravenous edrophonium, suggesting severely depleted vesicles in the pre-synaptic terminal (Connolly *et al.*, 1995). A similar pattern of decremental response from 3Hz repetitive nerve stimulation, as well as a reduction of CMAP, has been observed in Indian Krait bite victims with neuromuscular paralysis (Singh *et al.*, 1999).

Conventional electromyography has been used to study neuromuscular paralysis in snake envenoming to assess the extent of paralysis as well as treatment outcomes (Watt *et al.*, 1986; Singh *et al.*, 1999; Bell *et al.*, 2010). A reduction in the median-elicited CMAP has been observed in Indian Krait bite victims and thus electromyography was suggested as being useful in studying Krait venom induced paralysis (Singh *et al.*, 1999). In Papuan taipan bite victims, CMAP correlated well with the clinical progression and recovery of the neuromuscular paralysis, further suggesting the usefulness of the test (Connolly *et al.*, 1995).

Single-fibre electromyography (sfEMG) is an extremely sensitive test in assessing the function of neuromuscular junctions in-situ (Stalberg, Stålberg and Trontelj, 1997; Padua *et al.*, 2000; Stålberg and Sanders, 2009). This test is considered to be useful in diagnosing pre-synaptic neuromuscular conditions such as Lambert-Eaton myasthenic syndrome (Oh and Ohira, 2013), as well as post-synaptic conditions such as myasthenia gravis (Valls-Canals *et al.*, 2003; Padua *et al.*, 2014), with high sensitivity. sfEMG identifies action potentials from individual muscle fibres, rather than the whole muscle, and measures neuromuscular ‘jitter’ (i.e. irregularity of the transmission across the neuromuscular junction), which is the most

sensitive measure of neuromuscular transmission in-vivo (Stålberg and Sanders, 2009). Despite its high sensitivity to neuromuscular junction transmission, the use of sfEMG in clinical studies of neurotoxic snake envenoming is surprisingly rare. In the only such study reported so far, Connolly et al (Connolly *et al.*, 1995) observed markedly increased jitter and blocking in the sfEMG of three patients bitten by Papuan taipans, during the recovery phase.

5.3 Pathophysiology of neuromuscular paralysis

Toxins from many venomous animals, including snakes, target the neuromuscular junction, disturbing transmission and resulting in paralysis. This unique adaptive trait, shared by many evolutionarily distinct animals, provides an opportunity for the venomous animal to quickly neutralize the target animal, for the purpose of forage or defence (Harris and Goonetilleke, 2004; Harris, 2009). The primary target of the vast majority of neurotoxic venoms is the neuromuscular junction of the skeletal muscles, while smooth muscles are largely unaffected (Harris, 2009; Ranawaka, Lalloo and de Silva, 2013).

5.3.1 Neuromuscular junction

The neuromuscular junction (NMJ) is a specialized synapse between the nerve fibre and muscle fibre. The motor cortex governs the excitation of muscles through motor nerves, and the NMJ serves as the final relay station between the motor neuron and the target muscle. Therefore, given its high functional importance, the NMJ is vulnerable to many natural toxins (Harris, 2009). Figure 1 shows the structure of the NMJ and the potential target sites for snake venom toxins.

The motor nerve terminals have 20 to 100 branches and, of these, the terminal 30nm of the neurolema is demyelinated. Usually, each single nerve terminal branch innervates a single muscle fibre. All muscle fibres that are innervated by the branches of a motor nerve terminal

form a motor unit. The nerve terminal branches contain voltage gated K^+ and Na^+ channels which regulate the depolarization of the neurolema (pre-synaptic membrane).

Acetylcholine (ACh), the principal neurotransmitter at the skeletal muscle NMJ, is synthesized and stored within the vesicles arranged close to the neurolema of the nerve terminal branch near the release sites. These areas, called active zones in the pre-synaptic membrane, have a large number of voltage-gated Ca^+ channels and, with depolarization, these channels open resulting in an influx of Ca^+ into the axoplasm. This triggers the exocytotic release of ACh following fusion of the vesicles with the pre-synaptic membrane. The area of the sarcolema facing the pre-synaptic membrane forms clefts by invagination. These release sites are positioned over the synaptic clefts of the post-synaptic membrane (motor end plate), facilitating ACh molecules reaching their target site, usually within a few milliseconds. There is a high concentration of acetylcholine esterase (AChE), which hydrolyses ACh, within the synaptic clefts. About 50% of the released ACh is either hydrolyzed by AChE, or reabsorbed, before reaching the target site.

The post-synaptic membrane, which is a specialized area in the sarcolemma, forms secondary synaptic folds, which are concentrated at the target site of ACh, the nicotinic acetylcholine receptor (nAChR). In these areas, nAChR concentration is approximately 20,000μm^{-2}, which is 1000 times higher than in the rest of the sarcolemma (Hirsch, 2007). The nAChR is a transmembrane protein with five subunits surrounding a central pore which functions as a non-selective ion channel (Figure 1). In vertebrates, nAChRs are formed with different combinations of α1- α7, β1- β4, δ, ε and γ subunits, that contribute to the pentamer. The adult type muscle nAChR is composed of two α1, one β1, one δ and one ε subunits. In the immature (foetal) type nAChR, the ε subunit is replaced by a γ subunit (Nirthanan and Gwee, 2004; Albuquerque *et al.*, 2009). There are two agonist binding sites, which are hydrophobic pockets and are located between the α and δ subunits, and α and ε/γ subunits (Albuquerque *et*

al., 2009). The binding site at the α/δ interface is a high affinity binding site for agonists. Once both the binding sites of the nAChR are occupied by two ACh molecules or agonist molecules with affinity for this receptor, the resulting conformational change leads to the opening of the central pore, allowing an influx of Na^+ and Ca^{2+} and efflux of K^+. The change in membrane potential then results in the opening of voltage-gated Ca^+ channels. This leads to depolarization of the sarcolemma resulting in initiation of the muscle fibre contraction process (Albuquerque *et al.*, 2009).

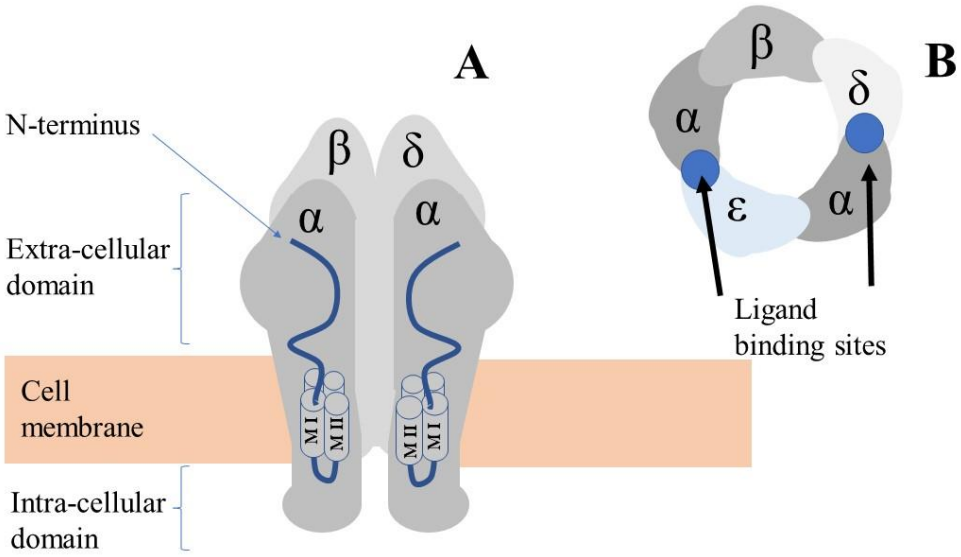

Figure 1: Structure of the adult type muscle nicotinic receptor (nAChR). A: nAChR is a pentamer of transmembrane homologous subunits arranged in the stoichiometry of 2α1: 1β1: 1δ: 1ε subunits. Subunit ε is not shown for the clarity. Each subunit has four helical transmembrane domains (M I-M IV) and M II domain of all five subunits line the central pore. The axis of the central pore is arranged perpendicular to the cell membrane. B: Top view of the receptor showing the two ligand binding sites. This figure was adopted from Nirthanan and Gwee, 2004.

5.3.2 Snake venom neurotoxins and their actions at the neuromuscular junction (NMJ)

Snake venom neurotoxins interfere with neurotransmission by acting on a range of target sites in the NMJ. Two sites in the NMJ are the major targets of most snake venom neurotoxins: the pre-synaptic nerve terminal and the nAChR.

Snake venom toxins acting pre-synaptically

Pre-synaptically acting neurotoxins have been isolated from many elapids and viperid snakes, and are important with regard to clinically evident neurotoxicity. Snake venom pre-synaptic toxins belong to group I and II phospholipases A_2 (PLA_2), a group of Ca^{2+}-dependent enzymes, abundant in the biological world. These snake venom PLA_2s possess similar molecular structure despite their diverse pharmacological actions which include pre-synaptic neurotoxicity, post-synaptic neurotoxicity, myotoxicity, hypotension, cytotoxicity, and anticoagulant effects. The PLA_2 activity of these toxins may, or may not, play a role in the pharmacological action of the toxin (Harris and Scott-Davey, 2013). As common to all secretory PLA_2s, the primary structure of these toxins is a single polypeptide of 115 to 125 amino acid residues with a molecular weight of 13 to 15 kDa (Harris and Scott-Davey, 2013). PLA_2s may appear as covalently linked multimers of up to six subunits. The classic example of a snake venom pre-synaptic neurotoxin is β-bungarotoxin, originally isolated from the venom of the many banded krait (*Bungarus multicinctus*) (Strong *et al.*, 1976). β-Bungarotoxin is a heterodimer, having covalently bound group I PLA_2 and a Kunitz-type proteinase inhibitor (Harris and Scott-Davey, 2013) . Notexin, a pre-synaptic toxin from Australian tiger snake (*Notechis scutatus*) venom, is a single polypeptide, whereas textilotoxin, a presynaptic toxin from Australian brown snake (*Pseudonaja textillis*) venom, is a hexamer(Harris, 2009). Some pre-synaptic toxins are also myotoxic.

The terminal part of the motor neurons, particularly the terminal boutons, is not protected with a blood-axonal barrier or a perineurium. This makes the pre-synaptic membrane more susceptible to pre-synaptic neurotoxins (Harris and Scott-Davey, 2013). The exact

mechanisms by which pre-synaptic toxins initiate their actions are not clear. However, it appears as though these toxins do not have an identifiable receptor in the pre-synaptic membrane to which they bind (Harris, 2009). Dixon and Harris (Dixon and Harris, 1999) demonstrated the depletion of synaptic vesicles followed by destruction of the motor nerve terminals of murine skeletal muscles exposed to β-bungarotoxin. Further, they demonstrated that the re-innervation process of the muscle fibres takes place 3 to 5 days after exposure to the toxin and, by 7 days, re-innervation is complete. Hydrolysis of phospholipids in the pre-synaptic membrane is believed to be the primary mode of action of the pre-synaptic PLA_2 neurotoxins, followed by the massive influx of Ca^{2+} into the motor nerve terminal. The increased Ca^{2+} is likely to provide the optimum condition for PLA_2 activity. The combination of these two events is likely to lead to exocytosis of the affected nerve terminals, with no endocytosis, leading to a depletion of synaptic vesicles in the nerve terminal (Dixon and Harris, 1999; Pungercar and Krizaj, 2007; Rigoni *et al.*, 2008; Harris, 2009; Harris and Scott-Davey, 2013). It has been shown that snake venom PLA_2 neurotoxins, once they have entered the motor nerve terminal, specifically bind to the mitochondria in the axoplasm, triggering depolarization and a subsequent change of morphology from an elongated shape to a swollen and rounded shape. This action has been shown to be a result of a specific action of snake venom PLA_2 neurotoxins on the permeability transition pores (PTP) in the mitochondrial membrane. Due to this, impairment of the mitochondrial activity is likely to further damage the motor nerve terminal (Rigoni *et al.*, 2008). This mechanism is evidently shared by at least the group Ia snake venom PLA_2s because notexin, taipoxin and textilotoxin all exert these effects, causing a similar pathophysiology at the pre-synaptic nerve terminal. In addition, the ability of snake venom PLA_2 neurotoxins to open the mitochondrial PTPs has been shown to be proportional to their relative neurotoxic potencies, indicating that this mechanism is likely to play a major role in the sustained inhibition of neurotransmission (Rigoni *et al.*, 2008). Interestingly, ammodytoxin A, a class IIa neurotoxic PLA_2 with pre-synaptic activity, isolated

from long nosed viper (*Vipera ammodytes ammodytes*) venom, induced neither damage to the pre-synaptic membrane nor significant depletion of synaptic vesicles at murine motor nerve terminals, suggesting a possible marked difference in the action of pre-synaptic neurotoxins compared to elapids (Logonder *et al.*, 2008). However, similar to the elapid pre-synaptic PLA_2s, ammodytoxin A induces swelling of mitochondria in the nerve terminals, which has been postulated to cause de-energization of the nerve terminal by inducing mitochondrial degeneration resulting in failure of ACh release (Logonder *et al.*, 2008).

In *in vitro* nerve-muscle preparations, particularly in mouse hemi-diaphragm nerve-muscle preparations, some pre-synaptically acting snake venom PLA_2 neurotoxins, such as β-bungarotoxin, crotoxin and taipoxin, exhibit a classic 'triphasic response'. This is particularly prominent when the safety factor of the neuromuscular junction is reduced by increasing Ca^{2+} concentration in the physiological salt solution, or increasing the frequency of indirect stimulation (Rowan, 2001; Hodgson, Dal Belo and Rowan, 2007). This triphasic response includes an initial transient inhibitory phase in twitch height, a prolonged facilitatory phase, followed by a progressive inhibitory phase, which lasts until the complete abolition of twitches. The first two phases are independent of PLA_2 activity, and the third phase is PLA_2 dependent (Rowan, 2001). However, the initial inhibitory phase is not induced by notexin or paradoxin.

Snake venom toxins acting post-synaptically

Most snake venom toxins that act post-synaptically belong to the Three Finger Toxins (TFTx) family. These toxin molecules possess three loops projecting out from a hydrophobic core. However, α-neurotoxins and TFTx are not synonymous. The TFTx family includes polypeptides with 60-72 residues which possess no enzymatic activity. These polypeptides have 4 to 5 disulfide bonds, four of which are essentially common to all members of this family (Kini, 2002). TFTx are flat molecules in their tertiary structure. The classic examples

of these toxins are α-bungarotoxin from many banded Krait venom and erabutoxin-b from the venom of the sea snake, *Laticauda semifasciata* (Barber, Isbister and Hodgson, 2013). Although most TFTx are curare-mimetic toxins, here are other toxin groups including muscarinic toxins, fasciculins and κ toxins that also belong to the TFTx family. Therefore, TFTx exhibit diverse pharmacological properties such as neuromuscular paralysis, effects on nerve terminals, myotoxicity, hypotensive effects, inhibition of enzymes such as AChE, and platelet aggregation (Hodgson and Wickramaratna, 2002; Kini, 2002; Nirthanan and Gwee, 2004)

Many TFTx, from diverse venomous animal groups, block neurotransmission by high affinity and selective binding to, and antagonism of the nAChR in the motor end plate (Nirthanan and Gwee, 2004). Collectively these are called "α-neurotoxins" or "curare-mimetic toxins" and these mimic the pharmacological action of d-tubocurarine but with a 15-to 20 fold higher affinity and poor reversibility (Nirthanan and Gwee, 2004; Barber, Isbister and Hodgson, 2013). Elapid venoms, including sea snakes, commonly contain α--neurotoxins.

Short-chain and long-chain α-neurotoxins *and their actions*

The TFTx with curare-mimetic activity are broadly categorized into two groups, depending on their molecular size. Short-chain curare-mimetic TFTx have 60–62 amino acids with four disulfide bonds whereas long-chain curare-mimetic TFTx have 66–74 amino acids with five disulfide bonds (Kini, 2002).

Short chain α-neurotoxins, typically those isolated from elapid venoms, have a similar amino acid sequences with cysteine residues in 3 and/or 4 positions. The notable exception is Pt syntx-1 and Pt syntx-2 isolated from Australian brown snake venom, which possess an amino acid sequence markedly different from other elapid α-neurotoxins (Barber, Isbister and Hodgson, 2013). Many long-chain TFTx share similar sequences with the short-chain TFTx's.

The extra disulfide bond of the long-chain TFTx is situated in loop 2, between Cys-30 and Cys-34 (Endo and Tamiya, 1991; Nirthanan, 2013).

Under normal physiological conditions, to achieve the conformational change that results in the opening of the ion channel, two agonist molecules need to bind to the two agonist sites located at the interfaces of the extracellular domains of α/δ and α/ε or γ subunits of the nAChR. The amino acid residues 185–196 of the α subunit are known to be the major determinants for the binding sites of the nAChR (Neumann *et al.*, 1986). Different agonists and antagonists may show some preference for one binding site over the other. Irrespective of their structural differences, both long- and short-chain α-neurotoxins bind to similar sites on the muscle type nAChR [$(\alpha)_2\beta\delta\gamma$] with the same affinity, despite differences in their functional sites (Servent *et al.*, 1997; Antil, Servent and Menez, 1999; Antil-Delbeke *et al.*, 2000). However, short-chain α-neurotoxins bind to the nAChR 6 to 7 times faster and dissociate 5 to 9 times faster than long-chain α-neurotoxins (Chicheportiche *et al.*, 1975). α-neurotoxins display a variety of types of interaction with the nAChR including reversible, pseudo-irreversible and irreversible binding in neuromuscular preparations. Long-chain α-neurotoxins are known to be generally irreversible compared to short-chain toxins and the reason for this has been attributed to the relative high proportions of hydrophobic amino acids found in the primary structure of the long-chain α-neurotoxins (Hodgson and Wickramaratna, 2002; Kini, 2002; Nirthanan and Gwee, 2004; Barber, Isbister and Hodgson, 2013). In addition, long-chain α-neurotoxins bind to the neuronal α7nAChR with high affinity, primarily because of the fifth disulfide bond (Servent *et al.*, 1997; Antil-Delbeke *et al.*, 2000). Since both binding sites on the nAChR need to be occupied by the agonist to lead to conformational change and the opening of the ion channel, even one binding site occupied by an α-neurotoxins will cause dysfunction leading to neuromuscular block. α-neurotoxins bind perpendicularly to the axis of the nAChR, with the 2nd loop ‘plugged’ into the binding site at

the interface between the two subunits. In addition, the C terminal and also the first loop may make contact with the receptor surface to facilitate binding (Nirthanan and Gwee, 2004).

Well known examples of long-chain α-neurotoxins are α-bungarotoxin from *Bungarus multicinctus* venom, and α-cobratoxin from *Naja kaouthia* venom. A well-known short-chain α-neurotoxin is Toxin-α from *Naja pallida* venom (Barber, Isbister and Hodgson, 2013).

Snake venom neurotoxins acting on other targets

There are several types of neurotoxins that act differently to the conventional pre-synaptic and post-synaptic toxins. Some elapid venoms possess κ toxins, which are dimeric TFTx with five disulfide bonds and bind to the neuronal $\alpha_3\beta_4$nAChR with high affinity. They do not bind to the muscle type nAChR (Kini, 2002). Muscarinic toxins, which were originally isolated from the venoms of African mambas (*Dendroaspis* sp.), exert agonist or antagonist activity on muscarinic AChR. These toxins are structurally similar to short-chain α-neurotoxins (Servent and Fruchart-Gaillard, 2009; Servent *et al.*, 2011). Fasciculins inhibit AChE in the neuromuscular junction, allowing ACh to accumulate. These toxins also have been isolated from the venoms of mamba species (*Dendroaspis* sp.). Once formed, the high affinity complex of fasciculin-AChE is very slow to dissociate (Marchot *et al.*, 1997). Dendrotoxins, isolated from the venom of several African black mamba species, block the voltage-gated K^+ channels in the nerve terminals resulting in continuous neurotransmitter release at vertebrate neuromuscular junctions. These toxins, when injected into the central nervous system, similarly facilitate neurotransmitter release (Anderson and Harvey, 1988; Harris, 2009).

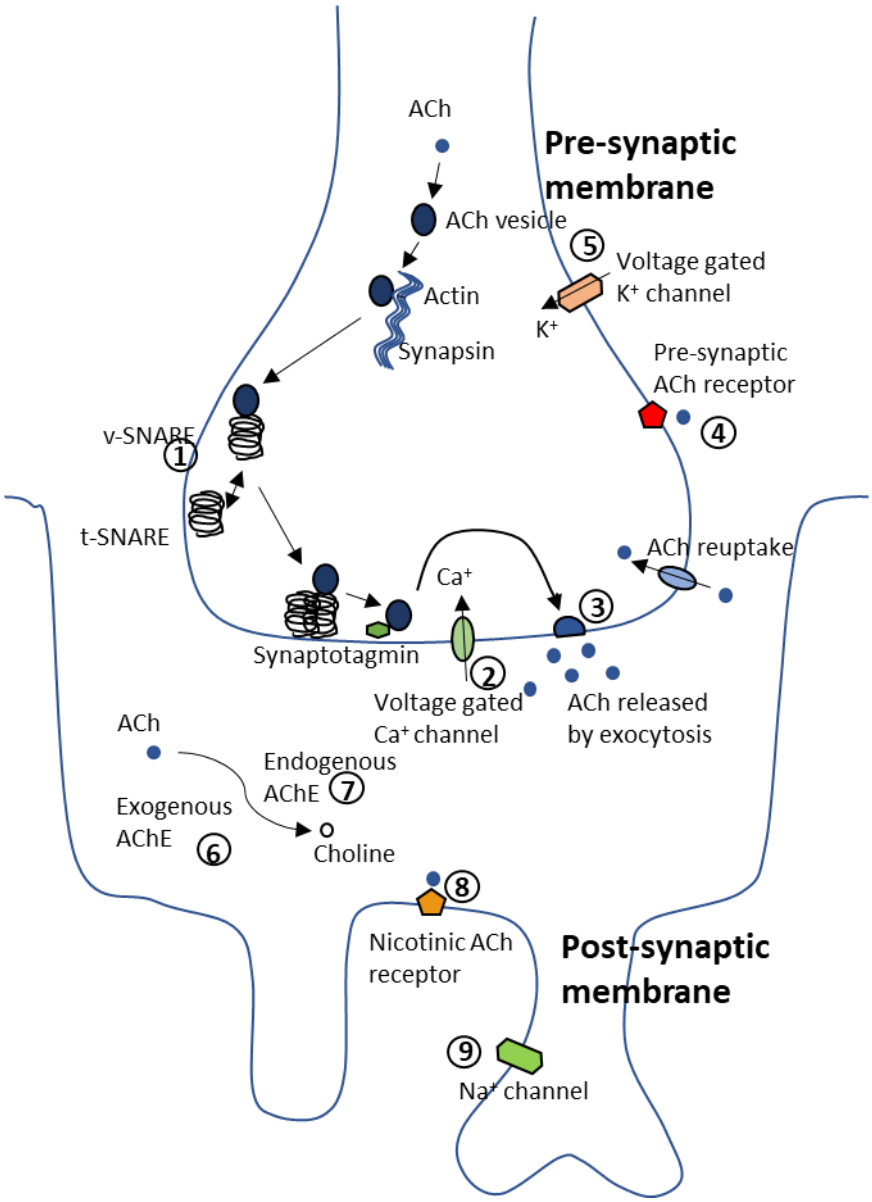

Figure 2: Sites of action of snake neurotoxins at the neuromuscular junction. Schematic representation of the neuromuscular junction showing different sites of action of snake neurotoxins.

(1) Synaptic vesicular proteins: β -bungarotoxin (*Bungarus* spp.), taipoxin (*O. scutellatus*); (2) Voltage-gated calcium channel: calciseptine (*Dendroaspis* spp.); (3) Pre-synaptic membrane: phospholipase A_2 toxins.; (4) Pre-synaptic ACh receptor: candoxin (*Bungarus candidus*); (5)Voltage-gated potassium channels: dendrotoxins (*Dendroaspis* spp.); (6) Acetylcholine: Lysis by exogenous acetylcholinesterase (AChE) in snake venom: cobra venom (*Naja* spp.); (7)AChE: Inhibitors of endogenous AChE in snake venom: fasiculins (Dendroaspis spp.); (8) Post-synaptic ACh receptors: α-bungaratoxin (*Bungarus* spp.), candoxin (*B. candidus*), azemiopsin (*A. feae*), waglerin (*T. wagleri*); (9) Voltage-gated sodium channels: Snake toxins: crotamine (*Crotalus* spp.).

Adopted from Ranawaka et. al. (2013)(Ranawaka, Lalloo and de Silva, 2013)

5.3.3 Isolation of neurotoxins from snake venoms

Isolation of neurotoxins from snake venoms is usually carried out using chromatographic techniques. Liquid chromatographic techniques such as size exclusion chromatography (SEC), reverse-phase high performance liquid chromatography (RP-HPLC) and ion-exchange chromatography are commonly used for this purpose. One chromatographic technique repeated a number of times or a combination of different techniques can be used to isolate neurotoxins from snake venoms depending on their chemical properties and abundance in the venom. Neurotoxins with similar pharmacological properties usually share similar structural (e.g. molecular weight, amino acid sequence) and physiochemical properties (e.g. charge, hydrophobicity, etc). Therefore, when the same HPLC column and method is used, the approximate elution times of these neurotoxins can be predicted (Barber, Isbister and Hodgson, 2013). For isolation of short-chain α-neurotoxins, multiple runs of reverse-phase HPLC can be used whereas a combination of size-exclusion chromatography and ion-exchange chromatography is often used to isolate long-chain α-neurotoxins (Kuruppu *et al.*, 2005; Kornhauser *et al.*, 2010; Barber, Isbister and Hodgson, 2013; Hart *et al.*, 2013). Pre-synaptic neurotoxins are commonly isolated using a combination of size-exclusion and reverse-phase or ion exchange chromatographic techniques (Huang and Lee, 1984; Gowda, Schmidt and Middlebrook, 1994; Tsai, Lu and Su, 1996; Barber, Isbister and Hodgson, 2012).

5.3.4 Pharmacological characterization of neurotoxins

In vitro pharmacological experiments, using isolated nerve-muscle preparations from different animal species, are a long-standing method to characterize snake venom neurotoxins. In common, this technique measures the effects of neurotoxins on the twitch response to indirect (i.e. motor nerve) electrical stimulation of a skeletal muscle preparation mounted in physiological salt solution. The most widely used nerve-muscle preparations, for the purpose

of characterizing snake venom neurotoxins, have been the isolated chick biventer cervicis nerve-muscle preparation and rat diaphragm-phrenic nerve preparation (Harvey *et al.*, 1994; Hodgson and Wickramaratna, 2002). Both preparations are mounted in an organ bath system containing physiological salt solution under resting tension, and the motor nerve is electrically stimulated with supramaximal voltage at regular intervals, normally every 10 s with rectangular pulses of 0.2 s duration (Hodgson and Wickramaratna, 2002; Hart, Isbister and Hodgson, 2013; Rusmili *et al.*, 2014). The diaphragm, along with the phrenic nerve, is dissected from mice/rats and the biventer cervicis muscles, with the nerve enclosed in the tendon, are isolated from young chicks. The chick biventer preparation has the advantage of possessing both focally- and multiply-innervated muscle fibres. The focally-innervated muscle fibres can be stimulated electrically and the multiply-innervated fibres can be stimulated by adding exogenous nicotinic agonists such as carbachol. This provides the opportunity to distinguish between the pre-synaptic and post -synaptic effects of neurotoxins (Hodgson and Wickramaratna, 2002). These *in vitro* tests are also useful in testing the efficacy of different antivenoms against neurotoxic activities of snake venoms and purified neurotoxins (Ramasamy, Isbister and Hodgson, 2004; Kuruppu *et al.*, 2005; Ramasamy, Fry and Hodgson, 2005)

Some studies have used the signs of neuro-muscular paralysis, such as hind limb paralysis seen in rodents during lethality experiments, as a way of demonstrating neurotoxicity (Jayanthi, Kasturi and Gowda, 1989; Maung-Maung-Twin *et al.*, 1995; Venkatesh *et al.*, 2013). However, this technique is highly subjective and non-specific in predicting the neurotoxic effects of venoms and neurotoxins.

6. Neuromuscular effects of snake envenoming and the role of antivenom as treatment: issues which require addressing

6.1 Clinical data with better resolution and authentication on the paralysis.

To understand the pathophysiology of snakebite-induced neuromuscular paralysis in humans, clinical studies should provide sufficient clinical details with accurate species identification and detailed, time-related descriptions of objective clinical parameters, to relate clinical data with experimental studies.

Snake venoms display remarkable variability, even resulting in different clinical outcomes for patients bitten by the same species of snake from different geographical populations. For example, Russell's viper (*Daboia russelli*) bites frequently cause clinically evident neurotoxicity in Sri Lanka and South India, but this has not been observed for the rest of its distribution across South Asia (Warrell, 1989). Due to this high variability, proper case authentication with accurate identification of the species involved in envenoming is essential in clinical toxinology. Proper case authentication includes evidence of an actual bite, collection of the animal specimen causing the bite and expert identification of the animal (a taxonomist or biologist), or if there is no animal specimen available, detection of specific venom antigens in the patient serum (Isbister, 2002). Many clinical studies on snake envenoming focus mainly on the clinical description and little attention is paid to accurate identification of the snake involved. Some important detailed clinical reports on neurotoxic snake envenoming have included cases authenticated using less reliable methods of snake identification such as "identified by the patient", "identified by medical staff" and "assumed based on the clinical signs of envenoming", or even not mentioning the method of authentication (Hung *et al.*, 2002; Kularatne, 2002; Bawaskar and Bawaskar, 2004; Khandelwal *et al.*, 2007; Hung, Du Thi and Hojer, 2009; Malina *et al.*, 2011). Since many neurotoxic snake groups such as kraits and cobras have several morphologically similar

species, and there are also non-venomous snakes which mimic the colour and behavioural patterns of venomous snakes within the same geographical areas (Wuster, 1996; Donnelly and Crother, 2003), proper case authentication helps to determine the clinical picture of envenoming by individual neurotoxic snake species.

Understanding the onset, progression and resolution of each feature of neurotoxic envenoming, would help in elucidating the pathophysiology of neuromuscular paralysis in snakebite victims. This is particularly important in distinguishing whether the victim has predominantly pre-synaptic or post-synaptic features of envenoming. Clinical case reports are usually detailed enough to understand the time course of the clinical features of neurotoxic envenoming (Sharma *et al.*, 2002; Khandelwal *et al.*, 2007; Faiz *et al.*, 2010; Gawarammana *et al.*, 2010; Vir *et al.*, 2010; Sodhi *et al.*, 2012; Malina *et al.*, 2013; Dayal *et al.*, 2014). However, single cases lack generalizability compared to clinical studies involving large number of patients, such as cohort studies and clinical trials. One major weakness with most of the published cohort studies and clinical trials describing neurotoxicity is that these studies generally describe the prevalence of each clinical feature within the cohort, with little or no description of the time course of individual clinical features (Theakston *et al.*, 1988; Kularatne, 2000; Kularatne, 2002; Hung, Du Thi and Hojer, 2009; Kularatne *et al.*, 2009; Isbister *et al.*, 2012; Johnston *et al.*, 2012).

It is difficult to eliminate the subjectivity of most clinical features of envenoming, because these are primarily observations made by individual clinicians. This subjectivity hinders our ability to compare the clinical description across different studies. This could be minimized by grading and describing the clinical features using a standardised method. For example, the occurrence of ptosis has been described as merely 'ptosis' in several studies (Watt *et al.*, 1988, 1989; Bucaretchi *et al.*, 2006; Hung, Du Thi and Hojer, 2009; Kularatne *et al.*, 2009; Johnston *et al.*, 2012), whilst this outcome could be graded as complete or partial ptosis to

indicate the severity. Occurrence of respiratory muscle paralysis is a critical point in the clinical status of the neurotoxic envenomed patient. Many studies have stated the occurrence of 'respiratory paralysis', without describing the clinical parameters they have been used in determining this outcome (Watt *et al.*, 1986, 1988, 1989; Kularatne, 2002; Bawaskar and Bawaskar, 2004; Hung, Du Thi and Hojer, 2009; Kularatne *et al.*, 2009; Faiz *et al.*, 2010; Johnston *et al.*, 2012). It could be assumed that this might indicate the point at which mechanical ventilation is required to sustain the life of the patient. However, it is solely a clinical decision based on an individual patient's overall status, hence may be difficult to generalize. In contrast, using objective parameters like maximum voluntary ventilation (using a spirometer) could be used in clinical studies to better define the respiratory function of the patient.

As previously discussed, neurophysiological techniques with high sensitivity, such as sfEMG, would be highly appropriate in clinical studies examining snakebite neuromuscular paralysis, to enable better documentation of the neuromuscular paralysis both clinically and sub-clinically. Although many venomous snakes can potentially lead to neurotoxic envenoming in humans, only a few species actually cause a significant number of envenomings across the globe. Unfortunately, most of the global burden of neurotoxic snake envenoming occurs in rural agricultural communities in the developing world (Kasturiratne *et al.*, 2008; Harrison *et al.*, 2009; Alirol *et al.*, 2010; Gutiérrez *et al.*, 2010; Williams *et al.*, 2010; Ranawaka, Lalloo and de Silva, 2013). Even within the developing world, snakebite is likely to be given a low priority in healthcare research because the commonly affected are mostly rural, poor and politically disadvantaged populations (Williams *et al.*, 2010). Leaving aside inaccessibility to adequate healthcare facilities including antivenoms in these areas, poor clinical reporting and, even if clinically reported, a lack of supporting laboratory experimental data to complement the clinical data, means there is a continued cycle of poor knowledge. In contrast, over the past decade, proper clinical reporting backed by appropriate quality laboratory data has

revolutionized our knowledge of the pathophysiology and treatment outcome of snakebite envenoming in developed settings such as Australia (Isbister *et al.*, 2008, 2013; Isbister, Duffull and Brown, 2009; Chaisakul *et al.*, 2010; Blacklow *et al.*, 2011; Allen *et al.*, 2012; Barber, Isbister and Hodgson, 2012; Isbister *et al.*, 2012; Isbister and Brown, 2012; Johnston *et al.*, 2012). Therefore, to improve the clinical management of snakebite victims, good quality clinical studies together with experimental studies on venom and the pathophysiology of envenoming is essential.

6.2 ***Role of pre- and post-synaptic toxins in snakebite-induced neuromuscular paralysis in humans.***

Many snake species that do not cause neurotoxicity in humans still have neurotoxins, particularly post-synaptic neurotoxins, in their venoms. Black snakes, at least all the species other than Papuan black snake (Lalloo *et al.*, 1994), cause neurotoxic envenoming in humans (Isbister *et al.*, 2006; Jansen *et al.*, 2007; Churchman *et al.*, 2010). However, the crude venoms of all the known black snakes show potent post-synaptic neuromuscular inhibition in chick biventer nerve-muscle preparations (Ramasamy, Fry and Hodgson, 2005; Hart, Isbister and Hodgson, 2013). Furthermore, the venoms of five of the black snake species that are non-neurotoxic to humans are actually more potent on the chicken tissue based on the time taken to induce 90% inhibition of the twitch height (Ramasamy, Fry and Hodgson, 2005). In addition, it was shown that avian nerve-muscle preparations are more susceptible to all black snake venoms, compared to murine preparations (Hart, Isbister and Hodgson, 2013). Similarly, further observations have been made using purified short-chain post-synaptic toxins from red-bellied black snake (*Pseudechis porphyriacus*) and Collett's black snake (*P. colletti*) venoms indicating different susceptibilities of nAChR from different animals (Hart *et al.*, 2013). The venom from Collett's black snake was shown not to affect the human nAChR although it binds to the avian nAChR (Hart, Scott-Davey and Harris, 2008). Nerve-muscle

preparations from frogs were resistant to several α-neurotoxins, compared to chick and mouse preparations (Lee, Chang and Chen, 1972). Studies on the binding of labelled α-bungarotoxin (long-chain) and labelled erabutoxin b (short-chain) with the nAChR from human, chimpanzee and mouse NMJ revealed that erabutoxin b does not bind to chimpanzee or human nAChR, but binds to mouse nAChR, whilst α-bungarotoxin binds to nAChR of all species tested (Ishikawa *et al.*, 1985). Furthermore, four non-labelled short-chain α-neurotoxins (i.e. erabutoxin b, toxin b from olive sea snake venom, toxin α from *Naja pallida* venom and toxin a from Stoke's sea snake venom) were unable to inhibit the binding of labelled α-bungarotoxin with human and chimpanzee nAChR, even at very high concentrations. However, these toxins were able to inhibit the binding of labelled α-bungarotoxin with mouse nAChR. In contrast, the same study showed that 3 unlabelled long-chain α-neurotoxins (i.e. Toxin b from yellow lipped sea krait venom, toxin c from Stoke's sea snake venom and α-bungarotoxin) concentration-dependently inhibited binding of labelled α-bungarotoxin to nAChR of human, chimpanzee and mouse origin. Therefore, this suggests that human and chimpanzee nAChR are potentially resistant to short-chain α-neurotoxins, compared to long chain α-neurotoxins, whilst mouse nAChR are susceptible to both types of toxins. Similar comparisons made between human and torpedo nAChR revealed comparatively unsuccessful binding of erabutoxin A (short-chain α-neurotoxin) with the human nAChR(Vincent, Jacobson and Curran, 1998).

Cobra species are traditionally believed to cause neurotoxicity in humans, predominantly via post-synaptic toxins (Watt *et al.*, 1986; Ranawaka, Lalloo and de Silva, 2013). This has primarily been assumed based on detailed clinical reports of envenoming by species like *Naja philippinus* (Philippine cobra) which indicate the rapid onset of severe neuromuscular paralysis, positive responses for AChEI (Watt *et al.*, 1986, 1988, 1989), as well as the isolation of several long- and short-chain α-neurotoxins from cobra venoms (Barber, Isbister and Hodgson, 2013). In 38 patients bitten by the Philippine cobra, the median time of onset of

neurotoxic features was 1 hour and the median time of onset of respiratory paralysis was 78 min, with some patients having respiratory paralysis 10-30 min after the bite (Watt *et al.*, 1988). Similarly, rapid development of neuromuscular paralysis has been described in cape cobra (*Naja nivea*) bite patients (Blaylock, Lichtman and Potgieter, 1985). However, clinical reports on neurotoxic envenoming by other species of cobras indicate neuromuscular paralysis of comparatively late onset (compared to *N. philippinus* and *N. nivea*) in humans. According to published case reports, the time gap between the bite and approximate time of the onset of neurotoxic features was comparatively higher in Thai cobra (*Naja kaouthia*) bites (Bernheim *et al.*, 2001) and Egyptian cobra (*Naja haje*) bites (Warrell, Barnes and Piburn, 1976), indicating possible presence of pre-synaptic toxins in some cobra venoms. Furthermore, clinical reports of envenoming by several other species of cobras, such as Black spitting cobra (*Naja nigricollis*) and Mozambique spitting cobra (*Naja mossambica*), do not report the occurrence of neurotoxicity (Warrell *et al.*, 1976; Tilbury, 1982). Envenoming by species such as the common cobra (*Naja naja*) and Chinese cobra (*Naja atra*) do not lead to neurotoxicity in most cases (Kularatne *et al.*, 2009; Wong *et al.*, 2010). Therefore, clinical evidence is insufficient to conclude that the neurotoxicity seen in humans due to cobra bites is primarily post-synaptic in origin.

Collectively, these studies suggest that the post-synaptic toxins, at least the short-chain toxins, are selectively toxic towards certain animal species. Further, these studies raise serious doubts about the importance of post-synaptic toxins in neuromuscular paralysis in humans.

6.3 Issues on antivenom in treating neuromuscular dysfunction in snake envenoming.

The failure of antivenoms to reverse, or prevent, neuromuscular paralysis from snake envenoming has often been attributed to geographical variations in the snake venoms leading to poor efficacy of the antivenom (Phillips *et al.*, 1988; Theakston *et al.*, 1990; Kularatne,

2002). In agreement with such clinical observations, poor neutralisation of the neurotoxic effects of Malaysian krait venoms by Thai monovalent and polyvalent antivenoms has been observed *in vitro* (Rusmili *et al.*, 2014). In contrast, some experimental studies suggest the ability of heterologous antivenoms to cross-neutralise different snake venoms (Leong *et al.*, 2012; Kornhauser *et al.*, 2013). While displaying diversification at the finer level, both α-neurotoxins and pre-synaptic neurotoxins have retained many key structural elements within the two groups (Kini, 2003, 2011; Harris and Scott-Davey, 2013; Jackson *et al.*, 2013). This suggests a possible, larger cross neutralisation within pre- and post-synaptic toxin groups, by antivenoms. A monoclonal antibody raised against toxin α (i.e. short-chain α-neurotoxin from *Naja nigricollis* venom) was able to recognize the functional site of several short-chain snake neurotoxins (Trémeau *et al.*, 1986). Therefore, it is necessary to study the cross-neutralisation ability of the commercially available heterologous antivenoms in neutralising different neurotoxins and neurotoxic venoms.

It is important to evaluate the existing clinical evidence for the antivenom as a treatment for neurotoxic snake envenoming. The question of whether the antivenom is effective in treating neurotoxic snake envenoming ideally needs to be addressed by randomized placebo controlled clinical trials. Since antivenom has been the standard treatment for snake envenoming throughout the world, it may not be practical on ethical grounds to test through a placebo controlled trial. However, in the absence of randomized placebo-controlled trials, even well designed detailed cohort studies with clinical and neurophysiological approaches would provide vital information on the effectiveness of antivenom. Therefore, a thorough review of the existing clinical literature may answer some of the questions associated with antivenom therapy.

6.4 Differential susceptibility of animals towards snake α-neurotoxins: Are rodents good models for the study of neurotoxic envenoming in humans?

Several animal species, including the mongoose and hedgehog, are known for their resistance against snake venom α-neurotoxins. It has been shown that aromatic amino acids at positions 187 and 189 of the ligand binding site of the α-subunit of nAChR are critical for binding with α-bungarotoxin. Interestingly, these resistant species, have evolved with non-aromatic amino acid residues in the two key positions, in order to acquire resistance against post-synaptic toxins (Barchan *et al.*, 1992, 1995). Interestingly, humans also have non-aromatic amino acid residues at these sites, similar to resistant species, while cat, shrew and mouse all have aromatic amino acid residues (Barchan *et al.*, 1995). In addition, the α-bungarotoxin binding site is conserved across the nAChR of all elapids (Takacs, Wilhelmsen and Sorota, 2000).

Based on the above evidence, it could be assumed that the short-chain toxins are unlikely to affect human nAChR whereas long-chain toxins should partially affect human nAChR. However, several weaknesses of these previous studies prevent us from arriving at this conclusion directly. Only a limited number of post-synaptic toxins have been used in these studies hence the ability to generalize across all venomous snake groups is difficult. In particular, the post-synaptic toxins from Australasian elapids have not been tested in these studies. Apart from α-bungarotoxin, the toxins used in these studies have originated from the venoms of snakes on which clinical data of envenoming in humans is lacking. Hence, future studies should be designed to overcome the above weaknesses.

Characterization of neurotoxic venoms, neurotoxins and testing the ability of the antivenoms to bind with these toxins are carried out using experimental animals or animal tissue. Lethality studies, the median lethality dose (LD_{50}) and the median effective dose (ED_{50}) with rodents are the current recommended standard assay tests for pre-clinical testing of antivenoms (World Health Organisation, 2010). These tests only look at the ability of the venom to kill rodents within a given time period and the ability of the antivenom to prevent death, irrespective of the cause of death. Given that rodents are more susceptible to α-

neurotoxins, and that α-neurotoxins have a much more rapid onset of action compared with pre-synaptic toxins, it could be assumed that α-neurotoxins are the most important toxins involved in causing death in these assays. With the growing evidence that snake venom α-neurotoxins are less relevant to the neurotoxic envenoming in humans, one could argue that these assays are testing something different to what they need to test (i.e. the effectiveness of antivenom against clinically relevant effects of snake venoms). With increasing concerns over animal ethics, the appropriateness of these tests should be re-evaluated through experimental studies and careful review of the literature.

7. Focus of this study

The present study is aimed at exploring the pathophysiology of the neuromuscular dysfunction of snake envenoming in humans using clinical and experimental data based on the clinically and epidemiologically most important snake species. For this, observational clinical studies supported with objective clinical and neurophysiological parameters, experimental studies and systematic literature reviews will be conducted. Snakes that commonly cause neurotoxic envenoming in Sri Lanka will be used as a model for clinical and experimental studies due to the high availability of the cases, underexplored pharmacology of the venoms, prevalent issues on antivenom effectiveness, efficacy and safety which could be generalizable across different settings. Furthermore, in order to increase the generalizability of the results, a wide range of neurotoxic venoms and post-synaptic toxins from clinically important snakes and a range of NMJ from different animal species, including humans, rodents and birds will be experimentally studied.

Specific objectives

1. Critically review the clinical evidence on the effectiveness of antivenom therapy in prevention or reversing snake venom-induced neuromuscular paralysis in humans.

2. Document the onset, progression and resolution of neuromuscular paralysis and the response to antivenom in Indian Krait (*Bungarus caeruleus*) and Russell's viper (*Daboia russelii*) envenoming in Sri Lanka, using objective neurological and neurophysiological parameters.

3. Investigate the pharmacology of the neuromuscular effects in Sri Lankan Russell's viper envenoming.

4. Study the ability of long-chain and short-chain post-synaptic neurotoxins from snake venoms to bind with the subtypes of nicotinic acetylcholine receptors (nAChR) found in rodent and human neuromuscular junctions.

5. Study the cross neutralisation of the neurotoxic venoms and their pre- and post-synaptic neurotoxins by different antivenoms.

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CHAPTER TWO

Critical review of current evidence on antivenom as a treatment for neuromuscular paralysis in snake envenoming.

Overview of the chapter

Antivenom therapy has been established as the standard treatment for neuromuscular paralysis in snake envenoming. However, a large body of clinical literature suggest that antivenom therapy is unable to prevent or reverse neuromuscular dysfunction in snake envenoming. This requires an evaluation of the current clinical evidence for antivenom therapy for neuromuscular dysfunction in snake envenoming through a critical review. The present Chapter includes a critical review on the use of antivenom therapy for neuromuscular paralysis in snake envenoming, based on a search carried out in MEDLINE and EMBASE for articles published over the last 70 years. This work highlights the lack of high quality clinical evidence globally for antivenom therapy in treating neuromuscular paralysis in snake envenoming.

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toxins

MDPI

Review

Antivenom for Neuromuscular Paralysis Resulting From Snake Envenoming

Anjana Silva [1,2,*], Wayne C. Hodgson [1] and Geoffrey K. Isbister [1,3]

[1] Monash Venom Group, Department of Pharmacology, Biomedicine Discovery Institute, Monash University, Clayton, VIC 3800, Australia; wayne.hodgson@monash.edu (W.C.H.); geoff.isbister@gmail.com (G.K.I.)
[2] Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura 50008, Sri Lanka
[3] Clinical Toxicology Research Group, University of Newcastle, Callaghan, NSW 2308, Australia
* Correspondence: anjana.silva@monash.edu; Tel.: [redacted]

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Abstract: Antivenom therapy is currently the standard practice for treating neuromuscular dysfunction in snake envenoming. We reviewed the clinical and experimental evidence-base for the efficacy and effectiveness of antivenom in snakebite neurotoxicity. The main site of snake neurotoxins is the neuromuscular junction, and the majority are either: (1) pre-synaptic neurotoxins irreversibly damaging the presynaptic terminal; or (2) post-synaptic neurotoxins that bind to the nicotinic acetylcholine receptor. Pre-clinical tests of antivenom efficacy for neurotoxicity include rodent lethality tests, which are problematic, and in vitro pharmacological tests such as nerve-muscle preparation studies, that appear to provide more clinically meaningful information. We searched MEDLINE (from 1946) and EMBASE (from 1947) until March 2017 for clinical studies. The search yielded no randomised placebo-controlled trials of antivenom for neuromuscular dysfunction. There were several randomised and non-randomised comparative trials that compared two or more doses of the same or different antivenom, and numerous cohort studies and case reports. The majority of studies available had deficiencies including poor case definition, poor study design, small sample size or no objective measures of paralysis. A number of studies demonstrated the efficacy of antivenom in human envenoming by clearing circulating venom. Studies of snakes with primarily pre-synaptic neurotoxins, such as kraits (*Bungarus* spp.) and taipans (*Oxyuranus* spp.) suggest that antivenom does not reverse established neurotoxicity, but early administration may be associated with decreased severity or prevent neurotoxicity. Small studies of snakes with mainly post-synaptic neurotoxins, including some cobra species (*Naja* spp.), provide preliminary evidence that neurotoxicity may be reversed with antivenom, but placebo controlled studies with objective outcome measures are required to confirm this.

Keywords: snake envenoming; paralysis; antivenom; neurotoxicity

1. Introduction

Snakebite is a major public health concern in the tropics. Although an accurate figure of the burden of global snakebite is unavailable, an estimate of 5.5 million annual snakebites across the globe is considered realistic [1,2]. South and Southeast Asia, sub-Saharan Africa and Latin America are the most affected regions, with more than two-thirds of the global snakebite burden reported to arise from Asia [1]. Neuromuscular paralysis due to snake envenoming is common, including envenoming by elapid snakes such as kraits (genus: *Bungarus*), cobras (genus: *Naja* and *Ophiophagus*), coral snakes (genus: *Calliophis* and *Micrurus*), taipans (genus: *Oxyuranus*), tiger snakes (genus: *Notechis*) and death adders (genus: *Acanthophis*). Snake venom induced paralysis becomes life threatening with progressive paralysis of the bulbar and respiratory muscles which requires prompt airway assistance and mechanical ventilation [3].

2. Neuromuscular Paralysis in Snake Envenoming

Snake venom neurotoxins primarily target the neuromuscular junction of skeletal muscles of which the motor nerve terminal (pre-synaptic) and the nicotinic acetylcholine receptor at the motor-end plate (post-synaptic) are the major targeted sites. This is well supported by clinical observations that neurotoxic snake envenoming almost exclusively results in flaccid paralysis [4–12] which is due to the blockade of neurotransmission at the neuromuscular junction by venom neurotoxins [13–16]. Neuromuscular paralysis in snake envenoming varies from mild to life threatening, depending on the degree of envenoming (i.e., quantity of injected venom reaching the circulation), the composition of the venom and potentially early therapeutic interventions. Neuromuscular paralysis is classically a descending paralysis which initially involves the eye-lid muscles, clinically manifesting as bilateral ptosis, usually within a few hours of the bite. This is followed by external ophthalmoplegia and diplopia, facial muscle weakness [4,5,7,17] with slurred speech and difficulty in mouth opening [7,18]. The paralysis then descends to the neck muscles and bulbar muscles. Involvement of bulbar muscles causes difficulty in swallowing and compromises airway protection. This is potentially life-threatening because of the risk of aspiration [19]. Neuromuscular paralysis then involves the respiratory muscles resulting in hypoventilation from decreasing tidal volumes which requires mechanical ventilation [4,9,20–22]. In severe neurotoxic envenoming, particularly by kraits, neuromuscular paralysis will eventually involve all limb muscles [4,23]. Recovery of neuromuscular function usually follows the reverse order of muscle involvement, with ptosis and ophthalmoplegia being the last neurological signs to disappear [4]. This unique descending sequence of muscle involvement and recovery is unlikely to be due to properties of the snake neurotoxins, but rather a combination of the size and the unique physiology of the muscles and motor nerve connections, including the redundancy of neuromuscular junctions [24,25].

Central neurological effects are rarely reported in snake envenoming, and are almost always due to indirect neurological effects of haemorrhagic or thrombotic toxicity in the central nervous system from snake venom procoagulant toxins [26–30]. Apparent deep coma reported after krait envenoming [23,31] suggests possible direct toxin-mediated central neurological effects. However, this may be explained as an extreme state of neuromuscular paralysis mimicking coma rather than actual coma [4,31–34].

3. Neuromuscular Junction: The Primary Target Site

The neuromuscular junction is a specialised synapse between the motor nerve and skeletal muscle fibre and serves as the final relay station in the motor pathways. The neurotransmitter, acetylcholine (ACh), is stored in vesicles within the motor nerve terminal. Depolarisation of the motor nerve terminal opens the voltage gated Ca^{2+} channels resulting an influx of Ca^{2+} into the motor nerve terminal, which triggers the fusion of vesicles to the neurolemma and release of ACh into the synaptic cleft via exocytosis. The nAChR is a pentameric transmembrane protein with a central pore which functions as a non-selective ion channel. Binding of two ACh molecules with the two agonist binding sites of the nAChR leads to a conformational change resulting in the opening ofthe central pore of the nAChR, allowing an influx of Na^{+} and depolarisation of the post-synaptic membrane. Subsequent opening of the voltage-gated Ca^{+} channels causes depolarization of the sarcolemma resulting in initiation of the muscle fibre contraction process [35,36]. Snake venom neurotoxins can disrupt the neurotransmission process at several points. Most snake neurotoxins act either on the motor nerve terminal (i.e., pre-synaptically) to prevent the release of ACh or at the nAChR on the motor-end plate (i.e., post-synaptically) by antagonising the receptor.

Most neurotoxic snake venoms contain both pre- and post-synaptic neurotoxins, and it is common for venoms to contain several different post-synaptic toxins. However, some snakes have venoms that contain only pre-synaptic (e.g., Sri Lankan Russell's viper, *Daboia russelii*) [37] or post-synaptic (e.g., King cobra, *Ophiophagus hannah*) toxins [38], rather than a combination of the two classes. With our rapidly expanding knowledge of snake venom proteomes and venom gland transcriptomes, many potential neurotoxins have been identified in snake venoms [38–42]. However, only a small proportion of these toxins have been isolated and functionally characterised.

Neurotoxins in Snake Venoms

Pre-synaptically acting neurotoxins have been isolated from many elapids such as Kraits, Tiger snakes, Taipans and also from some viperids, and appear to be important with regard to clinically evident neurotoxicity. Snake venom presynaptic toxins belong to group I phospholipases A_2 (elapid venoms) and group II phospholipases A_2 (Viperid venoms) (PLA_2), a group of Ca^{2+}-dependent enzymes. The exact mechanisms by which pre-synaptic toxins enter into the motor nerve terminal and initiate their actions are unclear. Hydrolysis of phospholipids in the neurilemma of the motor nerve is believed to be the primary mode of action of the pre-synaptic PLA_2 neurotoxins [16,43]. Based on experimental work using β-bungarotoxin, the major pre-synaptic neurotoxin in Chinese banded krait (*B. multicinctus*) venom, the key toxin-mediated event occurring in the motor nerve terminals is the necrotic degeneration of the terminal boutons [13,43,44]. In isolated nerve-muscle preparations, neuromuscular transmission failure caused by pre-synaptic toxins is usually tri-phasic. This includes an initial phase of weak inhibition of ACh release, a second prolonged phase of facilitated ACh release, and a third phase of progressive decline of neurotransmission due to necrotic degeneration of the presynaptic terminal [45]. The process of necrotic degeneration includes the depletion of synaptic vesicles through the impairment of vesicle recycling, degeneration of the mitochondria and fragmentation of the neurilemma of the nerve terminal [13,43,44,46]. Ultimately, the damaged nerve terminals withdraw from the synaptic trough resulting in empty synaptic troughs [13]. The above pathophysiological changes appear to occur for, at least, elapid PLA_2 pre-synaptic neurotoxins [47]. The process of necrotic degeneration occurs within the first 12 h of toxin exposure. Recovery of function by re-innervation takes 3 to 5 days after exposure to the toxin, and by 7 days, re-innervation is complete [13]. Many snake venoms that are known to cause severe paralysis in humans, such as Indian krait (*B. caeruleus*) [4], Malayan krait (*B. candidus*) [6], Chinese banded krait (*B. multicinctus*) [48], coastal taipan (*O. scutellatus*) [49] and tiger snake (*N. scutatus*) [11] venoms, contain potent pre-synaptic neurotoxins (Supplementary Table S1). A detailed account of these toxins is available in the review by Harris and Scott-Davey [16].

Most snake venom toxins that act post-synaptically belong to the three finger toxin family. Three finger toxins with curare-mimetic activity are broadly categorized into two groups: i.e., short-chain α-neurotoxins that have 60–62 amino acids with four disulfide bonds and long-chain α-neurotoxins that have 66–74 amino acids with five disulfide bonds. Irrespective of their structural differences, both long- and short-chain α-neurotoxins bind to similar sites on the nAChR with high affinity [50]. Short-chain α-neurotoxins bind to the nAChR 6 to 7 times faster, and dissociate 5 to 9 times, faster than long-chain α-neurotoxins [51]. α-Neurotoxins display a variety of types of interaction with the nAChR including reversible, pseudo-irreversible and irreversible binding in neuromuscular preparations. Long-chain α-neurotoxins are known to be relatively irreversible compared to short-chain α-neurotoxins. Both agonist binding sites of the nAChR need to be occupied by ACh (or agonist) to cause conformational change and the opening of the ion channel. Therefore, even if one binding site is occupied by an α-neurotoxin there will be dysfunction of the channel. Classic examples of long-chain α-neurotoxins are α-bungarotoxin from *B. multiscinctus*, and α-cobratoxin from *N. kaouthia*. A well-known short-chain α-neurotoxin is Toxin-α from *N. nigricollis* venom.

Recently published cobra venom proteomes (or "venomes") suggest a high relative abundance of α-neurotoxins in Thai cobra (*N. kaouthia*) [52], King cobra (*Ophiophagus hannah*) [38] and Indian cobra (*N. naja*) [53] venoms, but negligible amounts of phospholipaseA_2 toxins that are potentially pre-synaptic toxins. Although rare, life threatening neuromuscular paralysis has been reported in envenoming by all of the above cobra species, i.e., supporting the role of α-neurotoxins in neuromuscular paralysis in humans for at least some snakes. Detailed accounts of the structural and functional aspects of -neurotoxins are available in reviews by Barber et al. [15] and Nirthanan et al. [36]. There are other types of snake neurotoxins that are potentially important in the development of paralysis, but act at different sites compared to conventional pre-synaptic and post-synaptic toxins. Fasciculins inhibit AChE in the neuromuscular junction, allowing ACh to accumulate. These toxins

have been isolated from the venoms of mamba species (*Dendroaspis* sp.). Once formed, the high affinity complex of fasciculin-AChE is very slow to dissociate [54]. Dendrotoxins, isolated from several African black mamba species, block the voltage-gated K+ channels in the nerve terminals resulting in continuous neurotransmitter release at vertebrate neuromuscular junctions. These toxins, when injected into the central nervous system, also facilitate neurotransmitter release [55].

4. Antivenoms

Antivenoms are the only antidotal treatment available for snake envenoming and have been in clinical use for over a century. Antivenoms are a mixture of polyclonal antibodies which can be whole or fractionated, $F(ab)_2$ or F(ab) IgG, raised against one (i.e., monovalent) or several (i.e., polyvalent) snake venom(s) in animals such as horses, sheep, goats and donkeys [56]. Their polyclonal nature means that antivenoms consist of different antibodies against different toxin antigens in the venom. The antibody molecules bind with the toxins and (1) prevent the toxin-substrate interaction by blocking the active site, (2) form large venom-antivenom complexes preventing the distribution of the toxins from the central compartment, or (3) facilitate the elimination of toxins from the body [57,58]. Potential physico-chemical, pharmacokinetic and pharmacodynamic benefits of using monoclonal [59] and recombinant antibody [60] fragments raised against individual venom components has been experimentally explored. However, translation of such experimental antivenoms for clinical use has not yet occurred.

4.1. Antivenom Efficacy

The efficacy of antivenom against a particular venom is due to the ability of antivenom molecules to bind with toxins in the venom [61]. i.e., with respect to neurotoxicity, this is the ability of the antivenom molecules to bind with the neurotoxins in the venom. This is dependent on: (1) the avidity of the antivenom, which is a combined effect of the affinity constants of the different antibodies towards different toxins; (2) the relative abundance of antibodies in the antivenom against the individual neurotoxins; and (3) the relative abundance of the individual neurotoxins in the snake venom of interest. The ability of the antivenom molecules to bind with a specific venom can be quantified using an in vitro venom-antivenom binding assay, which provides useful insights into the overall ability of the antivenom to bind with the venom [62,63]. Immuno-depletion and, more recently, affinity chromatography based antivenomic approaches are useful tools in testing the ability of antivenoms to bind with specific neurotoxins or toxin groups in the venoms [64]. However, all of these approaches only demonstrate toxin binding and not neutralisation of neurotoxicity. In vitro pharmacological testing of antivenoms with chick biventer cervicis nerve-muscle preparations, frog rectus abdominis and rat phrenic nerve-hemidiaphragm preparations is useful in specifically testing antivenom efficacy towards the neurotoxic properties of the venoms [45]. Of these, the chick biventer nerve-muscle preparation is capable of differentiating post-synaptic neurotoxicity from pre-synaptic neurotoxicity [37,63,65,66]. In these experimental procedures, antivenom is first equilibrated in the organ bath that contains the tissue, and then the venom or toxin is added to the organ bath allowing the antivenom to have sufficient time to bind with the neurotoxins [45]. It therefore measures antivenom efficacy only because antivenom is present prior to venom being added. The ability of antivenoms to bind to, and prevent the neurotoxicity of both long- and short-chain post-synaptic neurotoxins as well as pre-synaptic neurotoxins has been extensively investigated in the chick biventer nerve-muscle preparation [67–70]. Some studies have also demonstrated the ability of antivenom to partially reverse neurotoxicity in the chick biventer nerve-muscle preparation [65]. This is supported by a study demonstrating the ability of antivenoms to reverse the binding of post-synaptic toxins with the nAChR [71,72]. However, neuromuscular blockade mediated by pre-synaptic toxins is not reversed by antivenom, even if the antivenom is able to bind with the toxin because of the irreversibility of pre-synaptic neurotoxicity [70]. The most widely used method for assessing antivenom efficacy is rodent lethality testing. This test calculates the dose of antivenom required to reduce the mortality of rodents by 50% defined as the median effective dose 50% (ED_{50}), when the animals are given five

times the median lethal dose 50% (i.e., LD_{50}) of the venom. From this test the ED_{50} of antivenom is calculated, which may be useful for inter-batch comparison of antivenoms or comparison of two antivenoms [73]. However, the outcome measure of this test is the death of the animal, which could be due to any, or a combination, of the toxin classes in the venom, not necessarily an effect of neurotoxicity, or even any toxic effect relevant to humans. Discrepancy between the rodent lethality based efficacy studies with in vitro venom-antivenom binding and nerve-muscle preparation based studies has recently been demonstrated [65]. In addition, the human muscle type nAChR is relatively resistant to snake venom short-chain post-synaptic neurotoxins compared to the rodent nAChR, suggesting possible inaccuracies with the use of rodents or their tissues as models for antivenom efficacy testing for neurotoxic venoms [74]. Therefore, the rodent lethality tests as a measure of antivenom efficacy for neuromuscular paralysis in humans is highly problematic. The ability of the heterologous antivenoms to cross-neutralise the neuromuscular effects of several other snake venoms and neurotoxins has been reported in in vitro pharmacological studies [66,75,76]. The remarkable ability of Asian antivenoms to cross-neutralise Australasian neurotoxic venoms and the similar ability of Australian antivenoms to cross-neutralise Asian neurotoxic venoms was shown recently [66]. This suggests the likely presence of common antigenic regions within the post-synaptic and pre-synaptic toxins indicating the potential to develop regional or even universal neurotoxic antivenoms [66].

4.2. Antivenom Effectiveness

The effectiveness of an antivenom can be defined as its ability to prevent the occurrence, or reverse the effects, of venom in the clinical setting [61]. With respect to neurotoxicity, the effectiveness of the antivenom is the ability of antivenom to prevent the occurrence of neurotoxicity or to reverse established neurotoxicity in snakebite patients. It is important to consider that in addition to the efficacy of the antivenom, many other factors will contribute to the effectiveness of an antivenom. These include: (1) factors that govern the severity and the rate of development of envenoming, such as the depth of the venom injection and amount of venom injected; (2) factors related to antivenom such as the delay from the bite to antivenom administration, antivenom dose, infusion rate, the ability of the antivenom to distribute to peripheral tissues; (3) the nature and extent of neurotoxin-mediated damage such as irreversible motor nerve injury due to pre-synaptic toxins; and (4), the geographical variations of the venom composition. The variability in these factors among patients makes it difficult to achieve an objective and pure measure of effectiveness of an antivenom in the clinical setting. Therefore, the interpretation of the evidence provided in clinical studies on the antivenom for neurotoxic envenoming should be interpreted with caution.

5. Clinical Studies of Antivenom for Neurotoxic Snake Envenoming

Well-designed, randomized placebo-controlled clinical trials are the highest quality evidence required to demonstrate antivenom effectiveness for neurotoxicity in humans. Non-placebo controlled trials, non-randomized comparative trials and well-designed cohort studies also provide useful evidence in the absence of the former. The effectiveness of an antivenom depends on diverse factors, including the inter- and intra-species variability of venom composition of the snake. Therefore, proper case definition is also of high importance in securing the data quality of the clinical studies [77].

5.1. Randomised Controlled Trials

Our literature search yielded no randomized, placebo-controlled clinical trials of antivenom for neuromuscular paralysis in snake envenoming. The search identified four randomized trials and one blinded cross-over trial, in which all arms in the trials received the same or different antivenom, without any placebo controls (Table 1).

Table 1. Randomized trials without placebo-control and cross-over trials that describe the antivenom effectiveness for neuromuscular paralysis in snake envenoming.

Study	Number in Each Arm	Snake Species	Authentication of Snake	Trial Arms	Blinded	Randomisation	Allocation Concealment	AV ** Dose Defined	Primary Outcome Defined	Neurotoxicity Measures for Outcome	Conclusion
Ariaratnam et al. 2001	23/20	*Daboia russelii*	Identification of snake specimen and ELISA * (n = 43); clinical features (n = 3)	2 different AV (2 arms)	No	Yes	Good	Yes	Multiple outcomes	Duration of neurological signs (ptosis, diplopia)	No difference between groups
Dart et al. 2001	16/15	North American Crotalids	Not stated	2 doses of AV (2 arms)	No	Yes	Good	Yes	Yes (Improvement of a defined severity score)	Weakness Fasciculations Dizziness Paraesthesia (included to the severity score)	Both doses equally effective
Sellahewa et al.	8/7	*Naja naja* (n = 3) *Daboia russelli* (n = 11) *Hypnale hypnale* (n = 1)	Identification of specimen (n = 10), clinical features (n = 5)	AV vs. AV + Intravenous immunoglobulin (IVIG)	No	Yes	Good	Yes	No	Duration of neurotoxic features (ptosis and ophthalmoplegia)	No clear difference except more re-dosing in AV group.
Tariang et al. 1999	31/29	'Cobra', 'viper'	Not stated	Two doses of AV	Yes	Yes	Not described	Yes	No	Not defined	Lower dose is effective than higher dose
Watt et al. 1989	2/2/4/8 (Cross-over)	Philippine cobra (*Naja philippinensis*)	ELISA (n = 5); Identification from pictures (n = 2); clinical features (n = 1)	Three different doses of AV and all patients received edrophonium	No	No	No	Yes	Multiple outcomes	Improvement of neurological signs	Tensilon is effective compared to AV

* ELISA: Enzyme-Linked Immunosorbent Assay; ** AV: antivenom.

Of the above five studies, three tested the effectiveness of an antivenom. One study compared a new monovalent antivenom with an existing polyvalent antivenom in Sri Lankan Russell's viper (*Daboia russelii*) envenoming [78] and the second compared two doses of the new CroFab antivenom in North American Crotalid envenoming in the United States [79]. In both studies, neuromuscular paralysis was not the primary outcome, but was part of the outcome assessment. Both studies showed no difference in the outcome between the trial arms. The snake species studied in both trials primarily caused venom-induced consumptive coagulopathy or local/regional effects, and neurotoxicity is non-life threatening for both snakes [5,80].

A third randomised blinded trial [81] conducted in South India tested two doses of Indian polyvalent antivenom against systemic envenoming. Although this study recruited patients with neuromuscular paralysis, the outcome measures for neurotoxic envenoming were not defined and the study included a heterogenous mix of snake types with the majority being unknown/unconfirmed. The study only had four versus three patients who developed neurotoxicity in the high and low dose groups, respectively. The study concluded that a low antivenom dose is more effective than the high dose, based on the obvious finding that the low dose group received less antivenom and that the low dose group had a shorter length of stay by one day.

The fourth randomised controlled trial [82] was a small pilot study of 15 patients with no predefined primary outcome, and no blinding of the treating doctors who were allowed to give repeat antivenom doses. In addition, the study included different types of snakes which were not balanced between groups.

A blinded randomised controlled trial [83] tested the effectiveness of edrophonium versus antivenom, only randomising patients to three different antivenom doses with all patients receiving edrophonium. The study was very small with only eight patients, had imbalanced randomisation and the time to antivenom varied between 3 h and 96 h, with a median of 24 h. This variability makes the results difficult to interpret, particularly the difference in time to antivenom because neurotoxicity usually develops over 24 h and then begins to recover. The fact that all patient responded to edrophonium suggests that this observed effect may not be directly related to the neurotoxicity.

All of the above studies lacked controlled arms and included small numbers of patients. In addition, individual studies had critical flaws such as lack of defined primary outcomes, authentication of the snake involved, and lack of blinding of the therapeutic interventions to the observer and the patient.

5.2. Non-Randomised Comparative Trials

Our search yielded three comparative trials (Table 2). One study compared the duration of neuromuscular paralysis in 27 patients with *Bungarus multicinctus* envenoming in Vietnam, treated with a new antivenom, with a historical control group of 54 patients who were not treated with antivenom [84]. The antivenom group had a shorter duration of mechanical ventilation, intensive care unit stay and other neurological signs, indicating the possible beneficial effects of antivenom in accelerating the recovery of neurotoxicity. However, there was no difference in the number of patients requiring mechanical ventilation between the two groups (>80%). This may have been due to the fact that antivenom was delayed by 19 $\pm$ 9 h. The un-blinded before and after nature of the study also meant that other changes to treatment may have occurred between the groups. Duration of ventilation and length of stay are not good objective measures and there was no pre-defined primary outcome for the study.

Table 2. Comparative clinical studies that describe the antivenom effectiveness for neuromuscular paralysis in snake envenoming.

Study	Number in Each Group	Snake Species	Authentication of Snake	Study Groups	Antivenom Dose Defined	Neurotoxicity Measures	Conclusion	Remarks
Agarwal et al. 2005	28/27	Not defined	Not defined.	High vs. low dose AV	Yes	Duration of the mechanical ventilation, duration of ICU stay	No difference in outcome between the two groups	Same AV loading dose was given to both groups. The difference was only the maintenance dose.
Hung et al. 2010	27/54	*Bungarus multicinctus*	Not defined	AV vs. no AV	Yes	Number of patients requiring mechanical ventilation, duration of mechanical ventilation, length of stay in the ICU, duration of a defined degree of muscle paralysis	AV group had shorter duration of ventilation, ICU stay and other neurological signs. No difference between the number of patients requiring ventilation.	The no AV group is a historical group; AV dose varied within the AV group; Bite-to-AV delay is 19 ± 9 h (range: 5–38 h).
Pochanugool et al. 1997	27/41	*Naja kaouthia* *Naja sumatrana*	Patient's description of snake or physician's identification of the snake	No AV vs. AV (three unbalanced dose groups within AV group)	Yes	Duration of respiratory failure	Two dose groups of antivenom (100 and 200 mL) had significantly lower duration of respiratory failure compared to no AV	The no AV group is a historical group; No definition of 'respiratory paralysis'.

A study of Thai cobra (*Naja kaouthia* and/or *N. sumatrana*) envenoming compared a group of patients who did not receive antivenom (historical) with three groups of patients who received a bolus of Thai cobra antivenom [85]. The antivenom-treated patient group had three sub-groups, each sub-group receiving a different antivenom dose (i.e., 50, 100 and 200 mL). The study showed a marked reduction in the duration of respiratory paralysis in the 100 and 200 mL groups compared to the no antivenom group. However, the study did not specify how respiratory paralysis was defined and the frequency of patients requiring ventilation was similar. The study also included a heterogenous group of patients with differing snake species and times of presentation to hospital. The study suggests that the post-synaptic neurotoxicity of Thai cobra envenoming is reversible, but a larger blinded, randomised and balanced study is required to confirm this.

The third study retrospectively compared the recovery of the snakebite patients with neurotoxicity who were treated with high dose versus low dose antivenom [86]. The study concluded that the lower dose was as effective as the higher dose. However, this could have been a result of the loading dose of antivenom being similar in both groups, while only the maintenance dose differed. All of these studies had major deficiencies in case definition as well as the objectiveness of the outcome measures.

5.3. Cohort Studies

The search also yielded numerous cohort studies that discussed the effects of antivenom on neuromuscular paralysis, including studies on envenoming by elapids such as Indian krait (*Bungarus caeruleus*) [4,18,87,88], multi-banded krait (*B. multicinctus*) [48,89], Malayan krait (*B. candidus*) [48,90], common cobra (*Naja naja*) [8,21], monocellate cobra (*N. kaouthia*) [91–94], Philippine cobra (*N. philippinensis*) [7], eastern coral snake (*Micrurus fulvius fulvius*) [95], coastal taipan (*Oxyuranus scutellatus*) [96], Papuan taipan (*O. canni*) [10,97,98], tiger snake (*Notechis scutatus*) [11,99], rough-scaled snake (*Tropidechis carinatus*) [100], Papuan death adder (*Acanthophis laevis*) [101], Australian death adders (*Acanthophis* sp.) [12], Papuan black snake (*Pseudechis papuanus*) [102], and viperids such as Sri Lankan Russell's viper (*Daboia russelii*) [5,103–105], Balken adder (*Viper berus bosniensis*) [17], southern tropical rattlesnake (*Crotalus durissus terrificus*) [106,107] and Mojave rattlesnake (*C. scutulatus scutulatus*) [108] and North American crotalids [109]. In addition, there was a large number of case reports on snake bite induced paralysis and the effect of antivenom.

Almost all the above cohort studies commented on the efficacy or effectiveness of the antivenom therapy for neuromuscular dysfunction. However, apart from seven studies [4,5,11,12,97,100,103], the remaining studies had no data on serial venom antigen concentrations to enable comment on the ability of antivenom to clear free venom (efficacy). This deficiency, together with a lack of serial, well-defined objective neurological or neurophysiological parameters, makes it difficult to interpret the findings of the vast majority of the studies on the effectiveness of the antivenom in preventing or reversing paralysis, given that there was no untreated group for comparison.

Several of the above observational studies showed that antivenom failed to reverse already established neurotoxicity, particularly in envenomings by taipans and kraits, whose venoms predominately possess pre-synaptic neurotoxins. A large cohort study of 166 Papuan taipan (*O. canni*) envenomings showed that antivenom did not reverse established neurotoxicity. In addition, neurotoxicity did not improve in any patients within 6 h, and 19% of patients developed their first signs of neurotoxicity and 37% deteriorated after antivenom [99]. A recent observational study of 33 common krait (*B. caeruleus*) envenomings in Sri Lanka found that Indian polyvalent antivenom, given as early as a median of 3.5 h after the bite, rapidly cleared circulating venom antigens from the blood. However, it did not reverse neurotoxicity and there was subsequent worsening of neuromuscular paralysis in the patients observed clinically and measured neurophysiologically [4]. These studies provide objective evidence for the poor effectiveness of antivenom in reversing the paralysis in krait and taipain envenoming, despite it being efficacious in binding free venom in krait envenoming.

A large cohort of 245 authenticated Russell's viper (*Daboia russelii*) bite patients from Sri Lanka found that neuromuscular paralysis continued to worsen, as evident clinically and

neurophysiologically, despite Indian polyvalent antivenom clearing circulating venom antigens [5]. These observations were largely consistent with the case reports that described the failure of antivenom therapy to prevent development or worsening of paralysis in common krait [110] and Cape cobra (*Naja nivea*) [111] envenoming.

A cohort study of 33 patients (18 envenomings) bitten by Papuan death adder (*Acanthophis* sp.) showed non-progression of the neuromuscular paralysis after antivenom therapy in 12 patients treated with death adder antivenom. In addition, a significant, rapid neurological improvement was observed in three patients [101]. In contrast, in a study of 29 definite death adder envenomings in Australia, 12 were treated with death adder antivenom and none showed a rapid improvement in paralysis despite the antivenom efficaciously clearing the venom antigens from the blood [12]. In contrast, there is evidence that early antivenom treatment decreases the frequency and severity of neurotoxic signs, and therefore prevents neurotoxicity developing in some snakes. In the study of 166 Papuan taipan envenoming, early administration of antivenom prevented intubation, with only 13.3% requiring intubation in patients given antivenom <4 h post-bite, compared to 63% in patients receiving antivenom >4 h post-bite [98]. Another study of 156 taipan envenomings also found that antivenom given within 4 h resulted in less patients requiring intubation [97]. A more recent cohort study in Australia of 40 coastal taipan (*Oxyuranus* spp.) envenomings demonstrated the efficacious binding of Australian polyvalent antivenom with the circulating venom. The same study found that early administration of antivenom was associated with a decreased frequency of neurotoxicity and requirement for intubation, indicating clinical effectiveness of the antivenom for preventing neuromuscular paralysis in taipan envenoming [96].

There were several case reports that reported spontaneous improvement with supportive treatment and no antivenom, in neuromuscular paralysis caused by *Naja haje* [112], *Naja kaouthia* [113], *Bungarus candidus* [114–116] and *Micrurus laticollaris* [117]. There were also numerous case reports that either suggested the beneficial effects of antivenom in accelerating the recovery of neuromuscular paralysis. None of these studies clearly demonstrated how antivenom therapy altered the clinical course of the paralysis, distinguishing it from the natural progression of the paralysis. Further, none of the reports provided circulating venom antigen data in-order to show the efficacious binding of the antivenom with the venom antigens.

6. Conclusions and Future Directions

Although antivenom use for neuromuscular paralysis in snake envenoming is established worldwide, there have been no randomized placebo-controlled clinical trials conducted on the effectiveness of antivenom therapy in preventing or reversing neuromuscular paralysis. Apart from a few studies, all the available randomized trials, comparative studies and cohort studies had deficiencies either in the case definition, study design, small sample size or in the objectivity of the measures of paralysis. A number of studies demonstrated the efficacy of antivenom in human envenoming by clearing circulating venom. Studies of snakes with primarily pre-synaptic neurotoxins (e.g., kraits, taipans) suggest that antivenom does not reverse established neurotoxicity, but early administration appears to prevent neurotoxicity or is associated with a decrease in severity for some snakes, mainly taipans. Small studies of snakes with mainly post-synaptic neurotoxins provide preliminary evidence that neurotoxicity may be reversed with antivenom, but placebo controlled studies with objective outcome measures are required to confirm this. The validity of the rodent lethality tests as pre-clinical tests of antivenom efficacy for neurotoxic envenoming in humans has been challenged and, in vitro pharmacological tests such as nerve-muscle preparation studies appear to provide more clinically meaningful information.

Future research needs to focus on well-designed studies investigating whether the early administration of antivenom will prevent neurotoxicity, both pre-synaptic and post-synaptic. This includes better case definition by using venom specific enzyme immunoassays to confirm envenoming [118], and improved objective measures of neurotoxicity, both clinical and

neurophysiological [4]. In addition, there needs to be carefully designed studies investigating the ability of antivenom to reverse neurotoxicity in snakes with almost exclusively post-synaptic neurotoxins, such as cobras and the king cobra.

7. Methods

A search was carried out in MEDLINE from 1946 and EMBASE from 1947 to 31 March 2017 and included studies of snake envenoming which reported neuromuscular paralysis and provided information on antivenom. The following keywords were used: "snakebite", "snake envenoming/ envenomation", "ophitoxaemia", "neurotoxicity", "paralysis", "ptosis", "antivenom/ antivenene" and "immunotherapy". Reference lists of retrieved articles were searched for additional relevant publications. Only articles in English were reviewed. After removing duplicates, we identified a total of 1136 studies of which 133 were included for review. (Figure 1) Articles that described in vivo and in vitro studies, reviews and epidemiological studies, clinical studies that did not report neurotoxic envenoming were excluded. There were four randomised comparative trials, one cross-over trial and 3 non-randomised comparative trials, 63 cohort studies and 62 case reports which described the effectiveness of antivenom.

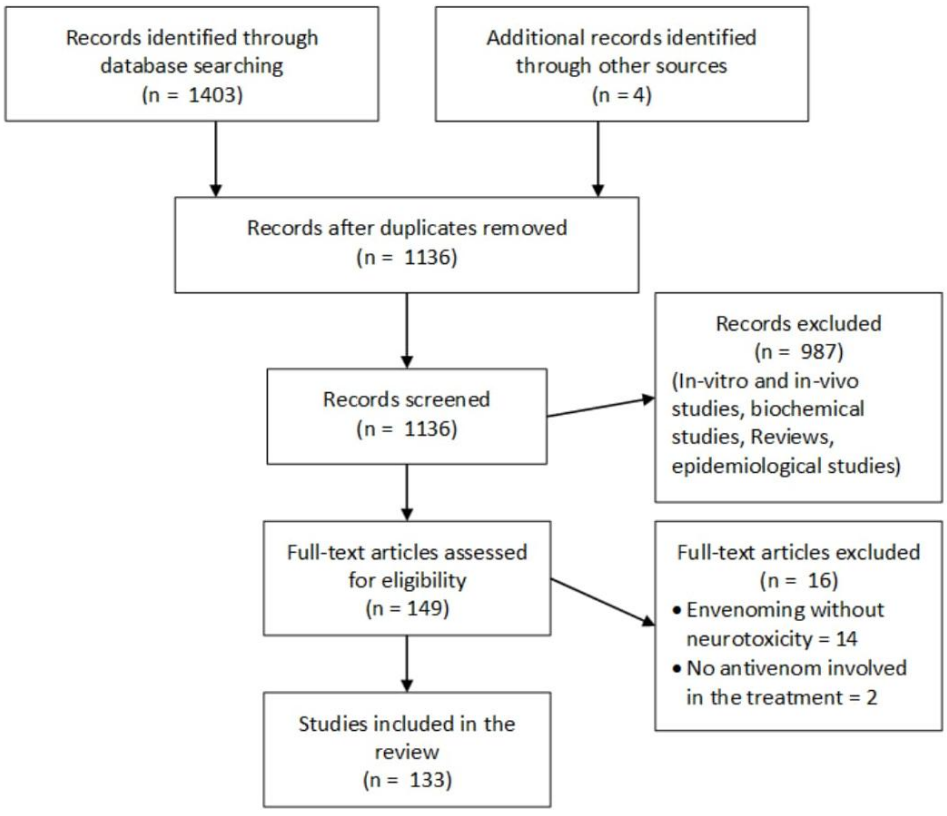

Figure 1. Selection of the studies for the review.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/9/4/143/s1, Table S1: Some pre-synaptic toxins isolated and pharmacologically characterised from snake venoms that cause paralysis in humans.

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CHAPTER THREE

Evolution and resolution of neuromuscular paralysis and the response to antivenom in snake bite patients in Sri Lanka.

Overview of the Chapter

The literature review in Chapter two indicated that there is a lack of high quality clinical evidence, by means of randomized placebo-controlled clinical trials, on the effectiveness of antivenom therapy for neuromuscular dysfunction following snake envenoming. Given that antivenom is the accepted treatment for envenoming globally, it is unrealistic to expect implementation of randomized placebo-controlled clinical trials on antivenom for envenoming in the future. Therefore, well-designed observational clinical studies are important in investigating the role of antivenom in preventing or reversing the neuromuscular dysfunction in snake envenoming. As indicated in Chapter Two, previous observational clinical studies had deficiencies including poor case authentication, lack of defined objective clinical and neurophysiological parameters, and a lack of serial venom concentration data. This Chapter describes an observational clinical study, conducted in Sri Lanka, to investigate neuromuscular dysfunction in snake envenoming and its response to antivenom therapy, designed to overcome the above highlighted deficiencies. The study included 33 authenticated envenomings by common krait (*Bungarus caeruleus*) and 245 authenticated envenomings by Russell's viper (*Daboia russelii*) and was published as two separate research papers. Common krait envenoming resulted in frequent life-threatening paralysis whereas Russell's viper envenoming resulted in mild, non-life-threatening paralysis. In both groups of patients, antivenom therapy resulted in a rapid drop in circulating venom antigens indicating the efficacious binding of antivenom with the venom antigens. However, this did not result in the prevention or reversal of paralysis.

The work contained in this Chapter was published in two research articles:

Silva A, Maduwage K, Sedgwick M, Pilapitiya S, Weerawansa P, Dahanayaka NJ, Buckley NA, Johnston C, Siribaddana S, Isbister GK. Neuromuscular Effects of Common Krait (*Bungarus caeruleus*) Envenoming in Sri Lanka. *PLoS Negl Trop Dis.* 2016;10 (2): e0004368.

Silva A, Maduwage K, Sedgwick M, Pilapitiya S, Weerawansa P, Dahanayaka NJ, Buckley NA, Siribaddana S, Isbister GK. Neurotoxicity in Russell's viper (*Daboia russelii*) envenoming in Sri Lanka: a clinical and neurophysiological study. *Clin Toxicol (Phila)*. 2016;54 (5):411-9.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Neuromuscular Effects of Common Krait (*Bungarus caeruleus*) Envenoming in Sri Lanka

Anjana Silva[1,2,3], Kalana Maduwage[3,4], Michael Sedgwick[3], Senaka Pilapitiya[2], Prasanna Weerawansa[2], Niroshana J. Dahanayaka[2], Nicholas A. Buckley[3,5], Christopher Johnston[4], Sisira Siribaddana[2], Geoffrey K. Isbister[3,4]*

1 Monash Venom Group, Department of Pharmacology, Monash University, Clayton, Victoria, Australia, **2** Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura, Sri Lanka, **3** South Asian Clinical Toxicology Research Collaboration, University of Peradeniya, Peradeniya, Sri Lanka, **4** Clinical Toxicology Research Group, University of Newcastle, New South Wales, Australia, **5** Clinical Pharmacology, University of Sydney, Sydney, Australia

* [redacted]

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Abstract

Objective

We aimed to investigate neurophysiological and clinical effects of common krait envenoming, including the time course and treatment response.

Methodology

Patients with definite common krait (*Bungarus caeruleus*) bites were recruited from a Sri Lankan hospital. All patients had serial neurological examinations and stimulated concentric needle single-fibre electromyography (sfEMG) of orbicularis oculi in hospital at 6wk and 6–9mth post-bite.

Principal Findings

There were 33 patients enrolled (median age 35y; 24 males). Eight did not develop neurotoxicity and had normal sfEMG. Eight had mild neurotoxicity with ptosis, normal sfEMG; six received antivenom and all recovered within 20–32h. Seventeen patients developed severe neurotoxicity with rapidly descending paralysis, from ptosis to complete ophthalmoplegia, facial, bulbar and neck weakness. All 17 received Indian polyvalent antivenom a median 3.5h post-bite (2.8–7.2h), which cleared unbound venom from blood. Despite this, the paralysis worsened requiring intubation and ventilation within 7h post-bite. sfEMG showed markedly increased jitter and neuromuscular blocks within 12h. sfEMG abnormalities gradually improved over 24h, corresponding with clinical recovery. Muscle recovery occurred in ascending order. Myotoxicity was not evident, clinically or biochemically, in any of the patients. Patients were extubated a median 96h post-bite (54–216h). On discharge, median 8 days (4–12days) post-bite, patients were clinically normal but had mild sfEMG

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abnormalities which persisted at 6wk post-bite. There were no clinical or neurophysiological abnormalities at 6–9mth.

Conclusions

Common krait envenoming causes rapid onset severe neuromuscular paralysis which takes days to recover clinically consistent with sfEMG. Subclinical neuromuscular dysfunction lasts weeks but was not permanent. Antivenom effectively cleared venom but did not prevent worsening or reverse neuromuscular paralysis.

Author Summary

Common krait bites cause muscular paralysis due to the venom disrupting communication between the nerves and muscles. This becomes life-threatening for the patient if there is paralysis of the muscles used for breathing. We studied the severity of paralysis, long term effects and the value of antivenom treatment in authenticated Indian krait bite patients from Sri Lanka. In addition to standard treatment with antivenom, the patients had single-fibre electromyography done, a sensitive neurophysiological test that detects the abnormalities of communication between the nerves and muscles. Half of the patients had severe paralysis and required mechanical ventilation, and the remainder had mild or no effects. Antivenom was given to all patients with severe paralysis and most with mild effects. However, despite antivenom binding all free venom after it was administered, it did not prevent or reverse already developed paralysis. Clinically evident paralysis resolved after a few days, but the neurophysiological abnormalities lasted for weeks. No permanent neurological damages were noted at 6 to 9 months after the snake bite.

Introduction

Globally snake envenoming is a major cause of morbidity and mortality in the rural tropics [1]. Neurotoxicity causing paralysis is one of the major clinical syndromes of snake envenoming, and occurs mainly with elapids (Australasian elapids, American coral snakes, Asian kraits and some cobra species)[2,3].Envenoming may result in prolonged hospital stay if ventilatory support is available or significant mortality where such medical resources are not available. Despite the magnitude of the health impact of neurotoxic snake envenoming, it continues to be associated with several unresolved issues of clinical importance[2].

Envenoming due to krait (Genus: *Bungarus*) bites is a common, serious health issue in South and South-East Asia. Common krait (*Bungarus caeruleus*) is distributed throughout South Asia, and is responsible for large numbers of cases of severe neurotoxic envenoming each year[4]. It results in a descending flaccid paralysis progressing to life threatening respiratory paralysis unless mechanical ventilation is available[5–7]. Krait bites typically occur at night and are not painful, so many patients do not notice the bite and continue sleeping[7–10], which delays medical care.

Neuromuscular paralysis in krait envenoming is characterized by progressive descending paralysis. Krait venom contains β-bungarotoxins, which are presynaptic neurotoxins with phospholipase A_2 activity and considered to be the major cause of paralysis[11]. The pre-synaptic action is irreversible and is the reason that once paralysis develops it is not reversed with antivenom[12].

The pathophysiology of neuromuscular paralysis in snake envenoming remains poorly understood. This is due to the lack of detailed clinical studies of authenticated bites that report the time course of paralysis, including the recovery of paralysis, and very few studies of neurophysiological function in snake envenoming[2]. There is also little evidence for the effectiveness of antivenom in reversing neuromuscular paralysis[13,14]. Neurophysiological testing could provide objective evidence of the progression and recovery of neurotoxicity, and the response to antivenom. Previous neurophysiological testing in krait bites with nerve conduction studies and repetitive nerve stimulation tests, provide some information on krait neurotoxicity[15]. Single fibre electro-myography (sfEMG) is a more sensitive test of neuromuscular function[16]. It has only been reported in one study of neuromuscular paralysis from Papuan taipan envenoming.[17]Better methods to measure neuromuscular dysfunction in neurotoxic envenoming will improve our understanding of neuromuscular paralysis and the clinical benefit of antivenom.

This study aimed to describe detailed neurological examinations in patients with krait envenoming, the use sfEMG to objectively measure the time related progression and recovery of neuromuscular paralysis and the relationship to serum venom concentrations.

Materials and Methods

Ethics statement

Ethical approval was granted by the research ethics review committees of the University of Peradeniya, Sri Lanka, the Rajarata University of Sri Lanka, and Monash University, Australia. Written informed consent was sought prior to the recruitment of all patients. For patients aged 16 and 17 years consent was also obtained from the patient's parent or guardian.

Study setting and the patients

This study is part of a prospective study of all snakebites admitted from April to October 2014 to the Teaching Hospital, Anuradhapura, a tertiary care centre and the largest hospital in the north central province of Sri Lanka. The region has one of the highest incidences of snakebite in Sri Lanka.

Patients aged 16 years or over with definite common krait (*B. caeruleus*) bites were recruited. Cases were authenticated as common krait bites by either expert snake identification or detection of krait venom in patient serum. All patients presenting with live or dead specimens of the offending snakes (Fig 1A), had the snake identified by one author (AS) who is a herpetologist. Patients with clinical suspicion of a common krait bite had serum tested for krait venom by venom-specific enzyme-immunoassay.

Data collection

All patients with suspected krait envenoming had a complete neurological examination on admission to hospital, then repeat examinations every 2h for the first 24h post-bite, and then every 4h for the remainder of their hospital stay. After discharge patients had a full neurological examination at six weeks and six months post-bite to detect residual neurological impairment. The Medical Research Council scale for muscle strength[18] was used wherever appropriate. For the assessment of respiratory muscles, tidal volume was measured using a spirometer. Ptosis was graded from grade I to III using a visual analogue scale, with complete ptosis being grade III. Weak or sluggish eye movements in one or all directions was considered to be partial ophthalmoplegia and absent eye movements in all directions, complete ophthalmoplegia. At each time point patients were specifically examined for features of autonomic neurotoxicity

Fig 1. Common krait. An adult common krait (Bungarus caeruleus) specimen, 92.5 cm in total length, that caused severe neuromuscular paralysis.

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(heart rate, blood pressure, lacrimation, sweating and salivation), central effects (level of consciousness and occulocephalic reflexes) and myotoxic effects (muscle pain and tenderness, both local and general).

Clinical assessments were done by one author (AS) or medically qualified clinical research assistants. All assessments performed by clinical research assistants were reviewed by AS and approximately one third were reviewed by another medically trained author (SS). In addition to a neurological assessment, all patients had a full clinical examination on admission, at 12h and 24h post-bite, and then daily until discharged. Local effects included pain, swelling, paraesthesia or regional lymphadenopathy and non-specific systemic effects were defined as headache, nausea, vomiting or abdominal pain. All assessments were recorded using a pre-formatted clinical data form.

Antivenom administration was decided by the treating physician. All patients who received antivenom in this study received 20 vials of Indian polyvalent antivenom as the first dose from VINS Bioproducts (batch numbers: 01AS11119, 01AS11121, 01AS13100, 01AS14025, 01AS14026, 01AS14035). No patient in this study received a second antivenom dose. The antivenom infusion was ceased briefly for 5–10min in patients who developed anaphylaxis. Antivenom reactions were treated according to the attending physician with adrenaline, antihistamines and corticosteroids.

During review visits patients had a routine physical and neurological examination. They were also questioned specifically about the presence of neuromuscular effects that prevented them performing routine daily work, and about any recovery of local effects.

Patients were classified into three groups based on the presence and severity of neurotoxicity. The first group included patients who developed no clinical evidence of neurotoxicity. The second group was patients with mild neurotoxicity defined as the presence of one of the following clinical features; ptosis, ophthalmoplegia or facial muscle weakness, but not bulbar, respiratory or limb weakness. The third group was severe neurotoxicity defined as patients developing paralysis that involved bulbar and respiratory muscles requiring mechanical ventilation.

Single-fibre electromyography (sfEMG)

Stimulated sfEMG of the orbicularis oculi muscle was performed using disposable concentric needle electrodes (diameter: 0.3mm) and a portable Medelec Synergy N2 EMG system (Medelec Synergy, UK). All tests were performed by AS at the bedside during the hospital admission and in a separate examination room during review visits at 6 weeks and 6 months. In order to establish the baseline values for stimulated sfEMG jitter for normal Sri Lankan subjects, 29 healthy individuals had sfEMG performed under the same conditions. The normal upper limits of jitter for individual fibres and individual subjects were established as 30μs and 20.6μs respectively[19].

Stimulation of the suprazygomatic branches of the facial nerve was by monopolar needle electrode at a frequency of 10Hz, with the stimulus delivered as rectangular pulses of 0.1ms. Stimulation intensity was increased until the appearance of visually discernible twitches of the orbital part of the orbicularis oculi muscle. Stimulus strength did not exceed 2mA. All recordings were done after the stimulus intensity had reached supra-threshold for the fibre being examined. Recording and analysis of the sfEMG followed Kouyoumdjian and Stålberg[20] and the jitter of individual fibres was expressed as a mean consecutive difference (MCD). Neuromuscular block was where there was no transmission across the neuromuscular junction in fibres on the sfEMG recording.

Common krait venom specific enzyme immunoassay

Blood samples from patients with suspected krait bites were collected on admission and at 1, 4, 8, 12 and 24h post-bite, and daily thereafter. All samples were immediately centrifuged, and serum aliquoted and frozen at -80°C. Venom was quantified using a sandwich enzyme immunoassay as previously described[21–23]. In brief, rabbit IgG antibodies (provided by University of Rajarata) were bound to microplates as well as conjugated to biotin for the sandwich enzyme immunoassay, detecting with streptavidin-horseradish peroxidase. The lower limit of detection for the assay was 0.2ng/ml.

Serum creatinine kinase assay

Creatine kinase (CK) activity was measured in the serum samples of patients obtained 24h post-bite, or a close to this time point as possible if the 24h sample was unavailable. Creatine kinase was measured using a commercially available assay kit using the CK-NAC Reageant (Creatine Kinase, activated by N-acetylcysteine, Thermo Scientific, Middletown, VA, USA).

Analysis

Analysis of clinical and sfEMG data was done using Prism, version 6.05 (GraphPad Software, Inc.). Continuous variables were reported as medians, range and interquartile range (IQR).

Jitter values and creatine kinase concentrations of different patient groups were analysed using one-way ANOVA followed by multiple comparison tests.

Results

During the study period 773 snakebite patients were admitted and 38 of these were suspected common krait bites. Thirty one patients had the offending snake positively identified as a common krait and two others were positive for krait venom in their blood. The remaining five cases were only suspected common krait bites based on circumstances of the bite, but had no features of local or systemic envenoming, despite having fang marks. None of these patients had a previous history of neurological disorders or therapeutic agents known to cause neurotransmission abnormalities. Table 1 provides the demographic features of the 33 confirmed cases of common krait bites.

Table 1. Demographic features of 33 definite Indian Krait bite patients.

Age (median, range)	35	16–78
Sex (male)	24	73%
Occupation (Farmer)	22	67%
(manual laborer)	5	15%
(security personnel)	2	6%
(student)	2	6%
(Other)	2	6%
Bite time (night: 1900–0600 hrs)	30	91%
Activity (Sleeping on the floor)	22	67%
(Sleeping on the bed)	3	9%
(household work)	4	12%
(Other)	4	12%
Place (home)	25	76%
(hut in the farmland)	4	12%
(Home garden)	2	6%
(other)	2	6%
Site of bite (lower limbs)	12	36%
(upper limbs)	12	36%
(Trunk)	4	12%
(head)	1	3%
(site uncertain)	4	12%
Under influence of alcohol when snake bite took place	4	12%
Distance from the place of bite to the nearest hospital (median, range; in km)	6	1.5–20
Mode of transport used to reach the nearest hospital (motorbike)	17	52%
(Three wheeler)	10	30%
(other)	6	18%
Time since bite to reach nearest hospital (median, range; in hours)	0.87	0.16–4.5
Time since bite to reach the study hospital (median, range; in hours)	3.3	1.5–7.0
First aid and home remedies (washed the bite site)	3	9%
(local application of lime juice)	3	9%
(application of tourniquet proximal to the bite site)	3	9%
(herbal decoctions)	3	9%
Previous history of snakebites	3	9%
Total length of the offending snake (median, range; in cm)	90.6	32.2–122.2
Sex of the offending snake (female)	15	58%

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Clinical effects during hospital stay

Eight patients who brought the offending snake and had evidence of a bite with fang marks, did not develop neurotoxic features during their hospital stay. None of these received antivenom (Table 2).

Eight patients had mild neurotoxicity and six of these received antivenom (Table 2). All these patients had partial ptosis on admission. In one patient, this progressed to complete ptosis with partial ophthalmoplegia and mild facial weakness within 12h of the bite, but 2h after antivenom was given. Two patients not receiving antivenom did not have any progression of neurotoxicity. All eight patients were fully recovered the next day and were discharged 20 to 32h post-admission.

Seventeen patients developed severe neurotoxicity with a range of neurotoxic clinical features on admission (Table 2). Three patients appeared to have altered consciousness and had no respiratory effort on admission with oxygen saturations $< 50\%$. These three were immediately intubated and ventilated. All 17 patients were given antivenom a median of 3.5h post-bite

Table 2. Comparison of the clinical effects and treatment of the patients who developed no neurotoxicity, mild neurotoxicity and severe neurotoxicity following common krait envenoming.

	No neurotoxicity	Mild neurotoxicity	Severe neurotoxicity
Number of patients	8	8	17
Hours from bite to hospitalization: median (range)	3.0 (1.5–6.0)	3.0 (1.0–6.0)	3.3 (1.5–7.0)
Days of hospital stay: median (range)	2 (1–3)	3 (2–3)	8 (4–12)
Neurotoxicity			
Maximum ptosis observed			
• Partial	-	7 (87.5%)	5 (29.4%)
• Complete	-	1 (12.5%)	12 (70.6%)
Maximum ophthalmoplegia observed			
• Partial	-	1 (12.5%)	3 (17.6%)
• Complete	-	0	14 (82.4%)
Strabismus	-	-	11 (64.7%)
Facial weakness	-	1 (12.5%)	17 (100%)
Neck flexion weakness (power <5)	-	-	15 (88.2%)
Difficulty in swallowing	-	-	17 (100%)
Low pitched voice	-	-	12 (70.6%)
Tidal volume <250ml	-	-	17 (100%)
Reduced upper limb power (Power <5)	-	-	12 (70.6%)
Reduced lower limb power (power <5)	-	-	5 (29.4%)
Diminished or absent deep tendon reflexes			9 (52.9%)
Intubation and mechanical ventilation	-	-	17 (100%)
Autonomic features	-	-	6 (35.3%)
Other features of envenoming			
Generalized myalgia	3 (32.7%)	2 (25%)	7 (41.2%)
Generalized muscle tenderness	2 (25%)	2 (25%)	7 (41.2%)
Local envenoming	5 (62.5%)	6 (75%)	9 (52.9%)
Non-specific systemic envenoming	-	5 (62.5%)	13 (76.5%)
Treatment			
Antivenom given	-	6 (75%)	17 (100%)
Hours from bite to antivenom: median(range)	-	7.5 (2.8–13.0)	3.5 (2.8–7.2)
Adverse reactions to antivenom	-	6 (100%)	13 (76.5%)

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(2.8–7.2h). Antivenom therapy did not stop progression of neurotoxic features in any patient and intubation and mechanical ventilation was required within 7h of the snake bite in all 17 patients. The clinical features of neurotoxicity progressed in a descending order from eyes to peripheral limbs (Fig 2A). Five patients developed complete limb paralysis with areflexia occurring within 8h of the bite (Figs 1C and 2A).

Neuromuscular function began to recover in all 17 patients on average 30h post-bite (22–51h). Muscle groups recovered in the reverse order to that in which paralysis developed. Fig 2B shows the time scale of the recovery of neurotoxic features. Patients were extubated a median of 96h post-bite (54–216 h).

One patient with severe paralysis and no motor function appeared to be in a deep coma with absent brainstem reflexes 12h post-bite. There was no recordable response on sfEMG done 10h and 16h post-bite. The patient had slight movement of the toes in response to a sternal rub 26h post-bite, but no occulocephalic reflex. The occulocephalic reflex recovered 6h later, followed by gradual ascending recovery of all motor function and sfEMG jitter.

All 33 patients were discharged with no clinically detectable neuromuscular paralysis. However, on discharge 23 patients had no pain, touch or temperature sensation in an area of 3–8cm diameter around the bite site. Of 23 patients who received antivenom, 19 developed an adverse reaction and six of these developed severe anaphylaxis with hypotension (systolic blood pressure <90mmHg) and tissue hypoxia (SpO_2<92%) that required resuscitation. Of these, three were intubated for the antivenom reaction before they developed respiratory muscle paralysis due to envenoming.

Clinical effects: Long term

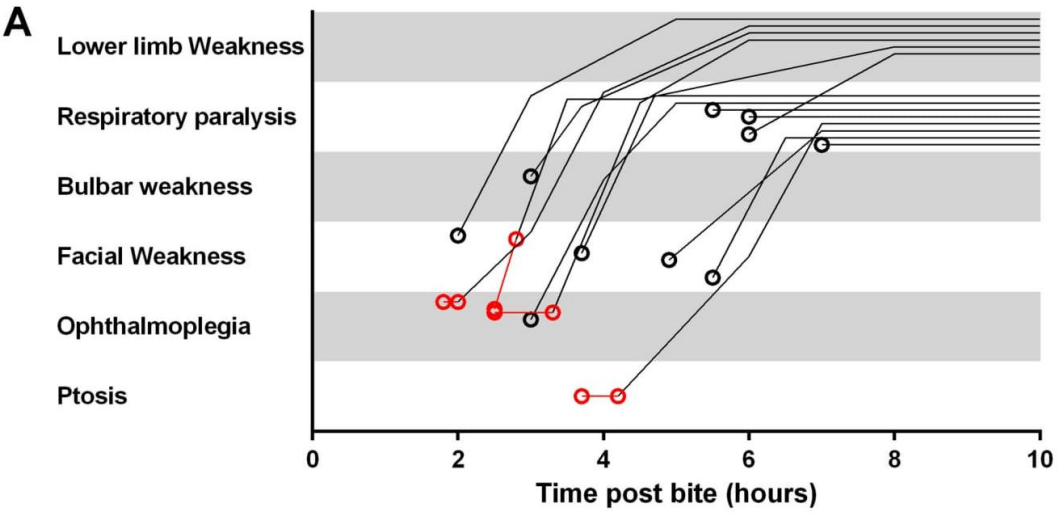
At 6 weeks, six of eight patients with no acute neurotoxicity, seven of eight with mild neurotoxicity and 14 of 17 with severe neurotoxicity were reviewed. None had clinically detectable neuromuscular paralysis. Sixteen patients (five mild and 11 severe neurotoxicity) still had absent pain, touch and temperature sensation around the bite site (4–7cm diameter area).

At 6 to 9 months after the krait bite, six with no acute neurotoxicity, five with mild neurotoxicity and ten with severe neurotoxicity were reviewed and there was no evidence of clinical neuromuscular dysfunction. All sensory abnormalities around the bite site had resolved and the patients stated the feeling of numbness around the bite site disappeared 2 to 3 months post-bite.

sfEMG

sfEMG were undertaken in seven non-envenomed patients, seven with mild neurotoxicity and ten with severe neurotoxicity. On admission, all patients with no neurotoxicity or mild neurotoxicity (n = 14) had a median MCD in jitter on sfEMG similar to normal controls (Figs 3A and 4A). Two patients with mild neurotoxicity had neuromuscular blockade in 3% and 12% of fibres respectively. The other patients had no neuromuscular block. Jitter was not markedly increased and no blocks were seen in the no neurotoxicity and mild neurotoxicity groups 6–12h post-bite (n = 5, n = 5; Fig 4B). The median MCD was also not different from normal on discharge, at 6 weeks and at 6 to 9 months in all patients in these two groups.

All patients with severe neurotoxicity had a high median MCD in jitter compared to normal, with most having neuromuscular blockade on admission (Figs 3B and 4A). At 6–12h post-bite, jitter was markedly increased and there was increased neuromuscular block in recorded fibres (Figs 4B, 5A and 5B). In two patients who had severe neurotoxicity, sfEMG recordings showed no response during this period (9 and 10.5h post-bite respectively), indicating complete neuromuscular blockade. Eleven patients received atracurium for rapid sequence


A
Lower limb Weakness
Respiratory paralysis
Bulbar weakness
Facial Weakness
Ophthalmoplegia
Ptosis
0
2
4
6
8
10
Time post bite (hours)

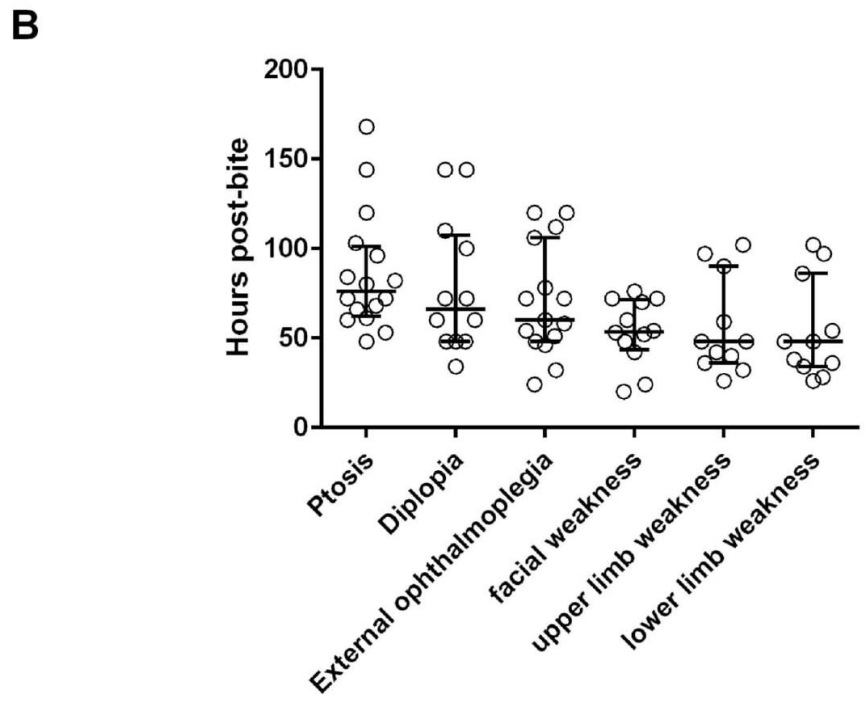

B
Hours post-bite
200
150
100
50
0
Ptosis
Diplopia
External ophthalmoplegia
facial weakness
upper limb weakness
lower limb weakness

Fig 2. Time related change of neuromuscular paralysis. A, Noodle plot showing the evolution of paralysis in 14 patients with severe neurotoxicity. Note the pre-antivenom events are in red; **B,** Scatter plots of the time taken for the complete resolution of five major clinical effects of neurotoxicity (muscle groups), including ptosis, diplopia, external ophthalmoplegia, facial weakness, upper limb weakness and lower limb weakness in the 17 patients with severe neuromuscular paralysis.

doi:10.1371/journal.pntd.0004368.g002

intubation. The post-intubation sfEMG for these patients was done a median of 5.8h (4.5–23.2h) after receiving the atracurium dose.

In all patients with severe neurotoxicity, sfEMG24–36h post-bite showed improved jitter (decreased median MCD) and decreased blocks compared to 6–12h post-bite (Fig 5A and 5B). On discharge, the median MCD in jitter on the sfEMG was still abnormally high in 11 patients. Of these, three had 3 to 10% fibres with blocks (10–12 days post-bite). At 6 weeks, seven of the

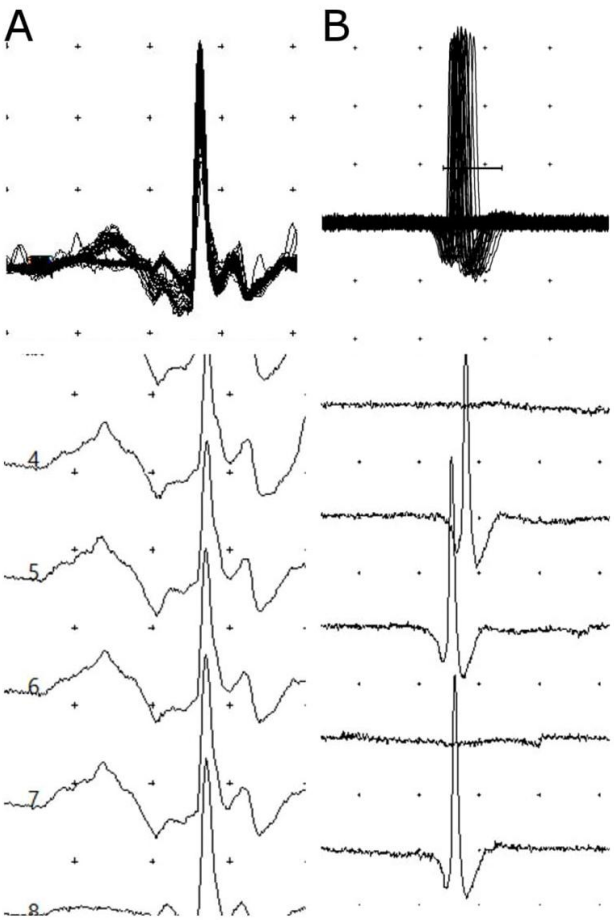


Fig 3. Superimposed and non-superimposed sfEMG recordings of the orbicularis oculi muscle of patients following krait bites. A, recordings of a patient on admission with no neurotoxicity indicating the normal jitter (14.5μs) and no blocks; **B,** high jitter (61.6μs) with intermittent blocks seen in a patient on admission with severe neuromuscular paralysis. (The distance between two dots represents 200μV vertically and 3ms horizontally.)

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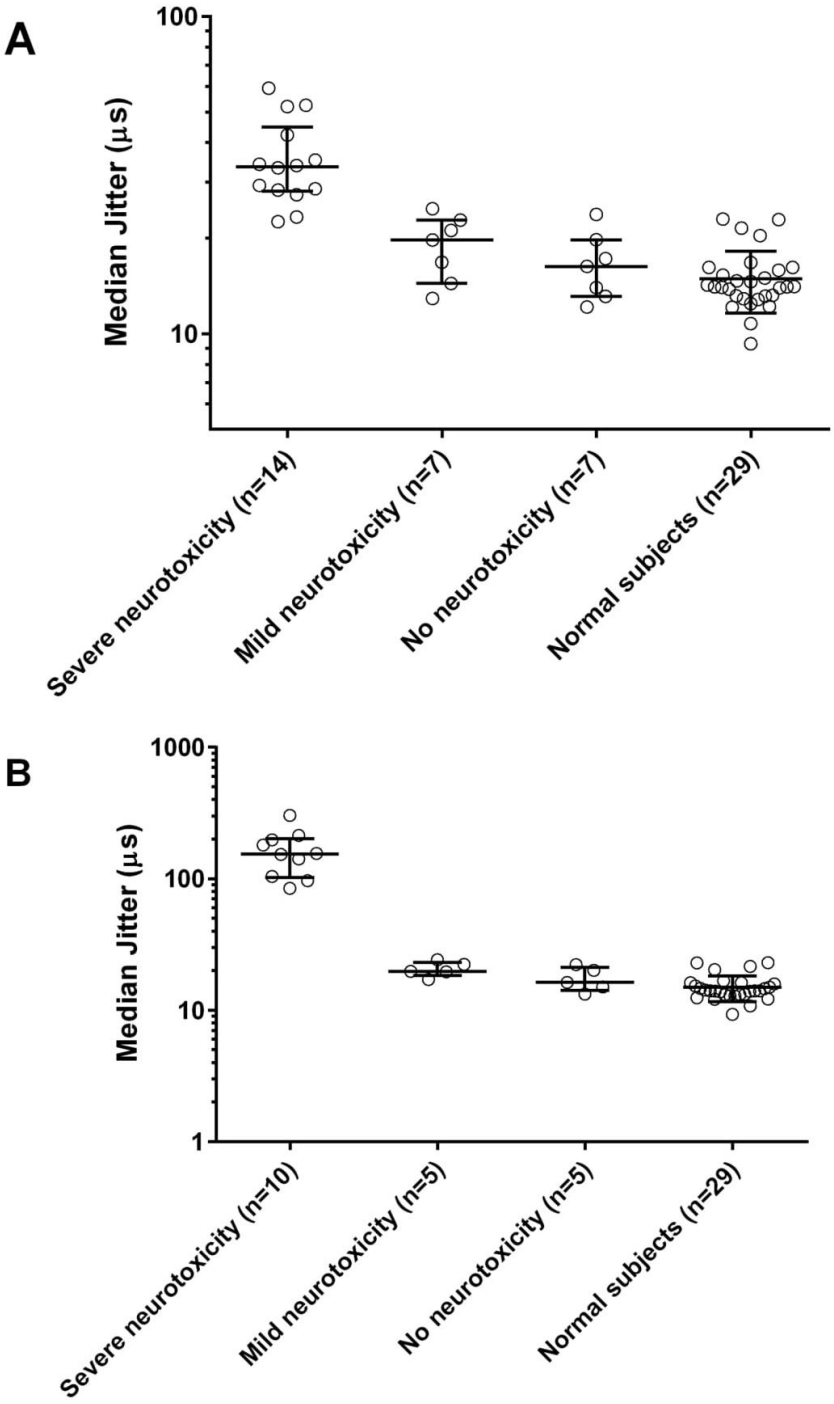
A
100
10
Median Jitter (μs)
Severe neurotoxicity (n=14)
Mild neurotoxicity (n=7)
No neurotoxicity (n=7)
Normal subjects (n=29)
B
1000
100
10
1
Median Jitter (μs)
Severe neurotoxicity (n=10)
Mild neurotoxicity (n=5)
No neurotoxicity (n=5)
Normal subjects (n=29)

Fig 4. sfEMG jitter comparison across no, mild and severe neurotoxicity groups. Scatter plots of the median MCD in jitter of the three groups of patients with common krait bites on admission **(A)** and 6–12 hours after the bite **(B)** compared to the median MCD in jitter values of normal subjects. Median and interquartile range is shown for each group in the graph. On both occasions, the severe neurotoxicity group has significantly high median jitter compared to the normal subjects (P<0.0001; one-way ANOVA followed by multiple comparison test).

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fourteen patients reviewed still had high MCD jitter values (>22.6μs) and six of them had blocks in 3 to 13% of the recorded fibres. At 6 to 9 months, ten of these patients were reviewed and all had normal jitter with no blocks recorded.

Measurement of venom concentrations

Krait venom was not detectable in the eight non-envenomed patients. Of the six patients with mild neurotoxicity who received antivenom, pre-antivenom blood samples of four patients were available but no venom was detected in any. The two patients with mild neurotoxicity who did not receive antivenom had no detectable venom in blood. Nine of the 17 patients with severe neurotoxicity had pre-antivenom blood samples available, and krait venom was detected in eight (0.3 to 52.2ng/ml) (Fig 6). None of the post-antivenom samples had detectable free venom, indicating rapid and persistent binding of venom by antivenom (Fig 6).

Serum creatine kinase concentrations

Serum samples were available in 32 of the 33 study participants and had a median creatine kinase concentration of 43 U/L (8 to 274 U/L). There were no significant differences in the creatine kinase concentrations between the mild, moderate and severely envenomed groups.

Discussion

In this cohort of patients with definite common krait envenoming, about half developed life-threatening neuromuscular paralysis that did not appear to be prevented by or respond to antivenom treatment. Patients who had sfEMG performed had increased jitter and increased neuromuscular block that correlated with the clinical severity. The neurophysiological abnormalities improved in line with clinical recovery but were still abnormal 6 weeks after the bite, despite the patients being clinically normal. The prolonged high jitter during the recovery phase may represent immaturity of the motor nerve terminals undergoing the re-innervation process. Excepting the three patients who were intubated due to antivenom reactions, all other patients were intubated due to bulbar weakness and/or respiratory paralysis, which developed within 7h of the bite. This demonstrates that severe neuromuscular paralysis develops rapidly. Based on this finding it appears that patients who do not develop bulbar weakness or respiratory paralysis within 12h of the bite, are highly unlikely to develop severe paralysis. These figures are largely in agreement with previous reports from Sri Lanka[7,10].

Although krait venoms contain both pre-synaptic neurotoxins (β-bungarotoxins) and post-synaptic neurotoxins (α-bungarotoxins), it is generally accepted that the pre-synaptic neurotoxins are more important in human envenoming[24,25]. Presynaptic neurotoxins cause irreversible injury. For example, the major pre-synaptic neurotoxin from the Chinese many banded krait (*Bunragus multicinctus*), β-bungarotoxin, causes depletion of synaptic vesicles followed by destruction of the motor nerve terminals in the isolated mouse phrenic nerve-hemidiaphragm preparation and the soleus muscle of the mouse hind limbs[12]. Common krait venom contains β_1–β_5-caerulotoxins, a group of toxins similar to β-bungarotoxin, that are most likely responsible for the paralysis in common krait bites.[26]

Ultrastructural damage and functional injury caused by β-bungarotoxin to the motor nerve terminals recovers over about 7 days[24]. In our study, clinically detectable neurotoxicity

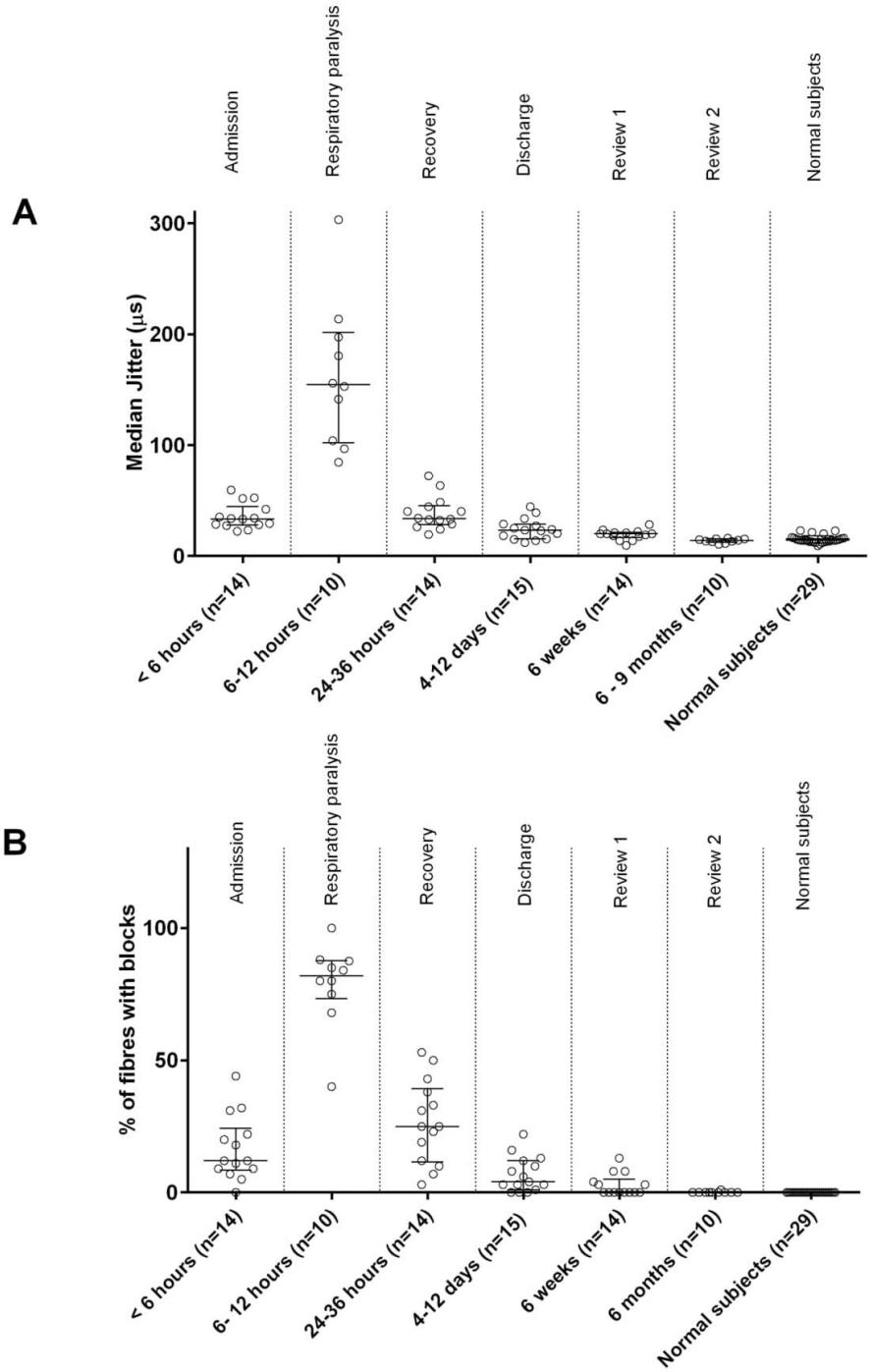
A
Admission
Respiratory paralysis
Recovery
Discharge
Review 1
Review 2
Normal subjects
Median Jitter (μs)
300
200
100
0
< 6 hours (n=14)
6-12 hours (n=10)
24-36 hours (n=14)
4-12 days (n=15)
6 weeks (n=14)
6 - 9 months (n=10)
Normal subjects (n=29)
B
Admission
Respiratory paralysis
Recovery
Discharge
Review 1
Review 2
Normal subjects
% of fibres with blocks
100
50
0
< 6 hours (n=14)
6- 12 hours (n=10)
24-36 hours (n=14)
4-12 days (n=15)
6 weeks (n=14)
6 months (n=10)
Normal subjects (n=29)

Fig 5. Time related change of sfEMG jitter and blocks in patients developed severe neurotoxicity. A, Scatter plots showing the time related change of the median MCD in jitter of the 17 patients with severe neurotoxicity, compared to normal subjects. Note the high median of the MCD in jitter values seen even at 6 weeks after the snakebite, compared to the normal subjects; **B,** scatter plots showing time related changes in the percentage of recorded fibres with neuromuscular blocks in these patients at the same times. Neuromuscular blocks are still present 6 weeks after the snakebite.

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resolved 4 to 12 days post-bite, but subclinical neuromuscular dysfunction remained for at least 6 weeks in some patients. This may be due to some motor nerve terminals taking longer to fully recover and re-innervate muscle, and the already re-innervated muscle fibres compensating for recovering fibres. sfEMG directly measures transmission across the neuromuscular junction by measuring security of transmission to individual muscle fibres, so is more sensitive than other neurophysiological investigations and likely to detect abnormal neurotransmission in recovering fibres. Therefore, sfEMG measurements are likely to give a more accurate measure of the recovery time of neuromuscular dysfunction in snake envenoming.

One previous study reported abnormalities in nerve conduction one year after the snake bite, particularly in patients presumed to be bitten by elapids[27]. However, this study by Bell et al. only reported nerve conduction studies one year post-bite, with no comparison at the time of the bite. In addition, these nerve conduction studies are not as sensitive as sfEMG, making it difficult to interpret their results. sfEMG recordings in our study demonstrate the complete recovery of neuromuscular function at 6 months, even in the patients who had severe neuromuscular dysfunction within 24h post-bite, suggesting the findings of Bell et al. one year post-bite are unlikely to be related to the snake bite.

In all 17 patients with severe neuromuscular paralysis, the peak or most severe effects, both clinical and neurophysiological, were observed during the first 24h after the bite. Patients then began to recover during day two, with considerable improvement in both neuromuscular jitter and neuromuscular block compared to day one. In contrast, animal experiments show that the initiation of re-innervation with motor nerve terminal sprouting occurs three to five days after

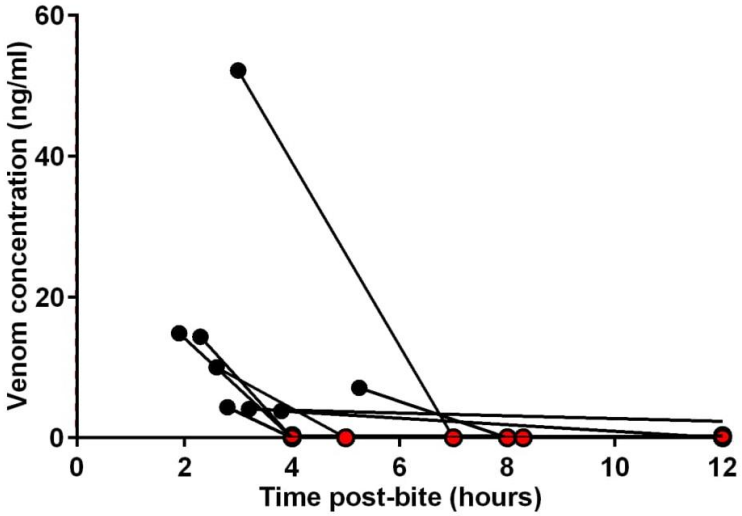

Fig 6. Serum venom concentrations of 9 patients with pre-antivenom blood samples, in relation to the time of initiation of the antivenom therapy. Pre-antivenom venom concentrations are indicated by filled black circles and post-antivenom concentrations by filled red circles.

doi:10.1371/journal.pntd.0004368.g006

venom inoculation [12,25]. The reason for the more rapid and marked improvement on the second day in humans is unclear. One possible reason is that those muscle fibres of the orbicularis oculi which are already denervated due to toxin induced injury by the second day, fail to produce any response with electrical stimulation. This means they are no longer part of the sampled fibres in the sfEMG recording. Motor nerve terminals that were less damaged are then the ones that make up the majority of the sampled fibres falsely improving the measurement. Although we are unable to exclude this on sfEMG, there was also clinical recovery in patients during the second day, based on the re-appearance of deep tendon reflexes and the plantar reflex. Further investigation is required to understand the pathophysiological basis of these observations in human recovery.

Serum venom concentrations depend on multiple factors including the venom dose delivered, the rate of venom absorption and therefore the time post-bite, individual patient factors that affect the pharmacokinetics of the venom (e.g. effect of patient size/weight, renal function) and the sensitivity of the assay. Kraits inject very small amounts of venom during their bite, explaining the low venom concentrations in our study and previous studies.[9,28] The eight patients who had no detectable venom in blood and had no features of envenoming, were 'dry bites', as previously seen among patients with common krait bites[7,9]. The absence of detectable venom in patients with mild neurotoxicity is more difficult to explain. The most likely explanation is that the assay is not sensitive enough to detect the small amount of such a potent elapid venom that can cause minor toxicity. A similar phenomena is seen with Australian brown snake (*Pseudonaja* spp) venom where < 0.2ng/mL can result in a mild coagulopathy [29]. An additional explanation is that this is due to late sampling times after the peak venom concentrations, when venom has distributed out of the central compartment. Either way these patients are likely to have had small venom doses from the bite. This is also the likely explanation for the two patients with mild neurotoxicity who did not receive antivenom and did not worsen. In the severe neurotoxicity group, there was also large variations in the venom concentrations (Fig 6).

Presynaptic neurotoxins cause irreversible nerve injury, so neurotoxicity is expected not to respond to antivenom once it has developed[24]. Despite most patients receiving early antivenom and antivenom rapidly clearing free venom in blood, the paralysis worsened and required mechanical ventilation in all 17 patients for several days. In the mildly neurotoxic patients one patient progressed despite antivenom and two patients who did not receive antivenom had similar outcomes to those receiving antivenom. Antivenom cannot reverse neuromuscular injury and recovery occurs through the natural nerve terminal repair[24,25]. These results demonstrate that Indian polyvalent antivenom is efficacious (binds venom) but is not effective for common krait envenoming in Sri Lanka, because of the irreversibility of the presynaptic neurotoxicity.

Antivenom was able to clear circulating free venom, so given early enough antivenom may still be beneficial in preventing progression of neuromuscular dysfunction. This has been demonstrated in studies of Papuan taipan bites where early antivenom (<6h post-bite) reduced the number of patients requiring intubation[17]. Unfortunately, the majority of patients (19/23) who received antivenom in our study developed acute adverse reactions, including some with life threatening anaphylaxis. Therefore, the safety and benefits of antivenom need to be carefully weighed up along with the clinical status of the patient, before deciding on antivenom therapy.

The majority of patients in this study reached a primary care centre early, but because of concerns about antivenom reactions, antivenom was not usually administered prior to transfer to the study hospital. If Indian polyvalent antivenom had a lower reaction rate, this would encourage primary care doctors to administer antivenom as early as possible, and before

transferring them to tertiary care hospital. Such an approach would help prevent neurotoxicity in the majority of cases, without risk of life-threatening adverse reactions.

Although generalized myalgia and muscle tenderness were observed in some patients, the normal serum creatine kinase concentrations in patients is consistent with common krait envenoming not causing myotoxicity. Mildly elevated serum myoglobin levels were previously reported in one envenomed krait patient in Sri Lanka,[28] but serum myoglobin is not a very specific marker of muscle injury. Myotoxicity has been reported in envenoming by other krait species, including *B. niger* [30], *B. multicinctus* [31] and *B. candidus* [32]. However, in the study of *B. candidus* there were only mild elevations of creatine kinase, and the study of *B. multicinctus* only reports myalgia.

Coma has been previously reported in common krait envenoming [7,33]. In one study, two patients with deep coma were reported to have electroencephalogram abnormalities, abnormal brain stem visual and auditory evoked potentials, leading to the conclusion that krait venom can cause cortical and brain stem effects [33]. However peptide and protein toxins are unlikely to cross the blood brain barrier making this theoretically unlikely. In the present study, one patient with severe paralysis had deep coma, absent brainstem reflexes and no sfEMG recordings. Interestingly, there was a period of time when the patient had absent brain stem reflexes but some motor function, suggesting that the patient was more likely to have had severe paralysis mimicking coma, rather than coma itself. Similar observations have previously been made in snakebite patients in India [34–37]. The altered consciousness observed in three patients on admission was most likely due to hypoxia secondary to respiratory muscle paralysis, rather than any direct central effect of the venom.

sfEMG jitter results can be influenced by pre-existing medical conditions that affect the peripheral nervous system, such as myasthenia gravis, diabetes mellitus and leprosy. None of the patients in this study had a history of any of these conditions. Two-thirds of the patients were farmers who may have had pre-existing neurotransmission abnormalities secondary to chronic exposure to organophosphates. However, we did not see a difference in the jitter values of the present cohort of patients at 6 months compared to the normal subjects, so this is unlikely.

A limitation of the study was that sfEMG was only performed on the orbicularis oculi muscle. This was done because it is one of the muscles affected earliest in snake bite paralysis and it is convenient to access. The neuromuscular jitter and blocking correlated well with the clinical picture indicating that this muscle is likely to be representative of the neurophysiology of the neuromuscular paralysis in snake envenomed humans. Another limitation of the study was that the recovery of certain muscles, e.g. neck extensors, buccinator, bulbar muscles, could not be assessed while patients were intubated. In addition, while the patients were sedated, assessment of the power of voluntary contractions of muscles was not possible. Hence the exact sequence of muscle involvement, particularly during the recovery, could not be documented in some patients. Finally, it is important to consider the risk of sfEMG in patients with coagulopathy.

Our study highlights the usefulness of sfEMG as a biomarker, particularly as a research tool on snakebite neurotoxicity. The sfEMG and clinical findings suggest that recovery occurs more rapidly than expected, based on animal studies, and further work is required to explain this. The study confirmed that antivenom did not reverse neurotoxicity and that if antivenom is going to prevent neurotoxicity, it must be given much earlier, prior to development of any neurotoxic effects. A placebo controlled trial is required to determine if antivenom hastens the recovery in established neurotoxicity.

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Author Contributions

Conceived and designed the experiments: AS KM MS NAB SS GKI. Performed the experiments: AS KM SP PW NJD CJ. Analyzed the data: AS NAB GKI. Contributed reagents/materials/analysis tools: KM CJ. Wrote the paper: AS NAB MS SS GKI.

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CRITICAL CARE

Neurotoxicity in Russell's viper (*Daboia russelii*) envenoming in Sri Lanka: a clinical and neurophysiological study

Anjana Silva[a,b,c], Kalana Maduwage[c,d], Michael Sedgwick[c], Senaka Pilapitiya[b], Prasanna Weerawansa[b], Niroshana J. Dahanayaka[b], Nicholas A. Buckley[c,e], Sisira Siribaddana[b] and Geoffrey K. Isbister[c,d]

[a]Monash Venom Group, Department of Pharmacology, Monash University, Clayton, VIC, Australia; [b]Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura, Sri Lanka; [c]South Asian Clinical Toxicology Research Collaboration, University of Peradeniya, Peradeniya, Sri Lanka; [d]Clinical Toxicology Research Group, University of Newcastle, Callaghan, NSW, Australia; [e]Clinical Pharmacology, University of Sydney, Sydney, NSW, Australia

ABSTRACT

Context: Russell's viper is more medically important than any other Asian snake, due to number of envenoming's and fatalities. Russell's viper populations in South India and Sri Lanka (*Daboia russelii*) cause unique neuromuscular paralysis not seen in other Russell's vipers. **Objective**: To investigate the time course and severity of neuromuscular dysfunction in definite Russell's viper bites, including antivenom response. **Methodology**: We prospectively enrolled all patients (>16 years) presenting with Russell's viper bites over 14 months. Cases were confirmed by snake identification and/or enzyme immunoassay. All patients had serial neurological examinations and in some, single fibre electromyography (sfEMG) of the orbicularis oculi was performed. **Results**: 245 definite Russell's viper bite patients (median age: 41 years; 171 males) presented a median 2.5 h (interquartile range: 1.75–4.0 h) post-bite. All but one had local envenoming and 199 (78%) had systemic envenoming: coagulopathy in 166 (68%), neurotoxicity in 130 (53%), and oliguria in 19 (8%). Neurotoxicity was characterised by ptosis (100%), blurred vision (93%), and ophthalmoplegia (90%) with weak extraocular movements, strabismus, and diplopia. Neurotoxicity developed within 8 h post-bite in all patients. No bulbar, respiratory or limb muscle weakness occurred. Neurotoxicity was associated with bites by larger snakes ($p < 0.0001$) and higher peak serum venom concentrations ($p = 0.0025$). Antivenom immediately decreased unbound venom in blood. Of 52 patients without neurotoxicity when they received antivenom, 31 developed neurotoxicity. sfEMG in 27 patients with neurotoxicity and 23 without had slightly elevated median jitter on day 1 compared to 29 normal subjects but normalised thereafter. Neurological features resolved in 80% of patients by day 3 with ptosis and weak eye movements resolving last. No clinical or neurophysiological abnormality was detected at 6 weeks or 6 months. **Conclusion**: Sri Lankan Russell's viper envenoming causes mild neuromuscular dysfunction with no long-term effects. Indian polyvalent antivenom effectively binds free venom in blood but does not reverse neurotoxicity.

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Introduction

Snakebite is a significant health problem, particularly in the developing world, with most affected people being from rural farming communities. Although exact numbers are unavailable due to poor reporting, recent estimates suggest that South Asia records the highest snakebite morbidity and mortality worldwide.[1] Moreover, poor availability of safe and effective antivenoms has been a major issue in this region, risking the lives of many snakebite victims.[2]

Of the two known species of Russell's vipers, the western species (*Daboia russelii*) ranges in South Asia and the Eastern species (*D. siamensis*) ranges in Southeast Asia. Local envenoming, venom-induced consumptive coagulopathy, acute kidney injury, neuromuscular paralysis, and myotoxicity are considered the common manifestations of the Russell's viper envenoming.[3] The clinical effects of Russell's viper bites differ in different geographical regions, which has been attributed to geographical variation in the venom composition.[3–5] A particularly unusual geographical variation is the neurotoxic effects reported with Russell's viper bites in Sri Lanka and the southern part of India, which has not been observed in other places that this snake ranges.[3,6] In Sri Lanka, neuromuscular effects are common and reported in 70–88% of Russell's viper envenoming cases.[7–11] The most commonly reported clinical effects are ptosis, external ophthalmoplegia, and blurred vision, with bulbar weakness being less common and neurotoxicity requiring ventilation only reported rarely.[9] Despite its common occurrence, the pathophysiology of the neuromuscular paralysis in Russell's viper envenoming in humans remains unclear, including whether it is pre-synaptic or post-synaptic in nature.

Most studies of Russell's viper bites from Sri Lanka and South India simply report the clinical presence of symptoms and signs of neurotoxicity, and none has reported

CONTACT Geoffrey K. Isbister geoff.isbister@gmail.com School of Medicine and Public Health, University of Newcastle, c/o Calvary Mater Newcastle, Edith Street, Waratah, NSW 2298, Australia

neurophysiological investigations. Repetitive nerve stimulation, nerve conduction studies, and electromyography were shown to be useful for investigating the pathophysiology of neuromuscular effects of snake envenoming; in the few studies they have been used.[12] More detailed studies of clinical neurotoxicity and neurophysiological investigation of neurotoxicity in Russell's viper bites is essential to improve our understanding of the pathophysiology, prognosis, and potential effect of antivenom. Single fibre electromyography (sfEMG) is considered to be the most sensitive tool for detecting neuromuscular dysfunction.[13,14] This technique was successfully used in investigating the neuromuscular paralysis in Papuan Taipan envenoming.[12] Recently, we used this technique in studying the paralysis in common Krait envenoming [15], where it was able to detect subclinical abnormalities in neuromuscular transmission.

The objective of this study was to investigate the severity, time-related change, response to antivenom as well as the long-term effects of the neuromuscular dysfunction in authenticated cases of Sri Lankan Russell's viper envenoming.

Methods

Design and setting

We undertook a clinical and neurophysiological study of neurotoxicity in confirmed Russell's viper bites recruited as part of a 14-month prospective cohort of snakebite patients. Ethics clearance for this study was granted by the Human Research Ethics Committees of the University of Peradeniya, Sri Lanka, Rajarata University of Sri Lanka and Monash University, Australia. Written informed consent was obtained prior to the recruitment of all patients.

The study was undertaken at the Teaching Hospital, Anuradhapura, which is a tertiary care centre and the largest hospital in the north central province of Sri Lanka. This is a geographical area that has one of the highest incidences of snakebite in the country.

Patients

Patients, older than 16 years, who were admitted with confirmed Russell's viper bites, were recruited for this study. Cases were confirmed as definite Russell's viper bites by either positive identification of the snake specimen involved as a Russell's viper (Figure 1) by AS, who is a herpetologist, or by detection of the specific Russell's viper venom antigens in blood samples of the patients.

Clinical assessment

All patients were subjected to a complete neurological assessment on admission to hospital. Serial neurological assessments were then done at 1, 4, 8, 12 and 24 h post-bite, and then every 24 h until discharge. A selected sample of patients, representing the full neurotoxic spectrum, were subjected to full neurological examination at 6 weeks and 6 months after the bite, to detect residual neurological damage.

Figure 1. A juvenile Russell's viper from Anuradhapura, Sri Lanka.

To maintain consistency of the clinical assessments, objective clinical parameters were used as much as possible. The Medical Research Council scale for muscle strength [16] was used wherever appropriate. All clinical assessments were done by AS or medically qualified clinical research assistants. All assessments performed by clinical research assistants were reviewed by AS. The entire clinical data collection was under the direct supervision of SS who reviewed approximately one-third of the cases. In addition to the neurological assessment, all patients had a full clinical examination on admission, at 12 and 24 h post-bite, and then daily until discharged. Local effects included pain, swelling, parasthesia, or regional lymphadenopathy. Non-specific systemic symptoms were defined as headache, nausea, vomiting, or abdominal pain. All assessments were recorded using a pre-formatted clinical data form.

Interventions

Antivenom

The majority of patients received antivenom and a small proportion of these received their first dose of antivenom at a primary care hospital, before being transferred to the study hospital. Indian polyvalent antivenoms from VINS® Bioproducts, Telangana, India (batch numbers: 01AS11118, 1119, 1121, 1123, 3100, 4001, 4025, 4026, 4031) and Bharat Serums and Vaccines Ltd., Mumbai, India (batch number: A5311018) were used throughout the study period. Antivenom treatment was determined solely by the treating physician based on the clinical and laboratory evidence of systemic envenoming. Patients were administered 10–20 vials of antivenom as a standard procedure. Antivenom was administered in normal saline and infused over 1 h. If an acute adverse reaction to antivenom occurred, the infusion was stopped for 5–10 min and treated with adrenaline, antihistamines, and corticosteroids, as per the treating clinicians.

Single fibre electromyography (sfEMG)

Stimulated sfEMG of the orbicularis oculi muscle was performed using a portable Medelec Synergy EMG system (Medelec Synergy, Surrey, UK) and disposable concentric

needle electrodes in a convenience sample of 50 patients. Tests were performed once during the first 24 h and once on discharge and once each during the two reviews at 6 weeks and 6 months. All tests were performed by AS at the bedside during the hospital stay and in a separate examination room during the review visits.

In order to establish the baseline values for stimulated sfEMG jitter for normal subjects, 29 healthy individuals were subjected to sfEMG, under the same conditions (tropical conditions with high humidity and temperature).

Stimulation of the zygomatic branch of the facial nerve was done using a monopolar needle electrode at a frequency of 10 Hz, with the stimulus delivered as rectangular pulses of 0.1 ms. The intensity of the stimulation was increased until the appearance of visually detectable twitches in orbicularis oculi, not exceeding 2 mA. All recordings were done after the examiner was satisfied that the stimulus intensity had reached supramaximal intensity. Analysis of the recordings followed Kouyoumdjian and Stålberg (2011) [17] and the jitter was expressed as the mean consecutive difference.

Russell's viper venom enzyme immunoassays

Blood samples were collected from patients on admission and then 1, 4, 8, 12 and 24 h post-bite, and daily thereafter. Upon collection, the samples were centrifuged, aliquoted, and stored at −80 °C. The samples were used for measurement of Russell's viper venom using a sandwich enzyme immunoassay.[18,19] Briefly, rabbit IgG was raised against Russell's viper venom from Sri Lanka. Antibodies were bound to microplates as well as conjugated to biotin as detecting antibodies with streptavidin-horseradish peroxidase. The lower limit of detection of the assay is 2.5 ng/ml. In cases where no venom was detected in pre-antivenom samples or pre-antivenom samples were not available, the post-antivenom samples were subjected to a glycine-HCl treatment to dissociate venom from antivenom, and then tested for venom using the same enzyme immunoassay.

Data analysis

Analysis of clinical data, snake length, venom concentrations, and sfEMG data was done using PRISM, version 6.05 (GraphPad Software, Inc., La Jolla, CA). Continuous variables were reported as medians, range, and interquartile range (IQR). Jitter values of different patient groups were analysed using one-way ANOVA followed by multiple comparison tests. Comparison of factors that predicted patients with neurotoxicity versus those without neurotoxicity was done using the Mann–Whitney test. Comparison of patients subjected to sfEMG versus the remainder was done using the Mann–Whitney test for the continuous variables (age) and Fisher's exact test for dichotomous variables (sex, the proportion with coagulopathy, and proportion with ophthalmoplegia).

Results

During the study period, 773 confirmed snakebite patients were admitted to the Teaching Hospital, Anuradhapura, of which 285 were suspected to be bitten by Russell's vipers. Of these, 245 were confirmed Russell's viper bites and included in the study; 69 of these patients brought in the offending snake which was identified as a Russell's viper and 176 had Russell's viper venom detected in their blood samples. Of the 40 patients excluded because of failure to confirm the snake, 23/40 (58%) had evidence of systemic envenoming. The characteristics of the study participants are provided in Table 1.

Table 1. Characteristics of the 245 definite Russell's viper bite patients enrolled for this study.

Age (median, range in years)	41	16–70
Sex (male)	171	70%
Bites during day time (08:00–18:00 h)	155	63%
Activity: farming activities	83	34%
Walking on footpath/road	114	47%
Other	48	19%
Site of bite: lower limbs – ankle or below	211	86%
Lower limbs – above ankle	29	12%
Upper limbs – hands	6	2%
Time since bite to reach the primary care hospital (median, IQR; in hours)	0.5	0.2–1.0
Time since bite to reach the study hospital (median, IQR; in hours)	2.5	1.75–4.0

All the study participants had clearly visible fang marks. Local effects were common with bite site pain and swelling in 244/245 patients, but none developed local tissue necrosis or gangrene. Clinical evidence of systemic envenomings, such as coagulopathy, neurotoxicity, non-specific systemic envenoming features, and acute kidney injury, was present in 199 (78%) patients. Coagulopathy occurred in 166 (68%), with bleeding manifestations in 82 (50%). Urine output was less than 400 ml/24 h in 19 (8%) patients of which five developed anuria for 24 h requiring haemodialysis. Myalgia and muscle tenderness were reported in the bitten limb of 177 (72%) patients. Generalised myalgia and muscle tenderness occurred in 36 (14%) patients.

Neurological features

There were 130 (53%) patients who developed clinically detectable neurotoxicity. All 130 patients had bilateral partial (110) or complete ptosis 20 (15%). Ptosis and blurred vision were the earliest features in most patients, followed by ophthalmoplegia. Blurred vision occurred in 93 (36%) and external ophthalmoplegia in 118 (45%). The latter was characterised by weak or absent external eye movements in all, diplopia in 25 and divergent strabismus in 12 (Figure 2). No facial, jaw, neck, bulbar, respiratory, or limb muscle weakness was found in any patient. The time course of each neurological feature is shown in Figure 3. Neurotoxicity developed within 8 h in all cases and in 80% had clinically resolved within 3 d, the remainder all recovering within 9 d.

Table 2 compares the characteristics of patients with and without neurotoxicity. Neurotoxicity was associated with bites by longer snakes ($p < 0.0001$, Mann–Whitney test) (Figure 4a). Non-specific and specific systemic features of envenoming were generally more prevalent among the neurotoxic group. Two patients who had no neurotoxicity died due to severe

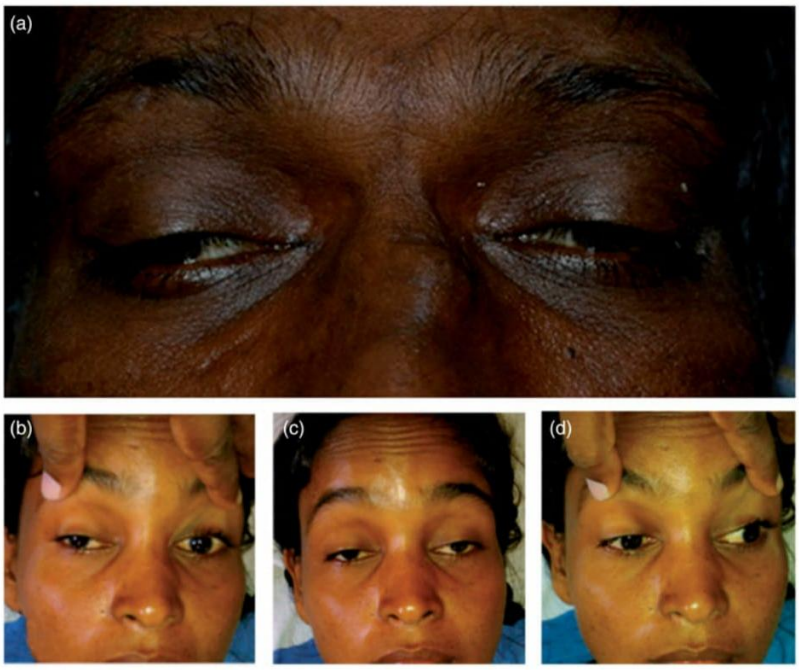

Figure 2. Neurological features of Sri Lankan Russell's viper envenoming. (a) Bilateral ptosis and divergent strabismus seen 24 h after a Sri Lankan Russell's viper bite. (b) A patient 36 h after being bitten by a Russell's viper: looking at an object in the right-hand side of the patient: note that the adduction of the left eye is impaired. (c) Neutral eye position of the same patient: note the divergent strabismus. (d) Looking at an object in the left-hand side of the patient: note that the adduction of the right eye is impaired.

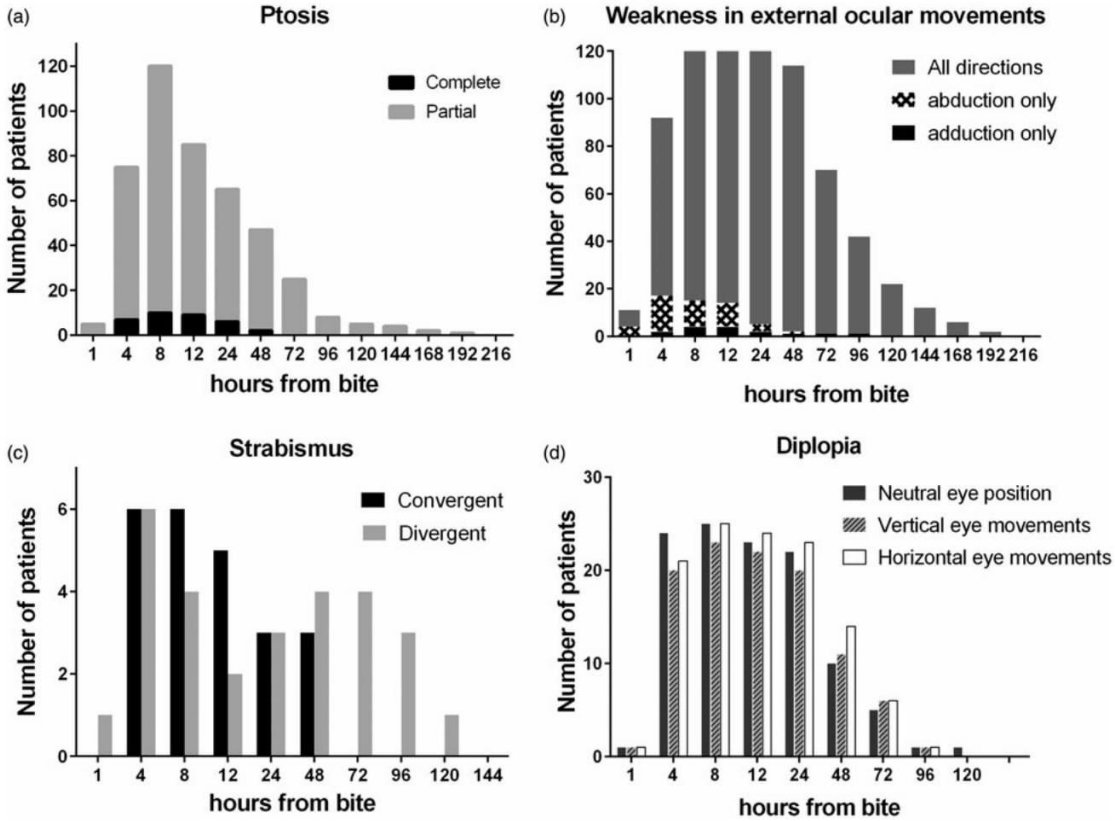

Figure 3. The time course of neurological features of 245 definite Sri Lankan Russell's viper bite patients[a]. (a) Complete and partial ptosis. (b) External ophthalmoplegia characterised by weak extraocular movements. (c) Divergent and convergent strabismus (d) and diplopia ([a]data available only for 20, 158 and 223 patients at 1, 4 and 8-h time points, respectively, due to the delay of hospital admission of individual patients).

Table 2. A comparison of the patients who developed neurotoxicity (n = 130) with the patients who did not develop neurotoxicity (n = 115) after Russell's viper bites.

	Neurotoxicity ($n = 130$)	No neurotoxicity ($n = 115$)
Length of the snake specimen: median (range)	64 (43–110) cm	31 (20–75) cm
Peak venom concentrations: median (range)	49 (2.5–2316) ng/ml (n=89)	12 (2.5–1478) ng/ml (n=41)
Time from bite to admission at study hospital: Median (IQR)	2.5 (1.5–4) h	2.75 (2–4) h
Effects of envenoming		
Local effects		
Local effects at the site of bite	130 (100%)	114 (99%)
Tender, enlarged regional lymph nodes	46 (35%)	45 (39%)
Non-specific systemic effects		
Nausea	118 (91%)	59 (51%)
Vomiting	86 (66%)	44 (38%)
Abdominal pain	73 (56%)	57 (50%)
Coagulopathy and bleeding manifestations		
Prolonged whole blood clotting time and INR >1.5	105 (81%)	61 (53%)
Haematuria	65 (50%)	13 (11%)
Gum bleeding	28 (22%)	5 (4%)
Haematemesis	12 (10%)	3 (3%)
Acute renal failure that required dialysis	5 (4%)	–
Muscle tenderness and myalgia	116 (90%)	61 (53%)
Local (in the bitten limb)	26 (20%)	9 (8%)
Generalised	6 (5%)	–
Treatment		
Antivenom received – total patients	130 (100%)	60 (52%)
First dose	130 (100%)	60 (52%)
Repeated doses	56 (43%)	21 (18%)
Time from snakebite to antivenom: median hours (IQR)	3.75 (2.75–4.75)	3.75 (2.75–5.25)
Outcome		
Length of the hospital stay: median days (range)	4 (2–31)	3 (2–8)
Deaths	4 (3%)[a]	2 (2%)[b]

Note: Antivenom reactions likely have played a role in the fatal outcomes of [a]three and [b]one patients.

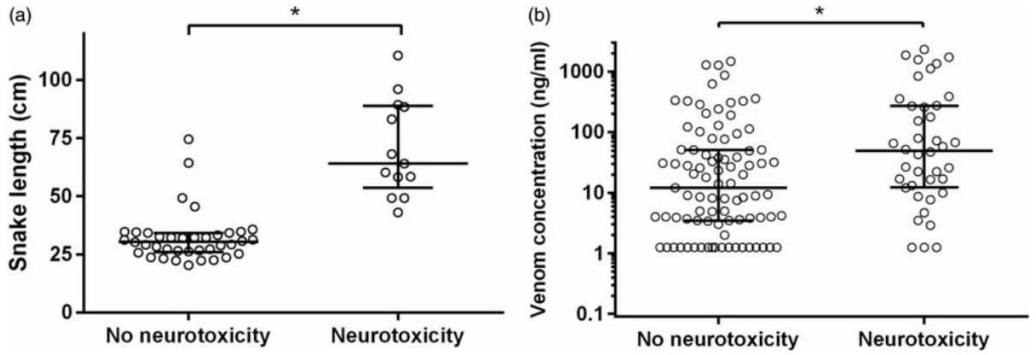

Figure 4. Association of neurotoxicity with the length of the snake specimen and the peak venom concentrations in blood samples. (a) Comparison of the lengths of the offending snake specimens involved in the envenoming of patients who developed ($n = 13$) and did not develop ($n = 38$) neurotoxicity. (b) Comparison of the peak venom concentrations in the blood samples of patients who developed ($n = 40$) and did not develop ($n = 89$) neurotoxicity. *$p < 0.05$ Mann–Whitney U-test.

coagulopathy and resulting massive gastrointestinal bleeding 3.5 and 58 h post-bite.

Twenty-seven patients who developed neurotoxicity and 23 who did not develop neurotoxicity were reviewed at 6 weeks and 6 months. None of the patients had clinically detectable neuromuscular abnormalities at these visits.

Treatment

All the neurotoxic patients and 52 non-neurotoxic patients (60%) received antivenom. Fourteen neurotoxic and eight non-neurotoxic patients received their first dose of antivenom at the primary hospital before they were transferred to the Teaching Hospital Anuradhapura. A total of 190 patients (78%) received antivenom with a median 20 vials (range: 10–90 vials). Repeat doses of antivenom were given to 77 (31%) patients. The presence of neurotoxicity on admission led to a more prompt administration of antivenom (Table 2). Bharat antivenom was used in 14 (7%) patients, and the rest received VINS® antivenom. More neurotoxic patients received repeat doses of antivenom. Of the 190 patients who received antivenom, 52 (27%) had no neurotoxicity at the time they were given antivenom. Of these, 31 (60%) still developed neurotoxicity which occurred over the subsequent 8 h.

Acute systemic hypersensitivity reactions occurred in 75/286 (26%) antivenom administrations and 68/190 (38%) patients receiving antivenom. Of this, 42 (22%) patients developed hypotension (systolic blood pressure <90 mmHg) and tissue hypoxia (oxygen saturation <92%) rapidly requiring resuscitation. Severe antivenom reactions (all caused by the

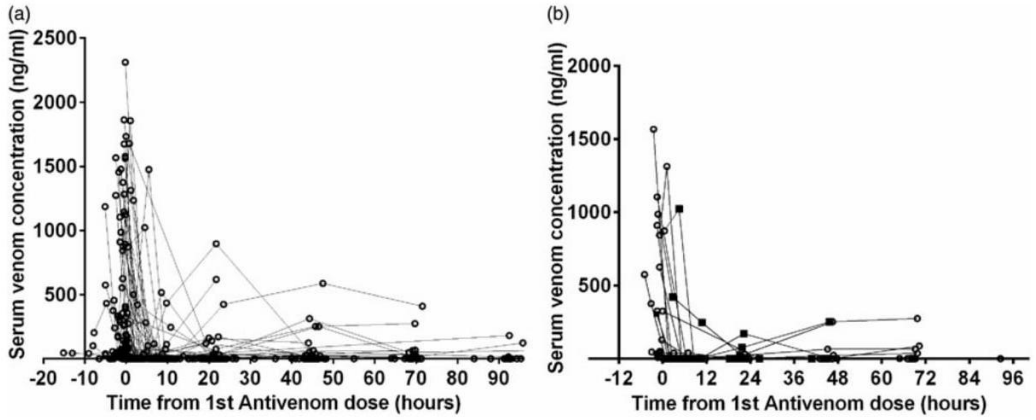

Figure 5. Serum venom concentrations in Sri Lankan Russell's viper bite patients. (a) Time course of serum venom concentrations with time zero being the time of first antivenom dose (N = 85 patients who had both pre and post-antivenom samples). (b) Time course of the venom concentrations for 19 patients who received antivenom before they developed clinically detectable neurotoxicity. The solid squares indicate the time points at which the neurological features were present. Open circles indicate the time points at which the neurological features absent.

VINS antivenom) have likely contributed towards fatal outcomes in three patients in the neurotoxicity group and one in the non-neurotoxicity group.

Venom concentrations

The median peak venom concentration was 25 ng/ml (range: 0.2–2313 ng/ml; IQR: 4–105 ng/ml) in 89 neurotoxic and 41 non-neurotoxic patients with blood samples positive for venom. 24 patients had very high venom concentrations, exceeding 1000 ng/ml. Those who developed neurotoxicity had higher peak venom concentrations compared to those who did not develop neurotoxicity ($p = 0.0025$, Mann–Whitney U-test) (Figure 4b). Following the first dose of antivenom, the venom concentrations decreased immediately and were undetectable in most patients (Figure 5a). Of the 31 patients who developed clinically detectable neurotoxicity post-antivenom, 18 patients had venom concentration data for multiple time points including at least a single pre-antivenom time point. In these 18 patients, the onset of clinically detectable neurotoxicity was often seen at time points with very low or zero venom concentrations (Figure 5b). In post-antivenom samples of 46 patients who had no pre-antivenom samples, Russell's viper venom was detected following venom-antivenom dissociation.

Single fibre electromyography

27 neurotoxic patients and 23 non-neurotoxic patients had sfEMG. There were no statistically significant differences in age, sex, and proportion with coagulopathy, between the 27 neurotoxic patients who had sfEMG and the remaining 103 neurotoxic patients. Similarly, there was no difference in age, sex, and proportion with coagulopathy between the 23 non-neurotoxic patients who had sfEMG and the remaining 92 non-neurotoxic patients. There was no difference in the proportion with ophthalmoplegia between the 27 neurotoxic patients who had sfEMG and the remainder. Slightly increased median jitter was observed in both neurotoxic and non-neurotoxic groups during the first 24 h of the snakebite (Figure 6a). Only 56/744 (7.5%) pooled fibres from neurotoxic patients and 41/542 (7.6%) pooled fibres from non-neurotoxic patients had neuromuscular jitter above the normal upper limit (30 μs). Neuromuscular blocks were not observed in any patient. On discharge, the median jitter values of both groups were similar to the normal subjects and also significantly reduced compared to day 1 (Figure 6b). No significant change in the median jitter values of the patients were recorded at 6 weeks and 6 months, compared to values of discharge.

Discussion

Based on a large cohort of definite cases, the present study found that neuromuscular paralysis developed in half the patients with Russell's viper bites, and was more often present in those with higher venom concentrations and other systemic features. Paralysis was non-life threatening with no cases of respiratory paralysis. It completely resolved in 80% of patients within 3 d with no residual effects. Early antivenom therapy reduced the venom concentrations but did not prevent or rapidly reverse neurotoxicity. Neuromuscular jitter was increased in both neurotoxic and non-neurotoxic patients compared to normal controls but did not apparently correlate with clinical neurotoxicity, and was much less than for krait bites.[15]

Patients with neurotoxicity had higher peak venom concentrations presumably due to bites by larger snakes delivering more venom. Other systemic features such as coagulopathy, bleeding manifestations, acute kidney injury were also more common and the hospital stay was also longer. Thus, neurotoxicity appears to be a characteristic of more severe envenoming. However, the absence of life-threatening paralysis, even in patients with very high venom concentrations suggests that the neurotoxins primarily responsible for the neurotoxicity in Sri Lankan Russell's viper envenoming are not very potent or account for only a very small proportion of the venom. The absence of neurotoxicity in patients bitten by small specimens (most likely juvenile Russell's vipers) might indicate a possible ontogenic variation in the venom

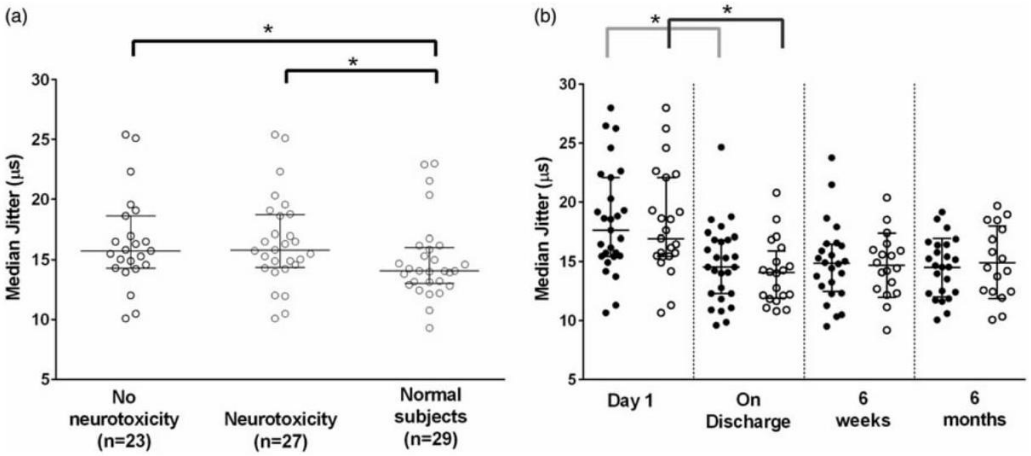

Figure 6. Stimulated single fibre electromyography of Sri Lankan Russell's viper bite patients. (a) Median jitter of the orbicularis oculi of 27 patients with neurotoxicity, 23 patients with no neurotoxicity during the first 24 h of envenoming and 29 normal healthy subjects. Note both neurotoxicity and non-neurotoxicity groups have high jitter compared to normal subjects. (b) Time-related change of the median jitter of the same patients. Note: the jitter on discharge at a median of 5 (range: 3–8) d in the neurotoxicity group and 3 (range: 2–7) d in the non-neurotoxic group is lower than jitter within the first 24 h. *$p < 0.05$ one-way ANOVA followed by multiple comparison tests.

Table 3. Time course of the clinical profile of Sri Lankan Russell's viper envenoming, as reported in observational clinical studies conducted previously.

	Jeyarajah [28]	Phillips et al. [11]	Kularatne [9]	Kularatne et al. [10]
Year	1980–1982	1985	1996–1997	2010
Number of cases	22	23	336	209
Definite cases (%)	NA	100%	21%	26%
Time from bite to hospital admission	Three within 5 h, eight within 48 h, 9 >48 h	Eight admitted a mean 8.4 h post-bite (rest NA)	72% patients reached the hospital within 5 h	NA
Time from bite to antivenom	NA	NA	NA	Median: 5.5 h
Neurotoxicity (%)	86%	82%	78%	70%
Bulbar and neck muscle paralysis	"Most of the patients" had bulbar weakness	Dysphagia in 55% of the 20 patients assessed	22%	Neck muscle weakness: 27%
Respiratory paralysis	–	–	2.8%	1%
Case fatality rate	23%	13%	2.6%	N/A

NA: not available.

composition of Sri Lankan Russell's viper, with adults having more neurotoxic venom compared to the juveniles. Both these issues need to be further investigated with *in vitro* pharmacological characterisation of the venom from Sri Lankan Russell's viper at various ages. Neuromuscular dysfunction is seen in neurotoxic snake envenoming ranges in severity from non-life-threatening mild paralysis (ptosis, ophthalmoplegia, and facial weakness) to life threatening severe paralysis (bulbar and respiratory paralysis requiring airway and ventilator support). In extreme cases paralysis involves all limb muscles.[15,20–22]. The severity of neuromuscular dysfunction depends on multiple factors including the potency of the individual toxins, the relative abundance of individual neurotoxins in the snake venom, the amount of venom injected during the bite, and the effectiveness of antivenom determined mainly by the delay to antivenom administration. Neurotoxic paralysis usually follows a descending order of muscle involvement with ptosis > ophthalmoplegia > facial weakness > neck and bulbar weakness > respiratory weakness > limb paralysis. Recovery usually follows the reverse ascending order.[15,23] The present study suggests that Russell's viper envenoming in Sri Lanka only causes mild paralysis within this spectrum, but still has the same descending order of involvement and ascending recovery. Therefore, the mild neuromuscular paralysis seen in the present cohort of Russell's viper bite patients is unlikely to be a "unique neuromuscular syndrome" that consists of only ptosis and ophthalmoplegia, but a milder form of the paralysis seen in all neurotoxic snake envenoming.

No clinical or neurophysiological abnormalities were detected up to 6 months post-bite, in the 50 Russell's viper envenomed patients we followed up. This indicates that the Sri Lankan Russell's viper bite induced neuromuscular dysfunction is limited to acute mild neurotoxicity.

The neuromuscular jitter of the orbicularis oculi was slightly high in both non-neurotoxic and neurotoxic patients during the first 24 h of the snake bite and became normal thereafter. Clinically, orbicularis oculi was unaffected in all Russell's viper bite patients studied. Therefore, the slightly higher jitter observed during the first day of envenoming in both neurotoxicity and non-neurotoxicity groups indicate a mild, transient sub-clinical neuromuscular dysfunction of the orbicularis oculi following Russell's viper envenoming. Further, no difference of the neuromuscular jitter was noted between neurotoxic and non-neurotoxic groups at any stage. This surprising finding suggests the clinically evident neurotoxicity is only a part of the venom induced neuromuscular dysfunction syndrome, which also

includes a sub-clinical neuromuscular dysfunction, within its spectrum.

Antivenom failed to prevent later neurotoxicity occurring in 60% of the patients who had no neurotoxicity at the time they were given antivenom. The Indian polyvalent antivenom reduced circulating venom concentrations rapidly in almost all patients studied in this study. The most plausible explanation is that at the time the patients received antivenom, the neurotoxins had already reached the neuromuscular junctions and an irreversible process leading to neuromuscular dysfunction had already begun. This is consistent with the predominately pre-synaptic type neuromuscular block caused by Sri Lankan Russell's viper venom *in vitro*.[24] Presynaptic neurotoxins in snake venoms are hypothesised to cause treatment resistant neuromuscular dysfunction in humans, by depleting the synaptic vesicles in the motor nerve terminal and ultimately leading to a destruction of the nerve terminal, which is unlikely to be reversed by antivenom therapy.[25] It is also worth noting that the time delay from the bite to the first dose of antivenom (median: 3.75 h) was much shorter in this study than previous studies, [9,26] hence, it is likely that this lack of prevention of neurotoxicity by the antivenom will be applicable to most hospital settings.

The prevalence of neurotoxicity in the present cohort (53%) was lower than previous Sri Lankan Russell's viper bite studies (70–88%) (Table 3).[8–11,27,28] This difference is largely explained by the greater extent to which subjects in previous studies have been diagnosed as Russell's viper bites based on the presence of features of systemic envenoming, rather than direct evidence of a Russell's viper bite such as identification of the snake specimen involved or presence of specific venom antigens in blood samples of patients. This is likely to have excluded many Russell's viper bite cases without systemic envenoming. In our study, 22% of patients had no systemic envenoming. The use of venom detection with enzyme immunoassays should reduce selection bias in cohort studies and enable a more precise estimate of the spectrum of clinical effects of envenoming.

There are several weaknesses in this study. Forty suspected Russell's viper bites (16%) had to be excluded due to the failure of confirming a definite bite. Although this is lower than previous studies [9,10], it still inevitably reduces accuracy in clinical feature descriptions. Second, more frequent sfEMG, particularly during the first day, might have added more information about the evolution of neuromuscular dysfunction. However, the perceived risk of bleeding from electrode insertion sites in patients with co-existing severe coagulopathy meant this was not done.

In previous studies conducted in Sri Lanka, neuromuscular dysfunction due to Russell's viper envenoming has resulted in bulbar, neck, and respiratory muscle paralysis, whereas the present study reports none (Table 3). The reason for this may be that patients in our study reached the hospital and received antivenom earlier. There have been considerable improvements in roads and transport in Sri Lanka. The earlier administration of antivenom in the present cohort presumably also reduced circulating free venom blood concentrations earlier and might have prevented more extensive damage that would otherwise have been caused by circulating neurotoxins. There has also been an apparent reduction in the incidence of severe acute kidney injury over the last three decades (Table 3). Thus, earlier treatment may be leading to a change in the clinical profile of Russell's viper envenoming in Sri Lanka, with it becoming less lethal than reported in historical studies.

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Disclosure statement

The authors report no declarations of interests.

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Errata

Page 106: The subtitle: ‘Serum Creatinine Kinase Assay’ should read: ‘Serum Creatine Kinase Assay’

CHAPTER FOUR

Pharmacological investigation of the neuromuscular effects of Sri Lankan Russell's viper venom.

Overview of the Chapter

As described in Chapter Three, Sri Lankan Russell's viper envenoming resulted in mild, non-life-threatening paralysis in humans, which was not prevented or reversed by antivenom therapy despite antivenom efficaciously binding with the circulating venom. This indicated a possible pre-synaptic type neurotoxicity. In addition, mild myotoxic effects have been observed in patients envenomed by Sri Lankan Russell's viper. Pre-synaptic neurotoxicity and myotoxicity are common properties of snake venom PLA_2 toxins. The neurotoxins and myotoxins responsible for these effects, and the pharmacological origin of the mild neuromuscular paralysis and the mild myotoxicity in Sri Lankan Russell's viper envenoming, have not been investigated. These were the focus of the current Chapter.

Using a combination of different liquid chromatographic techniques, two PLA_2 toxins were isolated from Sri Lankan Russell's viper venom and characterized using the isolated chick biventer cervicis nerve-muscle preparation. One toxin (i.e. U1-viperitoxin-Dr1a) showed weak pre-synaptic neurotoxicity and weak myotoxicity, while the other toxin (i.e. U1-viperitoxin-Dr1b) showed only weak myotoxic properties. This work was published as two research articles; one on the pharmacological basis of the neuromuscular paralysis:

Silva A, Kuruppu S, Othman I, Goode RJ, Hodgson WC, Isbister GK. Neurotoxicity in Sri Lankan Russell's Viper (*Daboia russelii*) Envenoming is Primarily due to U1-viperitoxin-Dr1a, a Pre-Synaptic Neurotoxin. *Nerurotox Res*. 2016; 31 (1): 11-9

The other is on the pharmacological basis of the myotoxicity of Sri Lankan Russell's viper envenoming. To complement the pharmacological investigation, clinical data on the

myotoxicity of the 245 authenticated Russell's viper envenomings described in Chapter 2 was included in the latter paper.

Silva A, Johnston CJ, Kuruppu S, Kniesz D, Maduwage K, Kleifield, O, Smith I, Siribaddana S, Buckley N, Hodgson WC, Isbister GK. Clinical and Pharmacological Investigation of Myotoxicity in Sri Lankan Russell's Viper (*Daboia russelii*) Envenoming. *PLoS Negl Trop Dis*. 2016; 10 (12): e0005172.

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ORIGINAL ARTICLE

Neurotoxicity in Sri Lankan Russell's Viper (*Daboia russelii*) Envenoming is Primarily due to U1-viperitoxin-Dr1a, a Pre-Synaptic Neurotoxin

Anjana Silva[1,2] · Sanjaya Kuruppu[1,3] · Iekhsan Othman[4] · Robert J.A. Goode[3] · Wayne C. Hodgson[1] · Geoffrey K. Isbister[1,5]

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Abstract Russell's vipers are snakes of major medical importance in Asia. Russell's viper (*Daboia russelii*) envenoming in Sri Lanka and South India leads to a unique, mild neuromuscular paralysis, not seen in other parts of the world where the snake is found. This study aimed to identify and pharmacologically characterise the major neurotoxic components of Sri Lankan Russell's viper venom. Venom was fractionated using size exclusion chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC). In vitro neurotoxicities of the venoms, fractions and isolated toxins were measured using chick biventer and rat hemidiaphragm preparations. A phospholipase A_2 (PLA_2) toxin, U1-viperitoxin-Dr1a (13.6 kDa), which constitutes 19.2 % of the crude venom, was isolated and purified using HPLC. U1-viperitoxin-Dr1a produced concentration-dependent in vitro neurotoxicity abolishing indirect twitches in the chick biventer nerve-muscle preparation, with a t_{90} of 55 ± 7 min only at 1 μM. The toxin did not abolish responses to acetylcholine and carbachol indicating pre-synaptic neurotoxicity. Venom, in the absence of U1-viperitoxin-Dr1a, did not induce in vitro neurotoxicity. Indian polyvalent antivenom, at the recommended concentration, only partially prevented the neurotoxic effects of U1-viperitoxin-Dr1a. Liquid chromatography mass spectrometry analysis confirmed that U1-viperitoxin-Dr1a was the basic S-type PLA_2 toxin previously identified from this venom (NCBI—GI: **298351762**; SwissProt: **P86368**). The present study demonstrates that neurotoxicity following Sri Lankan Russell's viper envenoming is primarily due to the pre-synaptic neurotoxin U1-viperitoxin-Dr1a. Mild neurotoxicity observed in severely envenomed Sri Lankan Russell's viper bites is most likely due to the low potency of U1-viperitoxin-Dr1a, despite its high relative abundance in the venom.

Keywords Russell's viper · Neurotoxicity · Pre-synaptic · Phospholipase A_2

✉ Anjana Silva
anjana.silva@monash.edu

1 Monash Venom Group, Department of Pharmacology, Biomedicine Discovery Institute, Monash University, Melbourne, VIC 3800, Australia

2 Department of Parasitology, Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura 50008, Sri Lanka

3 Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton Campus, Melbourne, VIC 3800, Australia

4 Jeffrey Cheah School of Medicine, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia

5 Clinical Toxicology Research Group, Faculty of Health and Medicine, University of Newcastle, Level 5, New Med Building, Calvary Mater Hospital, Edith Street, Waratah, NSW 2298, Australia

Introduction

Russell's vipers (Genus: *Daboia*) are snakes of significant medical importance in Asia, due to the high morbidity and mortality associated with envenoming and their wide geographical range (Myint-Lwin et al. 1985; Tun-Pe et al. 1991; Kularatne 2000; Hung et al. 2002; Kularatne et al. 2014). At least seven subspecies, based on the allopatric populations, have been described in the literature: *Daboia russelii pulchella* (Sri Lanka and South India), *D. r. russelii* (remainder of India, Bangladesh, Pakistan), *D. r. siamensis* (Myanmar, Thailand, South China, Cambodia), *D. r. limitis* (Indonesia), *D. r. formosensis* (Taiwan), *D. r. nordicus* (north India) and

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D. r. sublimitis (Java—Indonesia) (Warrell 1989; Wuster et al. 1992; Belt et al. 1997; Kularatne 2000; Thorpe et al. 2007). Currently, all these different populations of Russell's vipers are treated as two distinct species, *D. russelii* (all Russell's vipers distributed to the west of the bay of Bengal) and *D. siamensis* (all Russell's vipers distributed to the east of the Bay of Bengal) (Thorpe et al. 2007).

Based on the available clinical data, Russell's vipers almost universally cause venom-induced consumption coagulopathy following envenoming by species throughout their geographical range (Myint-Lwin et al. 1985; Warrell 1989; Tun-Pe et al. 1991; Kularatne 2000; Hung et al. 2002). Acute kidney injury is also reported throughout the geographical range (Myint-Lwin et al. 1985; Hung et al. 2002; Kularatne et al. 2014). In contrast, neuromuscular toxicity has remarkable geographical variation. The existing literature suggests that only Sri Lankan (Phillips et al. 1988; Kularatne 2000; Kularatne et al. 2014; Silva et al. 2016) and South Indian (Warrell 1989) populations of *D. russelii* cause clinically significant neurotoxicity in humans. This has not been reported in other geographical regions where Russell's vipers are found (Myint-Lwin et al. 1985; Tun-Pe et al. 1991; Belt et al. 1997; Mukherjee and Maity 2002; Hung et al. 2002, 2006). However, clinical studies involving authenticated Russell's viper bites from different parts of India, Bangladesh and Pakistan are lacking.

The neuromuscular dysfunction reported in Sri Lankan patients is mild, and life-threatening paralysis is rare (Phillips et al. 1988; Ariaratnam et al. 1999; Kularatne 2000; Kularatne et al. 2014; Silva et al. 2016). Our recent clinical and neurophysiological investigation of the neuromuscular dysfunction in 245 authenticated cases of envenoming by Sri Lankan Russell's viper (Silva et al. 2016) revealed that neurotoxicity (1) occurred only in about half of the patients, (2) occurred in patients with higher venom concentrations and those bitten by larger snakes and (3) was limited to mild neurotoxicity characterised by ptosis and extra-ocular weakness with no life-threatening paralysis. The study also found that although Indian polyvalent antivenom was able to bind with and clear the circulating venom antigens in patients, it failed to reverse neurotoxicity in the patients, suggesting a pre-synaptic mechanism of action (Silva et al. 2016).

D. russelii venom from India and Sri Lanka has been biochemically characterised, and the geographical variations in venom composition have been previously studied (Jayanthi and Gowda 1988; Tsai et al. 1996; Prasad et al. 1999; Mukherjee et al. 2000; Suzuki et al. 2010; Sharma et al. 2014). Several toxins with pre- and post-synaptic activities have been isolated from Russell's viper populations which have not been reported to cause neurotoxicity in humans, including the post-synaptic neurotoxins DNTx I, II and III isolated from *D. russelii* venom from eastern India (Shelke et al. 2002; Venkatesh et al. 2013) and "Russtoxin"/"Viperotoxin-F", a potent heterodimeric pre-synaptic PLA_2 toxin group isolated from Russell's vipers distributed in areas other than Sri Lanka and South India (Tsai et al. 1996). This latter group consists of PLA_2 toxins with an asparagine residue at the N-terminus (N-type). The PLA_2 toxins found in South Indian and Sri Lankan Russell's viper venoms are mostly basic, as opposed to the acidic toxins which predominate elsewhere. In addition, PLA_2 toxins from the venom of Sri Lankan and South Indian Russell's vipers include those with a serine residue at the N-terminus (S-type), in addition to the N-type PLA_2 toxins. A recent proteomic investigation demonstrated the pre-synaptic neurotoxic activity of the venom from Sri Lankan Russell's viper (Tan et al. 2015). However, little is known about which neurotoxins are responsible for clinical neurotoxicity or their pharmacological properties.

The aim of the present study is to isolate and pharmacologically characterise the major neurotoxin(s) from Sri Lankan Russell's viper venom and to study the ability of Indian polyvalent antivenom to bind and neutralise the neurotoxins in vitro.

Materials and Methods

Venom and Antivenom

Freeze-dried Russell's viper (*D. russelii*) venom (pooled from both sexes, both adult and juvenile) from Sri Lanka, donated by Prof. Ariaranee Gnanadasan (Faculty of Medicine, University of Colombo) was used for this study. Venom was dissolved in MilliQ water and stored at −20 °C until required. Protein quantifications of the venom, fractions and toxins were carried out using a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA), as per the manufacturer's instructions.

Indian polyvalent antivenom (Vins Bioproducts, Thimmapur, India; Batch No. 01AS14035) was used for this study. The antivenom was reconstituted in 2 mL of sterile injection water. According to the manufacturers, 1/10 of the antivenom in a single vial neutralises 0.6 mg of Russell's viper venom. For in vitro neurotoxicity neutralisation studies of the neurotoxins, the required amount of antivenom was calculated based on the relative abundance of the toxin in the venom.

High-Performance Liquid Chromatography (HPLC)

Fractionation was performed using a Shimadzu (Kyoto, Japan) system (LC-10ATvp pump and SPD-10AVP detector).

Size-Exclusion Chromatography

Crude venom (500 μg) was reconstituted in buffer A (ammonium acetate; 0.1 M, pH 6.8). Venom was then separated using a Superdex G-75 column (13 μm; 10 mm × 300 mm; GE Healthcare, Uppsala, Sweden) equilibrated with buffer A. Elution was performed at a flow rate of 0.5 ml/min and monitored at 280 nm. All fractions were freeze-dried immediately and later screened using the chick biventer cervicis nerve-muscle preparation (see "Chick Biventer Cervicis Nerve-Muscle Preparation" section below) to identify those with neurotoxicity.

Reverse-Phase HPLC (RP HPLC)

The fractions obtained from size exclusion chromatography, which displayed in vitro neurotoxicity, were subjected to further analysis using reverse-phase HPLC. Size exclusion chromatography fractions were reconstituted in solvent A (0.1 % trifluoroacetic acid [TFA]) and 2 mg of total protein was injected into a Phenomenex Jupiter semi-preparative C18 column (250 mm × 10 mm; 5 μm; 300 Å), equilibrated with solvent A. Fractions were eluted using the following gradient of solvent B (90 % acetonitrile [ACN] in 0.1 % TFA): 0–30 % over 10 min, 30–70 % for 10–50 min and 70–0 % for 50–55 min at a flow rate of 2.0 ml/min. Where indicated, the elution profiles of U1-viperitoxin-Dr1a, venom without U1-viperitoxin-Dr1a, and whole venom were compared on a Phenomenex Jupiter semi-preparative C18 column.

Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF) Mass Spectrometry

MALDI-TOF Mass Spectrometry analysis was performed with an Applied Biosystems (Forster City, CA, USA) 4700 TOF Proteomics Analyser, as described previously (Chaisakul et al. 2010). In brief, samples were mixed 1:1 with matrix (10 mg/ml a-cyano-4-hydroxycinnamic acid in 50 % Acetonitrile 0.1 % TFA) and spotted onto the MALDI target plate. Proteins were analysed in Linear mode with a mass range of 5–40 kDa. MALDI data were processed with 4000 Series Explorer version 3.0 with 15-point smoothing applied and externally calibrated against Myoglobin using 2-point calibrate masses, 16,952 and 8476 Da.

Amino Acid Sequence Determination by Liquid Chromatography–Mass Spectrometry (LC–MS)

Freeze-dried U1-viperitoxin-Dr1a dissolved in Milli-Q water was in-solution trypsin digested, and the digested peptides were loaded onto an Agilent C18 300 Å large capacity chip (Agilent Technologies, Santa Clara, USA). The column was equilibrated with 0.1 % formic acid in water (solution A), and peptides were eluted with an increasing gradient of 90 % ACN in water with 0.1 % formic acid (solution B) with the following gradient of 5–75 % for the first 30 min followed by a constant 75 % during 30–39 min. The polarity of the Q-TOF was set at positive, capillary voltage at 1745 V, fragmentor voltage at 1735 V, drying gas flow of 5 l/min and gas temperature of 325 °C. Spectrum was analysed in MSMS mode over a range of 110–3000 *m/z* using Agilent MassHunter data acquisition software (Agilent Technologies, Santa Clara, USA). Results were then analysed using PEAKS 7.0 software with the following filtering criteria: FDR 0.1 % and unique peptides ≥1. SwissProt and NCBI database (tax: serpentes) were used to identify the proteins.

PLA_2 Activity

PLA_2 activities of the venom and U1-viperitoxin-Dr1a were determined using a secretory PLA_2 assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The PLA_2 substrate used in this assay was the 1, 2-dithio analogue of diheptanoyl phosphatidylcholine. Free thiols generated following the hydrolysis of the thioester bond at the sn-2 position by PLA_2 were detected using DTNB [5, 5′-dithio-bis (2-nitrobenzoic acid)]. Colour changes were monitored every minute at 405 nm in a microplate reader (Perkin-Elmer, Waltham, MA, USA), sampling for a 5-min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolysed per min per mg of enzyme.

Chick Biventer Cervicis Nerve-Muscle Preparation

Male chickens (aged 4–10 days) were humanely killed by exsanguination following CO_2 inhalation. Biventer cervicis nerve-muscle preparations were dissected and then mounted on wire tissue holders under 1-g resting tension in 5-ml organ baths. Tissues were maintained at 34 °C, bubbled with 95 % O_2 and 5 % CO_2, in physiological salt solution of the following composition (mM); 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 11.1 glucose. Indirect twitches were evoked by stimulating the motor nerve (rate: 0.1 Hz; pulse duration: 0.2 ms) at supramaximal voltage (10–20 V), using a Grass S88 stimulator (Grass Instruments, Quincy, MA). Selective stimulation of the nerve was confirmed by the abolishment of twitches with D-tubocurarine (10 μM). Tissues were then repeatedly washed with physiological salt solution to restore twitch response to nerve stimulation. Contractile responses of the tissues to exogenous acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s) and KCl

(40 mM for 30 s) were obtained in the absence of nerve stimulation. The preparations were then stimulated for 30 min, before the addition of venom, U1-viperitoxin-Dr1a or venom without U1-viperitoxin-Dr1a. For antivenom experiments, the tissues were equilibrated with antivenom for 20 min before U1-viperitoxin-Dr1a was added. At the conclusion of the experiment, ACh, CCh and KCl were re-added as above.

Rat Phrenic Nerve-Hemidiaphragm Preparation

Adult male rats weighing 300–350 g were humanely killed by exsanguination following CO_2 inhalation. Two hemidiaphragms with intact phrenic nerves were carefully dissected and mounted at a resting tension of 1 g in 50-ml organ baths, under the same bath conditions used for the chick preparation. The electrical stimulation of the phrenic nerve and the recording was carried out as described for the chick preparation, except the supramaximal voltage used for stimulation ranged from 2 to 5 V. Selective stimulation of the motor nerve was confirmed by the abolition of twitches by d-tubocurarine (10 μM). Tissues were then washed, and once the twitches were fully recovered, the stimulation was carried out for another 20 min before the toxins were added to the organ bath.

Data Analysis and Statistics

The quantity of U1-viperitoxin-Dr1a in the venom was determined by measuring the area under the curve of the size-exclusion and RP-HPLC venom elution profiles. Nerve-mediated twitch responses and responses to exogenous agonists (ACh, CCh) and KCl were measured via a Grass FTO3 force displacement transducer and recorded on a PowerLab system (ADInstruments Pty Ltd., Australia). Responses were expressed as percentages of their pre-venom/toxin values. t_{90} values (i.e. time taken for 90 % inhibition of the maximum twitch response to occur) were determined for U1-viperitoxin-Dr1a. To compare the responses to exogenous agonists following the administration of venom, a one-way ANOVA was used. All ANOVAs were followed by Bonferroni's multiple comparison post-tests. Data presented are in the form of mean ± standard error of the mean (SEM) of n experiments. All statistical analyses and presentation of data were generated using GraphPad Prism 5.3 software. For all statistical tests, $P < 0.05$ was considered statistically significant.

Animal Ethics

All animal experiments used in this study were approved by the Animal Ethics Committee of Monash University (Approval No: MARP/2014/097).

Results

HPLC

Size-exclusion chromatography revealed eight major fractions (Fig. 1a) in the crude venom of Russell's viper. Peak E, eluting around 31–35 min showed marked pre-synaptic neurotoxicity in the chick biventer cervicis nerve-muscle preparation. Peak E was further analysed using RP-HPLC (Fig. 1b). In vitro neurotoxicity was confined to a single peak with a retention time of 38 min (Fig. 1c). This venom component was subsequently named U1-viperitoxin-Dr1a. U1-viperitoxin-Dr1a was found to constitute a mean of 19.2 % (SD 1.2; $n = 8$) of the crude venom, based on the area under the curve of the RP-HPLC profile of the venom (Fig. 1d).

Intact Protein Analysis with MALDI-TOF Mass Spectrometry

Analysis of U1-viperitoxin-Dr1a by MALDI-TOF mass spectrometry indicated a single mass with a mass/charge ratio of 13 641.0 Da (Fig. 2). A doubly charged ion with a mass/charge ratio of 6812.4 Da was also observed.

Amino Acid Sequence Determination by LC–MS

In both SwissProt and NCBI database, 93–77 % peptide coverage was found to align with the Basic Phospholipase A_2 (NCBI—GI: **298351762**; SwissProt: **P86368**, Entry name: PA2B3_DABRR) (Fig. 3).

PLA_2 Activity

The PLA_2 activities of *D. russelii* venom and U1-viperitoxin-Dr1a were determined as 870 ± 11 ($n = 3$) and 1625 ± 36 ($n = 3$) μmol/min/mg, respectively.

In Vitro Neurotoxicity

U1-viperitoxin-Dr1a produced concentration-dependent neurotoxicity in the chick biventer cervices nerve-muscle preparation (Fig. 4a). The effect of U1-viperitoxin-Dr1a on the exogenous agonists and KCl indicated a pre-synaptic mode of action (Fig. 4b). U1-viperitoxin-Dr1a (600 nM) only induced 80 % twitch inhibition over 180 min. However, U1-viperitoxin-Dr1a (1 μM) had a t_{90} value of 55 ± 7 min.

The inhibition of twitches by U1-viperitoxin-Dr1a (600 nM) was similar in the chick biventer and rat hemidiaphragm preparations (Fig. 4c). Neurotoxicity in the

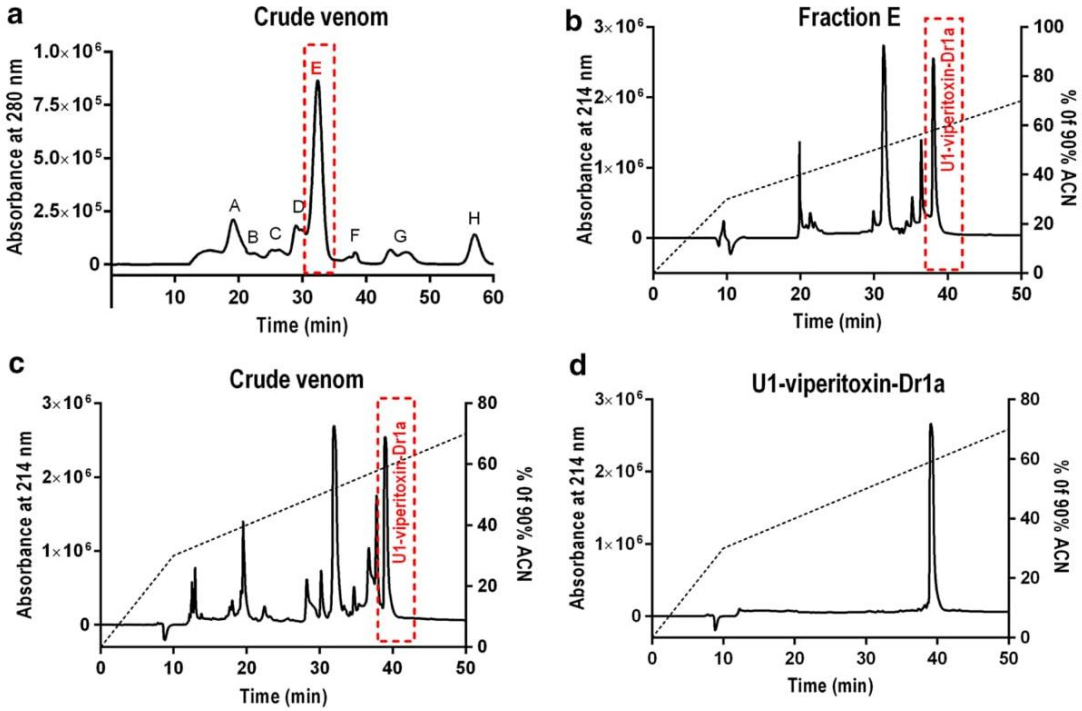

Fig. 1 Chromatograms of *D. russelii* venom and fractions from Sri Lanka. **a** Size-exclusion chromatogram of whole venom on Superdex G75 column; **b** reverse-phase high-performance liquid chromatogram (RP-HPLC) of the fraction 'E' on Jupiter C18 semi-preparative column; **c** RP-HPLC of the whole venom on Jupiter C18 semi-preparative column; **d** RP-HPLC of the purified U1-viperitoxin-Dr1a. *Note*: the peak eluting at 38–40 min in RP-HPLC chromatograms (panels **b**–**d**) is U1-viperitoxin-Dr1a

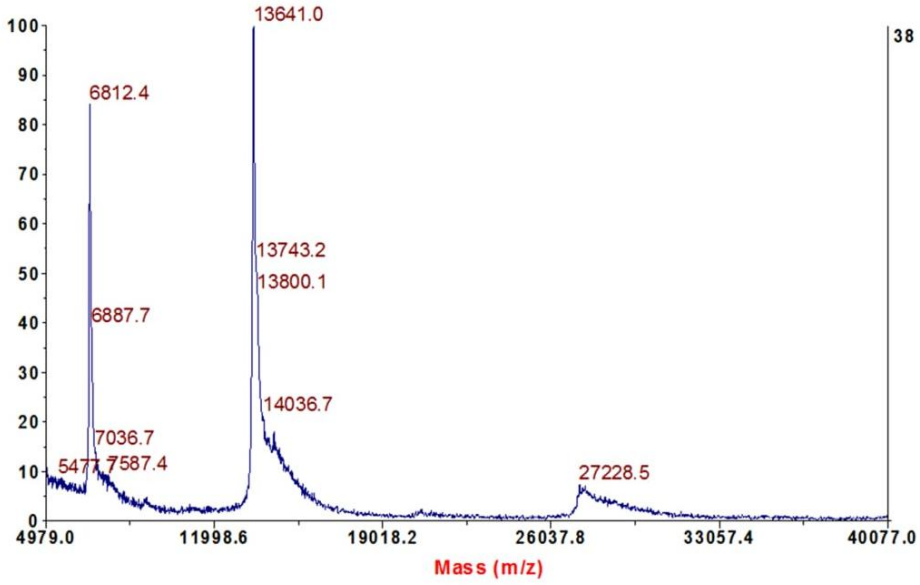

Fig. 2 Intact protein analysis chromatogram of MALDI-TOF: the intact mass of U1-viperitoxin-Dr1a is 13.641.0 kDa

chick biventer nerve-muscle preparation, induced by U1-viperitoxin-Dr1a (1 μM), was partially reversed by the Indian Polyvalent antivenom (Fig. 4a). Removal of U1-viperitoxin-Dr1a from the venom through size exclusion chromatography resulted in a significant loss of neurotoxicity (Fig. 4d).

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U1-viperitoxin-Dr1a  SP|P86368|PA2B3_DABRR ----------------------------SLLEFGMMILEE-TGKLAVPFYSSYGCYCGWGG 32
Crotoxin b           SP|P0CG56|PA2BB_CRODU ----------------------------HLLQFNKMIKFE-TRKNAVPFYAFYGCYCGWGG 32
Ammodytoxin A        SP|P00626|PA2BA_VIPAA -----MRTLWIVAVCLI------GVEGSLLEFGMMILGE-TGKNPLTSYSFYGCYCGVGG 48
Notexin              SP|P00608|PA2B_NOTSC  ----------------------------NLVQFSYLIQCANHGKRPTWHYMDYGCYCGAGG 33
Taipoxin α-chain     SP|P00614|PA2TA_OXYSC ----------------------------NLLQFGFMIRCANRRSRPVWHYMDYGCYCGKGG 33
β-bungarotoxin       SP|P00617|PA2B1_BUNMU MNPAHLLVLSAVCVSLLGAANIPPHPLNLINFMEMIRYTIPCEKTWGEYADYGCYCGAGG 60
                                                                *:::*  :*      .     *  ****** **

                     SP|P86368|PA2B3_DABRR KATPKDATDRCCFVHDCCYGNL---PDCNPKSDRYKYKRVNGAIVCE-QGTSCENRICEC 88
                     SP|P0CG56|PA2BB_CRODU QGRPKDATDRCCFVHDCCYGKL---AKCNTKWDIYRYSLKSGYITCG-KGTWCEEQICEC 88
                     SP|P00626|PA2BA_VIPAA KGTPKDATDRCCFVHDCCYGNL---PDCSPKTDRYKYHRENGAIVCG-KGTSCENRICEC 104
                     SP|P00608|PA2B_NOTSC  SGTPVDELDRCCKIHDDCYDEAG-KKGCFPKMSAYDYYCGENGPYCRNIKKKCLRFVCDC 92
                     SP|P00614|PA2TA_OXYSC SGTPVDDLDRCCQVHDECYGEAVRRFGCAPYWTLYSWKCYGKAPTCN-TKTRCQRFVCRC 92
                     SP|P00617|PA2B1_BUNMU SGRPIDALDRCCYVHDNCYGDAEKKHKCNPKTQSYSYKLTKRTIICYGAAGTCGRIVCDC 120
                                           .. * *  **** :** ** .      *      * :       *      * . :* *

                     SP|P86368|PA2B3_DABRR DKAAAICFRRNLNTYSKIYMLYPDFLCKGELKC- 121
                     SP|P0CG56|PA2BB_CRODU DRVAAECLRRSLSTYKNGYMFYPDSRCRGPSETC 122
                     SP|P00626|PA2BA_VIPAA DRAAAICFRKNLKTYNYIYRNYPDFLCKKESEKC 138
                     SP|P00608|PA2B_NOTSC  DVEAAFCFAKAPYNNANWN-IDTKKRCQ------ 119
                     SP|P00614|PA2TA_OXYSC DAKAAECFARSPYQNSNWN-INTKARCR------ 119
                     SP|P00617|PA2B1_BUNMU DRTAALCFGNSEYIEGHKN-IDTARFCQ------ 147
                                           *  ** *: .             *:
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Fig. 3 Alignment of U1-viperitoxin-Dr1a amino acid sequence with those of crotoxin b (*Crotalus durissus terrificus*), ammodytoxin A (*Vipera ammodytes ammodytes*), notexin (*Notechis scutatus*), α subunit of taipoxin (*Oxyuranus scutellatus*) and chain A of β-bungarotoxin (*Bungarus multicinctus*). Sequences were obtained from UniProt database and are presented with unique identification numbers and entry names. Within the regions highlighted in yellow, '*', ':' and '.' denote positions with, single fully conserved residue, conservation between groups of strongly similar properties (scoring >0.5 in the Gonnet PAM 250 matrix) and conservation between groups of weakly similar properties (scoring < 0.5 in the Gonnet PAM 250 matrix), respectively.

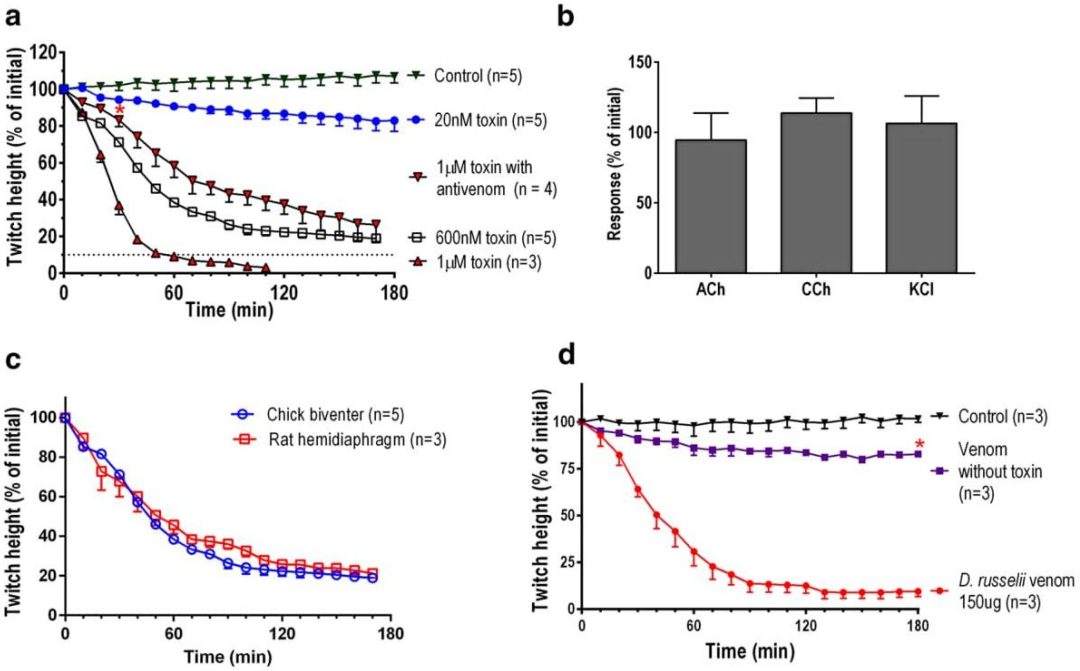

Fig. 4 In vitro neurotoxicity of U1-viperitoxin-Dr1a: **a** Concentration-dependent inhibition of indirect twitches in chick biventer nerve-muscle preparation by U1-viperitoxin-Dr1a. *Note*: Pre-addition of antivenom partially prevents the twitch inhibition of 1 μM U1-viperitoxin-Dr1a (* significantly different compared to no antivenom at all time points after 30 min; $P < 0.05$: unpaired *t* test). **b** Effect of U1-viperitoxin-Dr1a on responses to exogenous agonists ACh, CCh and KCl; **c** comparison of the twitch abolition in avian and rodent preparations with the presence of 600 nM U1-viperitoxin-Dr1a; **d** comparative twitch inhibition in chick biventer nerve-muscle preparation by 150 μg whole venom of Sri Lankan *D. russelii* and the venom without U1-viperitoxin-Dr1a and control: removal of the toxin significantly lowers the indirect twitch inhibition (* significantly different compared to whole venom at time = 180 min; $P < 0.05$: unpaired *t*-test)

Discussion

The present study demonstrates that the neurotoxicity of the Sri Lankan Russell's viper venom is primarily due to a pre-synaptic neurotoxin with PLA_2 activity, which we have named U1-viperitoxin-Dr1a. U1-viperitoxin-Dr1a constitutes 19.2 % of the crude venom and removal of the toxin from the venom led to an almost complete loss of in vitro neurotoxicity. U1-viperitoxin-Dr1a (1 μM) inhibited nerve-mediated twitches in the chick biventer by 90 % over 3 h. Further, the present study shows the ability of the Indian polyvalent antivenom to partially neutralise its neurotoxic properties.

Two monomeric PLA_2 toxins, VRV-PL-VIIIa and VRV-PL-V, isolated from *D. russelii* venom from South India have resulted paralysis features in envenomed rodents (Jayanthi et al. 1989; Kasturi and Gowda 1989). Tsai et al. (Tsai et al. 1996) isolated three monomeric PLA_2 toxins with pre-synaptic activity from '*D. r. pulchella*' venom (*D. russelii*—according to current nomenclature), although the exact geographical origin of the venom is unclear. Two of these toxins, P1 and P3 were suggested to be identical to VRV-PL-VIIIa and VRV-PL-V, respectively (Tsai et al. 1996). However, differences between the PLA_2 toxins from Russell's viper venoms from Sri Lanka and India have also been identified. Of five S-type PLA_2 toxins in Sri Lankan Russell's viper venom, '$PLA_2$3' had a unique sequence, previously not observed in *D. russelii* from other geographical locations (Suzuki et al. 2010). The amino acid sequence of $PLA_2$3 covered 93 % of the aligned trypsin-digested peptides of U1-viperitoxin-Dr1a, indicating that $PLA_2$3 and U1-viperitoxin-Dr1a are almost certainly the same toxin. Considering the importance of naming this toxin based on its function and the species identity, we have followed the widely accepted nomenclature (King et al. 2008) and named the toxin U1-viperitoxin-Dr1a for the sequence under the following accession numbers, NCBI—GI: **298351762**; SwissProt: **P86368**. The N-terminal sequence of the first 50 amino acids of P3 from *D. r. pulchella* venom (location not specified whether the sample is from South India or Sri Lanka) described in Tsai et al. (1996) matches 100 % with both U1-viperitoxin-Dr1a from Sri Lanka and VRV-PL-V from South India. The amino acid sequence of VRV-PL-V covered 89 % of aligned peptides of U1-viperitoxin-Dr1a. U1-viperitoxin-Dr1a differs from VRV-PL-V by having amino acid differences at 58, 61, 97 and 98 positions from the N-terminus. VRV-PL-V is present mainly in South Indian *D. russelii* venom and its relative abundance is minute in the western, eastern and northern regions of India. Although a thorough pharmacological investigation on VRV-PL-V is unavailable, it is likely to be responsible for the clinically detectable neurotoxicity in South Indian patients (Prasad et al. 1999).

The MALDI-TOF mass spectrometry showed a mass of 6812.4 Da, in addition to U1-viperitoxin-Dr1a. Despite the 8.1 Da mass difference, it is most likely a doubly charged ion of U1-viperitoxin-Dr1a. Further, the presence of another peptide was not indicated by LC–MS.

The potency of the pre-synaptic neurotoxicity of individual PLA_2 toxins, their relative abundance in the venom and the circulating toxin concentrations (i.e. degree of envenoming) are responsible for the occurrence and severity of the clinically detectable neurotoxicity. It has been previously suggested (Tsai et al. 1996) that, in general, S-type PLA_2s abundant in South Indian and Sri Lankan Russell's viper venoms likely lead to the clinically evident neurotoxicity seen in patients in these regions. However, this hypothesis needs further investigation based on both proteomic and pharmacological studies using the venoms and their PLA_2 toxins representing multiple populations of the Russell's vipers across its wide distribution.

U1-viperitoxin-Dr1a was found to be much less potent compared to the pre-synaptic toxins from known neurotoxic snakes previously characterised using the same chick biventer cervicis nerve-muscle preparation in our laboratory (Table 1) (Kuruppu et al. 2005; Hodgson et al. 2007; Chaisakul et al. 2010; Barber et al. 2012) Concentrations less than 100 nM of the pre-synaptic neurotoxins from all three taipan species and two death adder species were able to inhibit the indirect twitches with similar t_{90}'s compared to 1000 nM U1-viperitoxin-Dr1a, and 600 nm of U1-viperitoxin-Dr1a failed to fully abolish twitches within 3 h. Even after 3 h, 20 nM U1-viperitoxin-Dr1a did not inhibit the indirect twitches whereas 9 nM textilotoxin from the Brown snake inhibited 50 % of the twitch height (Barber et al. 2012). This indicates that U1-viperitoxin-Dr1a is likely to be one of the weakest pre-synaptic neurotoxic PLA_2 toxins from snake venoms.

Interestingly, the twitch inhibition caused by crude Russell's viper venom (i.e. 150 μg in a 5-ml organ bath which is equivalent to 428 nM U1-viperitoxin-Dr1a) was more pronounced compared to the twitch inhibition by 600 nM U1-viperitoxin-Dr1a (Fig. 4a, d). However, this discrepancy is likely to be due to the other toxins present in the venom contributing to the overall neurotoxicity or by synergistically acting with U1-viperitoxin-Dr1a.

Russell's vipers inject large amounts of venom in the majority of their bites. Of 130 patients envenomed by Sri Lankan Russell's viper, the median peak venom concentration was 25 ng/ml, but 24 patients had venom concentrations exceeding 1000 ng/ml (Silva et al. 2016). The presence of neurotoxicity was associated with having higher venom concentrations (Silva et al. 2016), but higher

Table 1 Comparison of the time taken for inhibition of the indirect twitches by 90 % (t_{90}) by different pre-synaptic PLA_2 toxins from snake venoms in the chick biventer cervicis nerve-muscle preparation compared to U1-viperitoxin-Dr1a

Toxin	Snake	Concentration (nM)	t_{90} mean (SD, min)	Reference
U1-viperitoxin-Dr1a	*D. russelii*	1000	54 (6.8)	–
P-EPTX-Ap1a	*Acanthophis praelongus* (Northern death adder)	20	96 (9.8)	Chaisakul et al. (2010)
		100	79.5 (8.0)	
P-EPTX-Ar1a	*A. rugosus* (Rough scaled death adder)	20	96 (9.8)	Chaisakul et al. (2010)
		100	79.5 (8.0)	
Taipoxin	*Oxyuranus scutellatus* (Coastal taipan)	45	~70	Barber et al. (2012)
Paradoxin	*Oxyuranus microlepidotus* (Inland taipan)	65	129 (8)	Hodgson et al. (2007)
Cannitoxin	*Oxyuranus scutellatus canni* (Papuan taipan)	66	<60	Kuruppu et al. (2005)

venom concentrations were not associated with more severe neurotoxicity. In the 24 patients with the highest venom concentrations, paralysis was still only limited to mild neurotoxicity characterised by ptosis and ophthalmoplegia and none had life-threatening paralysis. The absence of life-threatening paralysis despite high venom concentrations is likely to be due to the low potency of U1-viperitoxin-Dr1a. Further, in the above study, neurotoxicity in patients was associated with envenoming by larger snakes. The possible contribution of ontogenic variation of the venom towards the neurotoxicity in Sri Lankan Russell's viper envenoming cannot be excluded unless a comparative proteomic and pharmacological investigation is carried out using both juvenile and adult venoms.

In a patient with circulating venom concentrations of 1000 ng/ml, assuming that there is no significant difference in the pharmacokinetics of U1-viperitoxin-Dr1a compared to the antigenic components of the rest of the venom detected in the enzyme immunoassay, a 14.1 nM (192 ng/ml) concentration of U1-viperitoxin-Dr1a could be expected in the circulation, considering its relative abundance in the venom (i.e. 19.2 %). Assuming that the circulating U1-viperitoxin-Dr1a freely reaches the motor nerve terminal, 14.1 nM toxin concentration could be expected at the motor nerve terminal. In the present study, 600 nM of U1-viperitoxin-Dr1a was insufficient to inhibit the indirect twitches of both the avian and rat nerve-muscle preparations by 90 %, even after 3 h. Assuming that avian, rodent and human motor nerve terminals are similarly susceptible to the pre-synaptic neurotoxins, there is unlikely to be a high enough concentration of U1-viperitoxin-Dr1a to cause full disruption of neurotransmission, even in severely envenomed patients, which would explain the mild neurotoxicity seen in patients.

When the recommended antivenom concentration was pre-mixed with U1-viperitoxin-Dr1a in the organ bath, based on the proportion of U1-viperitoxin-Dr1a in the venom, the antivenom only delayed, but did not prevent twitch abolition by U1-viperitoxin-Dr1a. Unfortunately, an increased antivenom concentration could not be used because of significant alteration of solute concentration in organ bath with higher antivenom concentrations. Similarly, lowering the U1-viperitoxin-Dr1a concentration in the organ bath was not possible because U1-viperitoxin-Dr1a concentrations lower than 1 μM were unable to fully abolish twitches. It is therefore difficult to comment on the efficacy of the antivenom, beyond the conclusion that it does not completely prevent neurotoxicity by U1-viperitoxin-Dr1a at the recommended antivenom concentration. In a recent in vitro and in vivo study using Indian polyvalent antivenom (VINS), concentrations higher than recommended based on the use of 10 vials of antivenom were sufficient to fully bind and neutralise the neurotoxicity of the circulating Russell's viper venom antigens in envenomed humans (Maduwage et al. 2016).

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Clinical and Pharmacological Investigation of Myotoxicity in Sri Lankan Russell's Viper (*Daboia russelii*) Envenoming

Anjana Silva[1,2,3], Christopher Johnston[4], Sanjaya Kuruppu[1,5], Daniela Kneisz[1], Kalana Maduwage[3,4,6], Oded Kleifeld[5], A. Ian Smith[5], Sisira Siribaddana[2], Nicholas A. Buckley[3,7], Wayne C. Hodgson[1], Geoffrey K. Isbister[1,3,4]*

1 Monash Venom Group, Department of Pharmacology, Biomedicine Discovery Institute, Monash University, Melbourne, Victoria, Australia, **2** Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura, Sri Lanka, **3** South Asian Clinical Toxicology Research Collaboration, University of Peradeniya, Peradeniya, Sri Lanka, **4** Clinical Toxicology Research Group, University of Newcastle, Newcastle, New South Wales, Australia, **5** Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Melbourne, Victoria, Australia, **6** Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Peradeniya, Sri Lanka, **7** Clinical Pharmacology, University of Sydney, Sydney, Australia

*[redacted]

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Abstract

Background

Sri Lankan Russell's viper (*Daboia russelii*) envenoming is reported to cause myotoxicity and neurotoxicity, which are different to the effects of envenoming by most other populations of Russell's vipers. This study aimed to investigate evidence of myotoxicity in Russell's viper envenoming, response to antivenom and the toxins responsible for myotoxicity.

Methodology and Findings

Clinical features of myotoxicity were assessed in authenticated Russell's viper bite patients admitted to a Sri Lankan teaching hospital. Toxins were isolated using high-performance liquid chromatography. *In-vitro* myotoxicity of the venom and toxins was investigated in chick biventer nerve-muscle preparations. Of 245 enrolled patients, 177 (72.2%) had local myalgia and 173 (70.6%) had local muscle tenderness. Generalized myalgia and muscle tenderness were present in 35 (14.2%) and 29 (11.8%) patients, respectively. Thirty-seven patients had high (>300 U/l) serum creatine kinase (CK) concentrations in samples 24h post-bite (median: 666 U/l; maximum: 1066 U/l). Peak venom and 24h CK concentrations were not associated (Spearman's correlation; $p = 0.48$). The 24h CK concentrations differed in patients without myotoxicity (median 58 U/l), compared to those with local (137 U/l) and generalised signs/symptoms of myotoxicity (107 U/l; $p = 0.049$). Venom caused concentration-dependent inhibition of direct twitches in the chick biventer cervicis nerve-muscle preparation, without completely abolishing direct twitches after 3 h even at 80 μg/ml. Indian polyvalent antivenom did not prevent *in-vitro* myotoxicity at recommended concentrations. Two phospholipase A_2 toxins with molecular weights of 13kDa, U1-viperitoxin-Dr1a (19.2%

Competing Interests: The authors have declared that no competing interests exist.

of venom) and U1-viperitoxin-Dr1b (22.7% of venom), concentration dependently inhibited direct twitches in the chick biventer cervicis nerve-muscle preparation. At 3 μM, U1-viperitoxin-Dr1a abolished twitches, while U1-viperitoxin-Dr1b caused 70% inhibition of twitch force after 3h. Removal of both toxins from whole venom resulted in no *in-vitro* myotoxicity.

Conclusion

The study shows that myotoxicity in Sri Lankan Russell's viper envenoming is mild and non-life threatening, and due to two PLA_2 toxins with weak myotoxic properties.

Author Summary

There are many gaps in our knowledge of muscle damage caused by snake venoms. Russell's vipers are more medically important than any other snake in Asia. Sri Lankan Russell's viper (*Daboia russelii*) bites have been reported to cause muscle damage in humans, which is not reported for other Russell's vipers. The aim of the present study was to investigate the onset, severity and resolution of the muscle damage and to identify the toxins responsible for myotoxicity. For this, we studied muscle damage in 245 patients with confirmed Sri Lankan Russell's viper bites. Patients reported local muscle pain in 72% of cases and generalised muscle pain in 15%. None had severe muscle damage and the symptoms resolved in 80% of patients within 4 days. Measurement of biomarkers of muscle damage in patient blood was consistent with only mild muscle injury, even in patients with symptoms. Two toxins were isolated from Sri Lankan Russell's viper venom that had similar myotoxic activity to whole venom in chick muscle preparations. Both toxins were weak myotoxins, consistent with what was seen in patients.

Introduction

Snake bite is a significant public health issue in the tropics [1]. Coagulopathy, neuromuscular paralysis, acute kidney injury and local effects are the most important clinical syndromes of snake envenoming [2]. Russell's viper bites cause a large number of envenomings across Asia, and are more medically important than any other snake in the region [3,4]. Both species of Russell's vipers, i.e. *D. russelii* (found in Sri Lanka, India, Pakistan, Nepal, Bangladesh) and *D. siamensis* (found in some parts of southeast and east Asia such as Cambodia, Myanmar, Thailand, Taiwan, South China and, some parts of Indonesia including East Java), commonly cause venom-induced consumption coagulopathy, acute kidney injury and mild local effects throughout their distribution [3,5]. However, there is geographical variation in the clinical effects of Russell's viper envenoming with neuromuscular paralysis and rhabdomyolysis only reported from Sri Lanka and South India [3,6–9]. In a recent clinical and neurophysiological investigation of neuromuscular paralysis in Sri Lankan Russell's viper envenoming, we showed that the paralysis is mild, non-life threatening with no long term effects [6]. Clinical evidence of myotoxicity, including local and generalized myalgia, muscle tenderness, and dark red or black coloured urine suggestive of myoglobinuria, has been reported in cases of Russell's viper envenoming in Sri Lanka [7–10]. Elevation of plasma and urinary myoglobin concentrations were reported in 19 Russell's viper bite patients from Sri Lanka further suggesting the existence of myotoxicity in Sri Lankan Russell's viper patients [10]. A recent study of Sri Lankan

Russell's viper venom injected into mice reported an elevation in creatine kinase, also suggesting that the venom is myotoxic *in-vivo* [11].

Myotoxicity is an important effect of snake envenoming and can manifest locally and systemically [12]. Local muscle necrosis is a component of the local necrotic effects [12,13]. Systemic myotoxicity has been reported following envenoming by some vipers [14,15], sea snakes [16–18], Australasian elapids [19,20] and some Asian kraits [21,22]. Systemic myotoxicity ranges in severity. Loss of functioning muscle cells due to myotoxicity can aggravate co-existing weakness due to neuromuscular block caused by neurotoxins. More importantly rhabdomyolysis can cause secondary acute kidney injury [23,24] and itself can result in life-threatening hyperkalaemia due to extensive muscle cell damage [15].

Although many cytotoxic components in snake venoms may contribute to the development of myotoxicity, the most important snake venom myotoxins are phospholipase A_2 (PLA_2) toxins [12,25]. Three types of PLA_2 myotoxins, commonly referred as Asp49, Ser49 and Lys49 PLA_2, have been characterised in viperid venoms. Despite structural similarity, the latter two types lack catalytic activity, and are referred to as 'PLA_2 like' peptides [26–28]. It is important to note that the enzymatic activity and the myotoxic activity of PLA_2 myotoxins are independent [29]. Myotoxic PLA_2s cause muscle damage primarily by destruction of the sarcolemma [12,30]. Several PLA_2 toxins have been isolated from Sri Lankan Russell's viper venom and biochemically characterised, [31,32] Some of these PLA_2 toxins contain a unique Serine residue at the N-terminus (S-type), while others have an Asparagine residue at the N-terminus (N-type) [32]. We have recently shown that the pre-synaptic neurotoxicity of the Sri Lankan Russell's viper venom is primarily due to one of the S-type PLA_2 toxins, which we named U1-viperitoxin-Dr1a [33].

There are several gaps in our understanding of the myotoxicity associated with Sri Lankan Russell's viper envenoming. It is unclear if Sri Lankan Russell's viper envenoming causes severe myotoxicity and whether any resultant myotoxicity can be treated with Indian Polyvalent antivenom. Further, the *in vitro* myotoxicity of Sri Lankan Russell's viper venom has not been pharmacologically studied. This requires isolation and pharmacological characterisation of the myotoxins from the venom. The present study aims to investigate the clinical severity of myotoxicity from Russell's viper envenoming and isolate the toxins responsible for this activity.

Methods

Clinical study

Ethics, study setting and patients. We carried out a prospective cohort study of confirmed Russell's viper bite patients aged over 16 years admitted to the Teaching Hospital Anuradhapura, Sri Lanka for a 14 month-period. The hospital is a tertiary care hospital which admits over 1000 suspected snake bite cases annually. Ethics clearance for this study was granted by the Human Research Ethics Committees of the University of Peradeniya (Approval No: 2012/EC/63), Sri Lanka, Rajarata University of Sri Lanka (Approval No: ERC 2013/019) and Monash University, Australia (Approval No: CF14/970–2014000404). Written informed consent was obtained prior to the recruitment of all patients. For patients 16 and 17 years of age, consent was also obtained from the patient's parent or guardian. The recruitment of patients to this cohort study has been previously described. [6]

Case authentication. Cases were authenticated by either positive identification of the snake specimen involved as a Russell's viper by AS, who is a herpetologist, or by detection of the specific Russell's viper venom antigens in blood samples of the patients using enzyme-immunoassay (EIA).

Clinical data collection and blood collection. Clinical examination of all patients was undertaken on admission to hospital, 1 h, 4 h, 8 h, 12 h and 24 h post-bite, and then every 24 h until discharge. In particular, muscle pain and tenderness, in the bitten limb (local myotoxicity) and the other limbs (generalized myotoxicity) were assessed as part of the clinical examinations. Blood samples were collected from patients on admission and then 1 h, 4 h, 8 h, 12 h and 24 h post-bite, and daily thereafter. All blood samples were immediately centrifuged, serum aliquoted and frozen initially at -20˚C before transfer to -80˚C storage until they were analysed.

Antivenom. Indian polyvalent antivenoms from VINS Bioproducts, India (batch numbers: 01AS11118, 1119, 1121, 1123, 3100, 4001, 4025, 4026, 4031) and BHARAT Serums and Vaccines Ltd, India (batch number: A5311018) were used throughout the study period. In the present cohort, the majority of patients received antivenom and a small proportion of these received their first dose of antivenom at a primary care hospital, before being transferred to the study hospital. The decision to administer antivenom was made solely by the treating physician, based on clinical and laboratory evidence of systemic envenoming. Patients were administered between 10 to 20 vials of antivenom which is the standard dose. Antivenom was administered in normal saline and infused over 1h. If an acute adverse reaction to antivenom occurred, the infusion was stopped for 5 to 10 min and the patient treated with adrenaline, antihistamines and corticosteroids, as per the treating clinicians.

Russell's viper venom enzyme immunoassays. Measurement of venom in the samples was carried out using a Russell's viper venom specific Enzyme-Immunoassay (EIA) which has been previously described [6,34,35]. In brief, rabbit IgG was raised against Russell's viper venom from Sri Lanka. Antibodies were bound to microplates as well as conjugated to biotin as the detecting antibodies with streptavidin-horseradish peroxidase. The lower limit of detection for the assay was 2.5 ng/ml. In cases where no venom was detected in pre-antivenom samples or pre-antivenom samples were not available, the post-antivenom samples were subjected to heat dissociation treatment and then tested for venom using the same EIA [35].

Serum creatine kinase (CK) assay. Creatine kinase (CK) was measured using the Thermo Scientific Ltd CK-NAC reagent kit on stored frozen serum samples. Patient serum samples were thawed and 15 μL added to 300 μL of reagent. After 120 s, the samples were read in a plate reader (340 nm, 37˚C) for 3 min. An abnormal CK was defined as a concentration greater than 300 U/l. A screening assay was done on all 24 h post-bite samples, or as close to this time point as possible. If the screening assay was abnormal (> 300 U/L), then serial CK assays were carried out for all samples available for that patient.

Data analysis. Continuous variables were reported as medians, range and interquartile range (IQR). Associations between venom concentration and CK measurements were assessed by Spearman's correlation test. The CK concentrations between groups of patients was compared with the Kruskal-Wallis test. Analysis of clinical data was done using PRISM, version 6.05 (GraphPad Software, Inc.).

Pharmacological study

Venom and antivenom. Freeze-dried Russell's viper (*D. russelii*) venom from Sri Lanka (pooled from both sexes, including juveniles) donated by Professor Ariaranee Gnanadasan (Faculty of Medicine, University of Colombo, Sri Lanka) was used for this study. Venom was dissolved in MilliQ water and stored at -20˚C until required. Protein quantification of the venom, fractions and toxins was carried out using a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA), as per the manufacturer's instructions. The relative abundance of the two isolated toxins was determined by the area under the chromatogram of the whole

venom. Isolation of the two toxins was performed as described, from whole venom, using the two chromatographic techniques. Here, the two toxins separately and the venom without the two toxins were collected by multiple HPLC runs and pooled separately. Then, the three samples (i.e. venom without both toxins, and the two toxins) were dissolved separately. Based on the protein assay, the protein/toxin amounts in the samples were determined. Then the different combinations of the venom with and without individual toxins were made by mixing the venom without the two toxins with appropriate toxin according to the proportion present in the venom.

Indian polyvalent antivenom (VINS Bioproducts, Thimmapur, India; batch No: 01AS14035) was used for the study. The antivenom was reconstituted in 2 mL of sterile injection water. According to the manufacturer, 1/10 of the antivenom in a single vial neutralises 0.6 mg of Russell's viper venom. For *in-vitro* studies examining the neutralisation of myotoxins, the required amount of antivenom was calculated based on the relative abundance of the toxin in the venom.

In-vivo myotoxicity studies. Male Sprague–Dawley rats (300-360g) were anaesthetised with sodium pentobarbital (60–100 mg/kg, i.p.) with additional anaesthetic being administered during the experiment as required. An incision was made in the cervical region, the carotid artery was cannulated for blood sample collection and the trachea intubated for artificial respiration, if required. The rats were kept on small animal surgery tables with their body temperature maintained at 37˚C. Venom (250 μg/kg in 50 μl 0.9% saline, n = 4) was injected into the gastrocnemius muscle of the left hind-limb. For control rats (n = 4), 50 μl 0.9% saline was injected in the same location. Blood samples (500 μl) were collected into MiniCollect LH/Gel separation tubes (Greiner bio-one), through the carotid cannula immediately before the venom/saline injection and at 1.5, 3, 4.5 and 6 h post-injection. At 6 h, rats were humanely killed with an overdose of sodium pentobarbitone. For the baseline measurements of CK, blood samples of an additional seven 'control' (i.e. not envenomed) rats were used. Measurement of CK was done using the same CK assay method used for human samples.

Liquid chromatography. Fractionation was performed using a Shimadzu (Kyoto, Japan) system (LC-10ATvp pump and SPD-10AVP detector).

Size-exclusion chromatography: Crude venom (500 μg) was reconstituted in buffer A (ammonium acetate; 0.1 M, pH 6.8). Venom was then separated using a Superdex G-75 column (13 μm; 10 mm × 300 mm; GE Healthcare, Uppsala, Sweden) equilibrated with buffer A. Elution was performed at a flow rate of 0.5 ml/min and monitored at 280 nm. All fractions were freeze-dried immediately and later screened using the chick biventer cervicis nerve-muscle preparation (see 2.2.6 below) to identify those with myotoxicity.

Reverse-Phase HPLC (RP-HPLC): The fractions obtained from size exclusion chromatography, which displayed *in vitro* myotoxicity, were subjected to further analysis using reverse-phase HPLC. Size exclusion chromatography fractions were reconstituted in solvent A (0.1% trifluoroacetic acid [TFA]) and 2 mg of total protein was injected into a Phenomenex Jupiter semi-preparative C_{18} column (250 mm ×10 mm; 5 μm; 300 A˚), equilibrated with solvent A. Fractions were eluted using the following gradient of solvent B (90% Acetronitrile in 0.1% TFA): 0–30% over 10 min, 30–70% for 10–50 min, and 70–0% for 50–55 min at a flow rate of 2.0 ml/min.

Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF) Mass Spectrometry. MALDI-TOF Mass Spectrometry analysis was performed in order to determine the intact mass of the toxins, with an Applied Biosystems (Forster City, CA, USA) 4700 TOF TOF Proteomics Analyser, as described previously (Chaisakul et.al.). [36]

Amino acid sequence determination by Liquid Chromatogarphy-Mass Spectrometry (LC-MS). Freeze-dried U1-viperitoxin-Dr1b dissolved in 100 mM ammonium bicarbonate buffer, 2 mM DL-Dithiothreitol, incubated for 20 min at 65˚C and alkylated with 5 mM

chloroacetamide. The protein mixture was digested with trypsin at 1:100 (w/w) ratio for 18 h at 37˚C. The resulting peptides were desalted with Pierce C18 Spin columns (ThermoFisher Scientific), dried, resuspended in 2% Acetronitrile, 0.1% TFA and analysed by LC-MS/MS analysis as previously described [37]. The raw data acquired by the mass spectrometer was converted into a centroided peaklist file using ProteoWizard (version 3.0.9967) [38]. The MS/MS results were analysed with PMI-Byonic software (Protein Metrics, ver 2.8.14) against Uniprot serpents (taxID:8570) protein sequences (update 07/2016) using the following search parameters: trypsin digestion with up to 2 missed cleavages, fixed cysteine alkylation, variable methionine oxidation and asparagine and glutamine deamidation, precursor mass tolerance 10 ppm and fragment mass tolerance 20ppm.

PLA_2 activity. PLA_2 activity of the venom and U1-viperitoxin-Dr1b was determined using a secretory PLA_2 assay kit (Cayman Chemical, Ann Arbor, MI, USA) as described previously [33] and according to the manufacturer's instructions.

Chick biventer cervicis nerve-muscle preparation. Male chickens (aged 4–10 days) were humanely killed by exsanguination following CO_2 inhalation. Biventer cervicis nerve-muscle preparations were dissected and then mounted on wire tissue holders under 1 g resting tension in 5 ml organ baths. Tissues were maintained at 34˚C, bubbled with 95% O_2 and 5% CO_2, in physiological salt solution of the following composition (mM); 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 11.1 glucose. Direct twitches were evoked by stimulating the muscle belly (rate: 0.1 Hz; pulse duration: 2 ms) at supramaximal voltage (20–30 V) with the two electrode loops directly encircling the muscle belly, using a Grass S88 stimulator (Grass Instruments, Quincy, MA). Contractile responses of the tissues to KCl (40 mM for 30 s) was obtained in the absence of muscle stimulation. Following that, direct stimulation of the muscle was resumed. Selective stimulation of the muscle was ensured by abolishment of any indirect twitches by the continual presence of adding d-tubocurarine (10 μM) to the organ bath. The preparations were then stimulated for 30min, before the addition of venom or toxins. For antivenom experiments, the tissues were equilibrated with antivenom for 20 min before the venom or toxin was added. The preparation was exposed to the treatment/control until abolishment of direct twitches or for a period of 3 h. At the conclusion of the experiment, KCl was re-added as above.

Data analysis and statistics. The quantity of the toxins in the venom was determined by measuring the area under the curve of the size-exclusion and RP HPLC venom elution profiles. Direct twitch responses and responses to KCl were measured via a Grass FTO3 force displacement transducer and recorded on a PowerLab system (ADInstruments Pty Ltd., Australia). Responses were expressed as percentages of their pre-venom/toxin values. T_{90} values (i.e. time taken for 90% inhibition of the maximum twitch response to occur) were determined for the two toxins. To compare the responses to exogenous agonists following the administration of venom a one-way ANOVA was used. All ANOVAs were followed by Bonferroni's multiple comparison post-tests. Data are presented in the form of mean ± standard error of the mean (S.E.M.) of n experiments. All statistical analyses and presentation of data were generated using GraphPad Prism 6.05 software. For all statistical tests $p < 0.05$ was considered statistically significant.

Animal ethics. All animal experiments used in this study were approved by the Monash University Animal Ethics Committee (Approval No: MARP/2014/097).

Results

Myotoxicity in authenticated cases

Clinical features of myotoxicity. Of the 245 authenticated Russell's viper bite patients recruited to the study, 177 (72.2%) had myalgia and 173 (70.6%) tenderness in the bitten limb

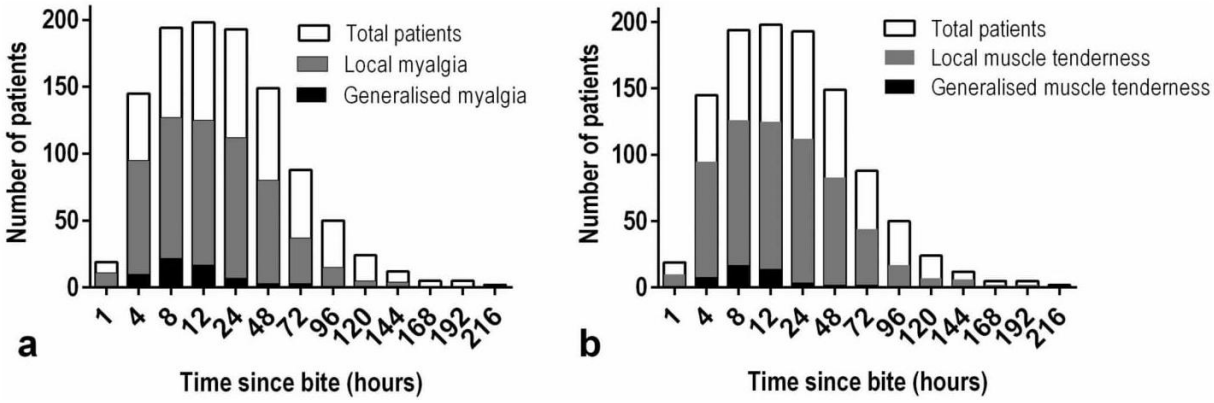

Fig 1. Clinical features of myotoxicity in 245 confirmed Sri Lankan Russell's viper envenomings (a), the presence of localised and generalised myalgia over time; (b), the presence of localised and generalised muscle tenderness over time. Note: data of all 245 patients were not available at any given time because some patients were admitted later than the time point or discharged before the time point, or the clinical assessment of the patient was not possible at that time point because the patient was sedated, unconscious or transported away for investigations.

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(local myotoxicity). Thirty-five patients (14.2%) and 29 patients (11.8%) had generalized myalgia and generalized muscle tenderness, respectively. Changes in local and generalized myalgia and muscle tenderness over time among the study participants is shown in Fig 1A and 1B. Myalgia and muscle tenderness resolved in over 80% of the patients by day 4. Six patients (2.4%) had dark coloured urine observed between 12 and 48 h after the bite. The clinico-epidemiological data related this cohort of patients is described in the Table 1.

Serum creatine kinase concentrations. Serum CK concentrations in 24 h post-bite samples of 219 patients ranged from 2 to 1019 U/l (median: 110 U/l). Samples of the remaining 26 patients were unavailable. Of the 219 patients, 37 patients (16.8%) had CK concentrations above 300 U/l. The median 24 h post-bite CK concentrations of patients who had no clinical features of myotoxicity was 58 U/l (IQR: 34–183 U/l), compared to 137 U/l (IQR: 56–285 U/l) in those with features of local myotoxicity, and 107 U/l (IQR: 71–226 U/l) in patients who had features of systemic myotoxicity ($p = 0.049$; Kruskal Wallis test; Fig 2A). There was no statistically significant association between 24 h serum CK concentration and peak venom concentrations (Spearman's correlation; $p = 0.48$; Fig 2B). Similarly, there was no relationship between the time delay from the snakebite to first dose of antivenom and 24 h serum CK (Fig 2C). Of

Table 1. Clinical and epidemiological data of the 245 patients recruited for the present study.

Age (Median, range in years)	41 (16–70)
Sex (males)	171 (70%)
Length of the snake specimen (median, range)	32.3 (20.4–110.5) cm
Time since bite to reach the study hospital (Median, IQR)	2.5 (1.75–4.0) h
Peak venom concentration (median, range)	25 (2.5–2316) ng/ml
Number of patients with coagulopathy	166 (68%)
Number of patients with neurotoxicity	130 (53%)
Number of patients received antivenom	190 (78%)
Time from snakebite to antivenom (Median, IQR)	3.75 (2.75–5.0) h
Number of antivenom vials received (median, range)	20 (10–90)
Length of hospital stay	3 (2–31) days

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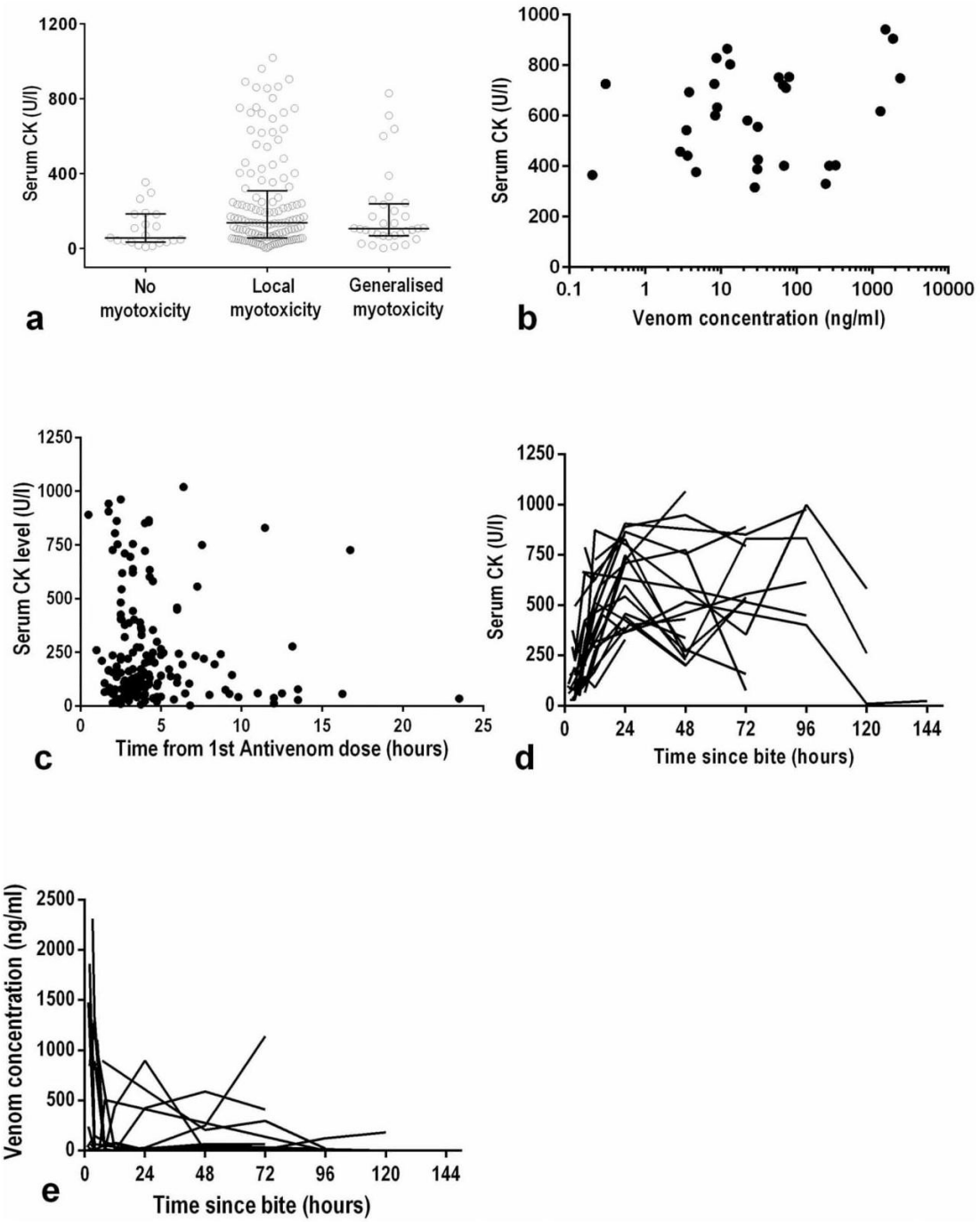

Fig 2. Serum creatine kinase (CK) concentrations in 219 study participants: (a) Scatter plot comparing the CK concentrations (at 24 h post bite) of patients with no features, patients with features of local myotoxicity (myalgia or tenderness) and patients with features of systemic myotoxicity (*p = 0.049; Kruskal-Wallis test*); (b), peak serum concentrations of CK versus venom in 37 patients who had CK >300U/l at 24 h post-bite (*Spearman's correlation; p = 0.48*); (c), CK concentration at 24 h versus time from the snakebite to the first antivenom dose in 178 patients who received antivenom (note: 12 patients who received antivenom had no CK due to the unavailability

of the sample at 24 h); (d), plots of the CK concentrations versus time since bite for the 37 patients; (e), plots of the venom concentrations versus time for the 37 patients.

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the 190 patients who were given antivenom, CK concentrations at 24 h were available in 178 patients. Of these, 135 patients were given antivenom within 6 h and 31 of these developed a CK > 300 U/l at 24 h, compared to 31 patients who received antivenom after 6 h with 6 developing a CK > 300 U/l at 24 h.

Only three of the six patients with dark coloured urine had CK concentrations above 300 U/l (404, 633 and 856 U/l,). However, red blood cells were reported in three of these six patients (S1 Table). The dark coloured urine in Russell's viper envenoming could be due to myoglobinuria, haemoglobinuria, haematuria or a combination of all these.

The serial CK concentrations of the 37 patients with high CK had a median peak value of 666 U/l (maximum: 1066). In most patients, CK reached a peak in 24 h after the bite (Fig 2D). By the time CK started to rise, the venom concentrations in these patients had already decreased, and in most cases was lower than the level of detection (Fig 2E).

In-vivo myotoxic effects in anaesthetised rats

The plasma CK concentrations of rats injected with Sri Lankan Russell's viper venom 250 μg/kg (i.m.) were not different tocontrol rats and were within the normal range until 6 h of the venom injection (S1 Fig).

Isolation and biochemical characterisation of U1-viperitoxin-Dr1a and U1-viperitoxin-Dr1b

Liquid Chromatography. Eight major fractions were identified with size-exclusion chromatography of the crude venom of Russell's viper (Fig 3A). Peak E, eluting around 33 min showed marked myotoxicity in the chick biventer cervicis nerve-muscle preparation. Peak E was further analysed and fractionated using RP-HPLC (Fig 4B). *In vitro* myotoxicity was largely confined to two peaks with retention times of 31 min and 38 min, respectively (Fig 3B). The former venom component eluting at 31 min was subsequently isolated as U1-viperitoxin-Dr1b, and constituted 22.7% (SD: 1.2; n = 10) of the crude venom, based on the area under the curve of the size exclusion chromatography and RP-HPLC profiles of the venom. The component eluting at 38 min was named U1-viperitoxin-Dr1a, a PLA_2 toxin which has been previously characterised biochemically and found to be the major neurotoxin (with a relative abundance of 19.2%) in Sri Lankan Russell's viper venom [33].

Intact protein analysis of U1-viperitoxin-Dr1b by MALDI-TOF. Analysis of U1-viperitoxin-Dr1b by MALDI-TOF mass spectrometry indicated a single species with a mass/charge ratio of 13564 Da (Fig 4). A doubly charged ion with a mass/charge ratio of 6779.8 Da was also observed.

LC-MS/MS identification of U1-viperitoxin-Dr1b by LC-MS. LC-MS/MS analysis of U1-viperitoxin-Dr1b identified it as Basic phospholipase. The obtained sequence coverage was 100% showing that the toxin is exactly identical to Basic phospholipase A_2 VRV-PL-VIIIa (SwissProt: **P59071**, Entry name: PA2B8_DABRR) (see S2 Fig and S2 Table).

PLA_2 activity of U1-viperitoxin-Dr1b. PLA_2 activity of U1-viperitoxin-Dr1b was 1139 (+/- 19) μmol/min/mg.

Pharmacological investigation of the myotoxicity. Sri Lankan Russell's viper venom caused concentration-dependent inhibition of direct twitches in the chick biventer nerve muscle preparation (Fig 5A) and inhibited the response to 40 mM KCl (Fig 5B). However, high

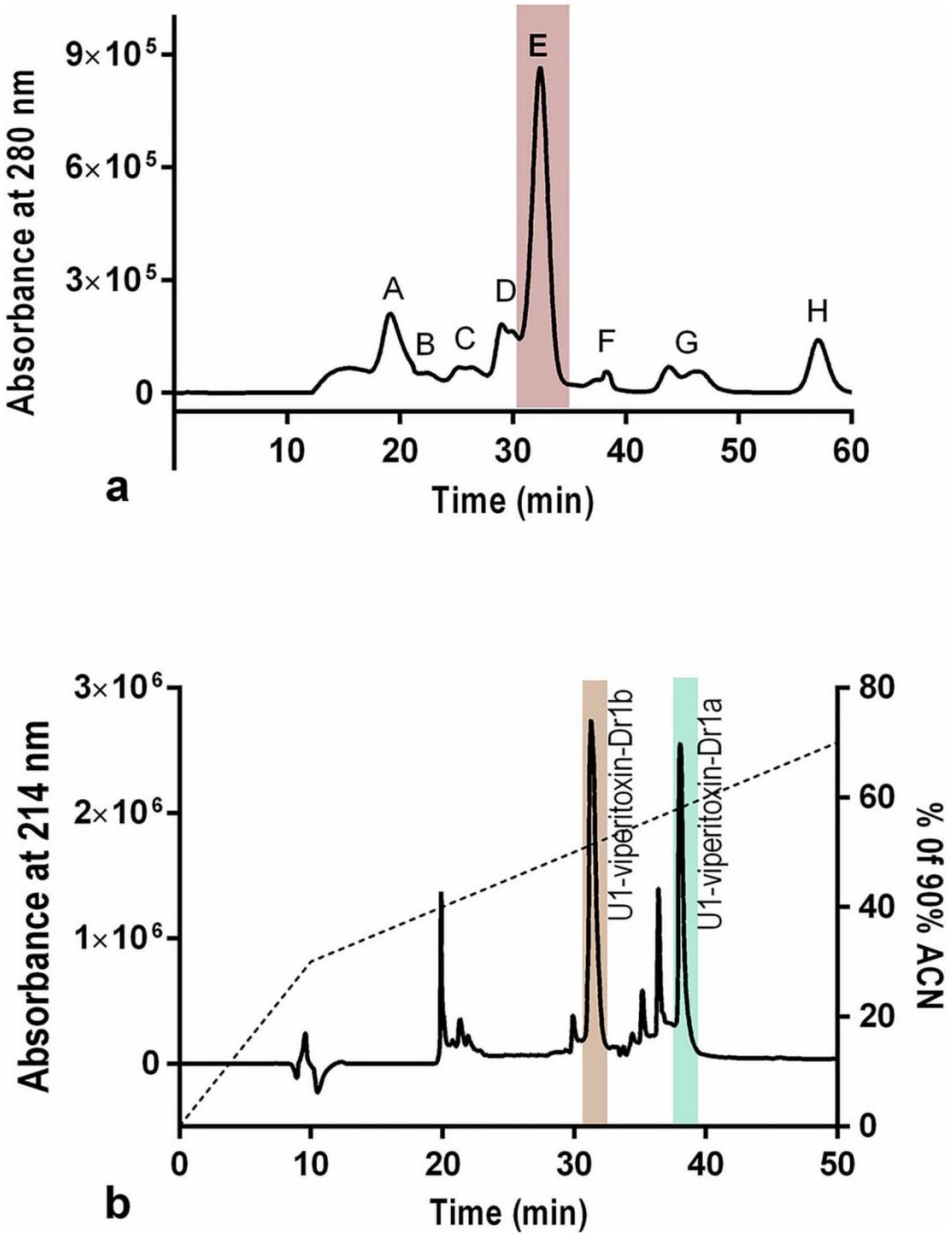

Fig 3. Chromatograms of Sri Lankan Russell's viper venom and fractions (a), Size-exclusion chromatogram of the whole venom on Superdex G75 column; (b), Reverse Phase High Performance Liquid Chromatogram (RP-HPLC) of the fraction 'E' on Jupiter C_{18} semi-preparative column. Note: the peaks eluting at 31 and 38 min in RP-HPLC chromatograms are U1-viperitoxin-Dr1b and U1-viperitoxin-Dr1a, respectively.

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venom concentration such as 80μg/ml did not completely abolish either the direct twitches or the response to KCl, even after 3 h. U1-viperitoxin-Dr1b (Fig 5C) and U1-viperitoxin-Dr1a (Fig 5D) concentration-dependently inhibited the direct twitches and also inhibited the

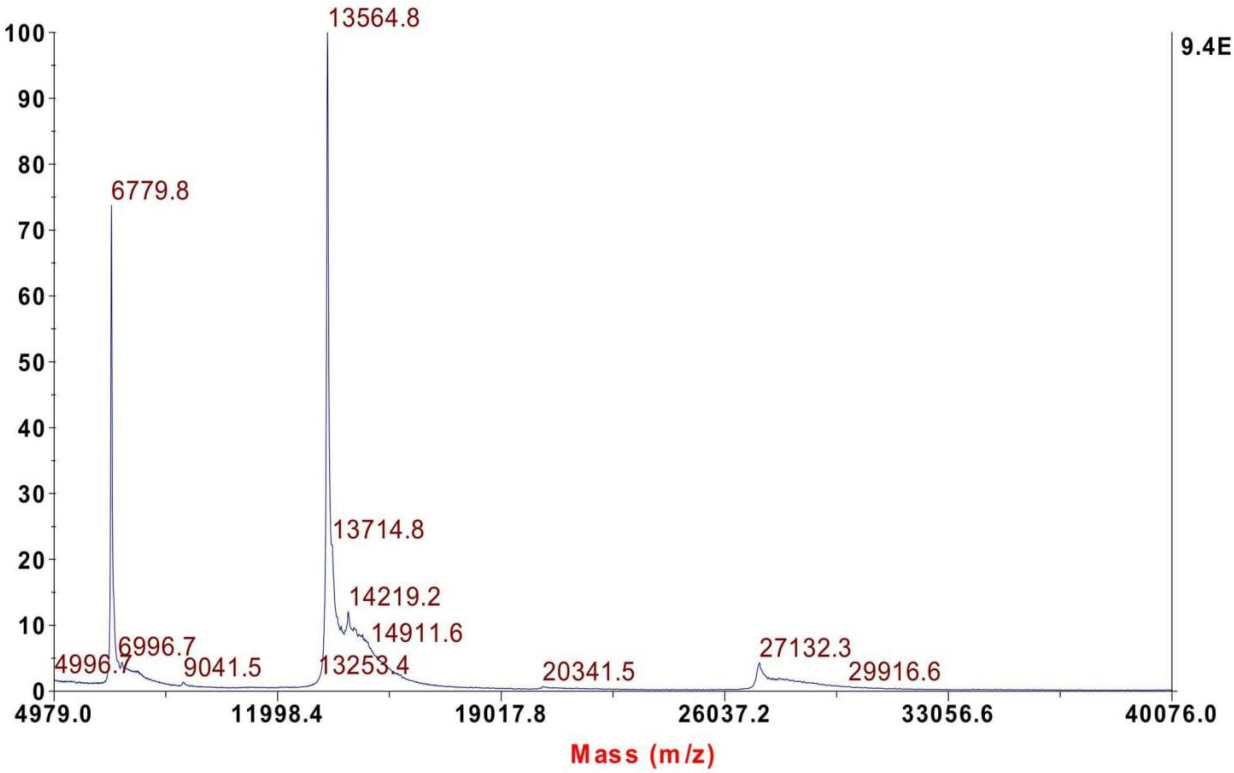

Fig 4. Intact protein analysis chromatogram of MALDI-TOF: the intact mass of U1-viperitoxin-Dr1b is 13.564 kDa.

doi:10.1371/journal.pntd.0005172.g004

response to KCl (Fig 5E). When the response to KCl was compared between the crude venom 50μg/ml, each of the two toxins of the same amount of venom individually, a combination of the two toxins, and the whole venom without individual or both toxins, removal of the two toxins led to a loss of all myotoxicity from the venom (Fig 5F). Antivenom, at the recommended concentrations, did not neutralise the myotoxic effect of the venom (Fig 5B)

Discussion

The present study demonstrates that myotoxicity seen in humans following Sri Lankan Russell's viper envenoming is mild, based on the relatively low frequency of generalized myotoxic features and only a very mild elevation in CK. There were small but statistically significant association between clinical features of myotoxicity and the 24 h CK measurements. The rats injected intramuscularly with Sri Lankan Russell's viper venom did not show an elevation of CK within the first 6 h. Although Sri Lankan Russell's viper venom displays concentration-dependent myotoxic effects *in-vitro*, very high venom concentrations (i.e. >50 μg/ml) are required to completely inhibit direct twitches in the chick biventer nerve-muscle preparation and the response to KCl. Antivenom at recommended concentrations was unable to neutralise the myotoxic properties of the venom *in-vitro*. The myotoxicity of the whole venom appears to be due to two abundant PLA_2 toxins in the venom, U1-viper1toxin-Dr1a and U1-viperitoxin-Dr1b (named herein), which both have *in vitro* myotoxic activity, but only at very high concentrations (3 μM).

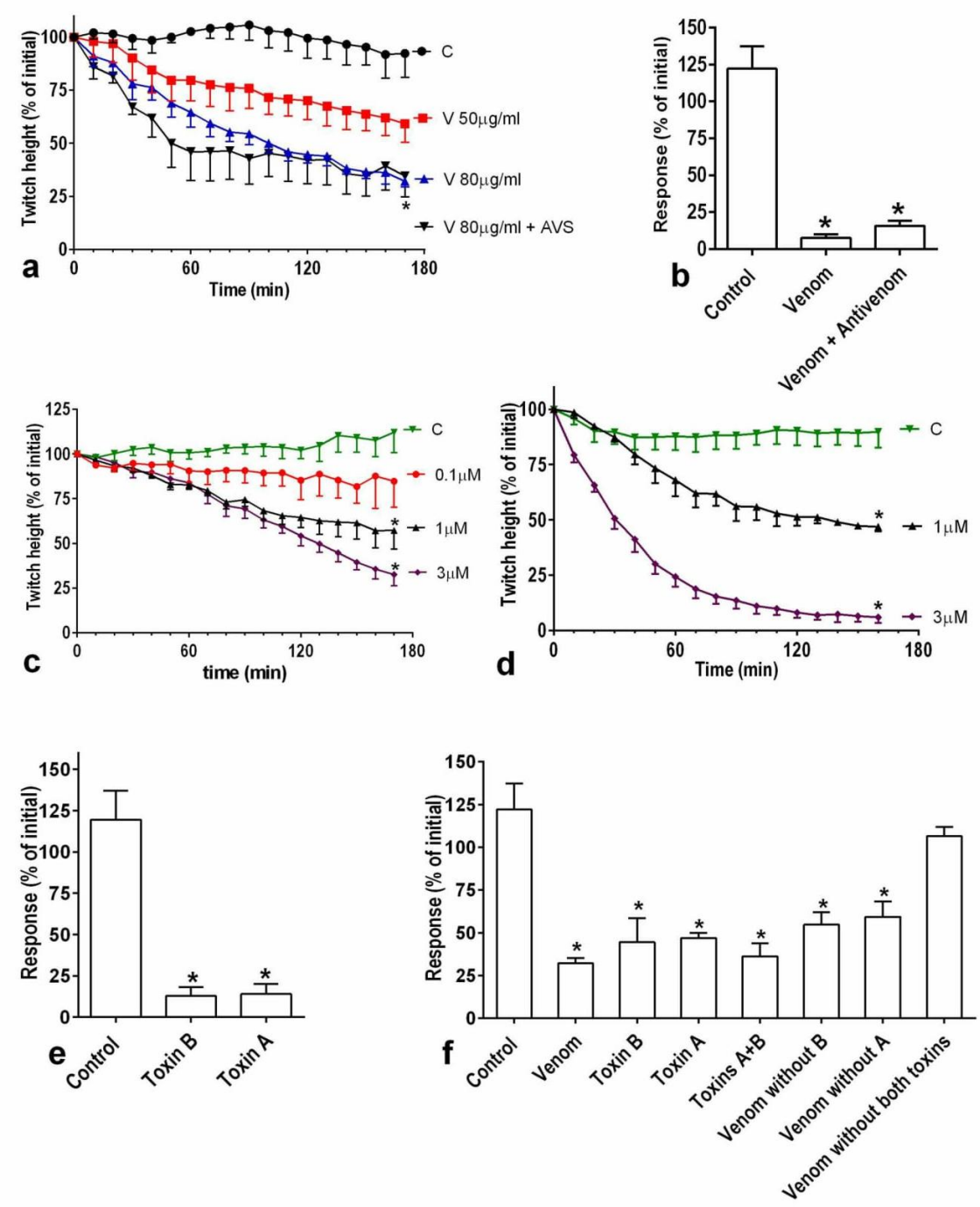

Fig 5. *In-vitro* myotoxicity of whole venom (Venom) of Sri Lankan Russell's viper, U1-viperitoxin-Dr1a (Toxin A) and U1-viperitoxin-Dr1b (Toxin B) compared to controls: **(a)**, Concentration-dependent inhibition of direct twitches in chick biventer nerve-muscle preparation by whole venom. (* the 80μg/ml venom group is significantly different from 50 μg/ml group as well as the control group at 170 min; *p<0.05*: *One-way ANOVA followed by Bonferroni's post-hoc test*, *n = 4*.). **(b),** Effect of 80μg/ml venom alone and in the presence of antivenom on the

response of the muscle to 40mM KCl. (* significantly different compared to the response to KCl obtained prior to the addition of venom; *p<0.05*: *paired t-test*) **(c)**, Concentration-dependent inhibition of the direct twitches in chick biventer nerve-muscle preparation by U1-viperitoxin-Dr1b. *(twitch height significantly lower than the control group: *p<0.05*, *one-way ANOVA followed by Bonferroni's post-hoc test; n = 3–5*); **(d)**, Concentration-dependent inhibition of the direct twitches in chick biventer nerve-muscle preparation by U1-viperitoxin-Dr1a. (* twitch height significantly lower than the control group: *p<0.05*, *one-way ANOVA followed by Bonferroni's post-hoc test; n = 3–5*); **(e)**, Effect of 3 μM U1-viperitoxin-Dr1a (B) and U1-viperitoxin-Dr1b (M) towards the response of the muscle for 40 mMKCl. (* the KCl response of the venom with and without antivenom, at 170min were significantly reduced compared to the initial responses; *p<0.05*: *paired t-test*); **(f)**, Effect of 50μg/ml whole venom versus U1-viperitoxin-Dr1a, U1-viperitoxin-Dr1b, venom without U1-viperitoxin-Dr1a, venom without U1-viperitoxin-Dr1b, both toxins together, venom without both toxins of a same amount of venom in 5ml organ bath towards the response of the muscle for 40 mMKCl. * (* the KCl response is significantly lower compared to the control group at 170 min; *p<0.05*: *one-way ANOVA followed by Bonferroni's post-hoc test*; n = 3–5).

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Features of systemic myotoxicity such as generalized muscle tenderness and myalgia, and dark coloured urine were observed in less than 15% of the patients. In addition, three of the six patients with dark coloured urine did not have 24 h CK concentrations outside the normal limit, so the dark urine was more likely due to macroscopic haematuria (due to haemorrhage) which occurs with Russell's viper envenoming. Peak CK concentrations were abnormally high in less than 17% of patients, and even in these cases there were only modest elevations with the median and maximum being 666 and 1066 U/l, respectively. In comparison, the reported median peak CK values following envenoming by mulga snakes and tiger snakes in Australia were 3100 U/l [20] and 4749 U/l [19], with severe myotoxicity being associated with CK values over 100,000 U/L. This means that myotoxicity in Russell's viper envenoming is uncommon and mild when it occurs compared to other myotoxic snakes. The median 24 h CK concentrations were higher in symptomatic patients demonstrating that the clinical features were consistent with biochemical evidence of mild muscle injury.

Anaesthetised rats injected with venom showed no significant increase in serum CK concentrations compared to saline injected rats, even 6 h after the venom injection. Assuming that the blood volume of a rat with a body weight of 330g is approximately 25 ml, the maximum serum venom concentration that this venom dose (i.e. 250 μg/kg), with 100% systemic absorbance, could give rise to is 3300 ng/ml. This venom concentration is greater than the maximum venom concentration observed in the Russell's viper envenomed patients in this study, hence the venom dose used for the envenoming is clinically relevant. The absence of a significant CK rise in the envenomed rats during the 6 h observation period may be due to a combined effect of the weak myotoxicity of the venom and the period of observation being too short to observe any CK elevation. These results are not in agreement with the previously reported high CK concentrations (mean: 16,000 U/l) in mice, 3 h after the intramuscular injection of 5 μg (250 μg/kg dose in 20 g mouse) of Sri Lankan Russell's viper venom [11], despite similar venom doses in the two studies. The reason for the discrepancy is unclear but such a large early increase in CK concentrations is unusual and the mouse model has not previously been validated.

The clinical findings were consistent with the *in vitro* studies in which two low potency myotoxins were found to be relatively abundant in the venom. Very high venom concentrations up to 80 μg/ml (80 times higher than the maximum peak venom concentration observed in envenomed patients) were required to decrease the direct twitch force by two thirds within 3 h. The low potency of both myotoxins was evident from the fact that high concentrations (3 μM) were required for significant *in-vitro* myotoxic activity. Venom with these two toxins removed did not cause myotoxicity confirming that these toxins are the only major myotoxins in Sri Lankan Russell's viper venom.

Three previous studies between 1984 and 2000 report much higher rates of myotoxicity, based mainly on myalgia and muscle tenderness. In 1984, Jeyarajah [9] reported myotoxic

symptoms in 77% of the cases but no biochemical confirmation of myotoxicity. This study included only severe envenoming with 19/22 with acute kidney injury and 6 deaths. In 1988, Phillips et. al. [10] reported myotoxic symptoms in 32% of the cases, but again this only included more severe envenoming (4/23 died). The mean venom concentration of 375 ng /ml (range: 16.5–702 ng/ml) [10] was much higher compared to the median peak venom concentration of 25 ng/ml (range:2.5–2316 ng/ml) in our study [6]. Phillips et al. reported serum and urine myoglobin, which are difficult to interpret because no studies have correlated these measurements with outcomes. They detected myoglobin in the plasma of all 19 patients tested (range: 100->8000; median: 2745, normal value:<50 ng/ml) and in the urine in 14 of 18 patients (110 to >16 000; median: 4000; normal: <21 ng/ml)[10]. Although these appear to be high, much higher values are reported in Australian mulga snake envenoming, where urinary myogolobin values in three patients were 4129, 28200 and 127000 ng/ml[20]. In a larger study of 336 patients in 2000 by Kularatne et al [7] there were 47 patients (14%) with myotoxicity based on generalised muscle tenderness. This is consistent with our study in terms of clinical effects but they did not provide any biochemical confirmation. Our 245 patients are typical of current patients. They had high circulating peak venom concentrations with a median of 25ng/ml, with 24 patients having venom concentrations >1000 ng/ml [6]. Further, nearly all had local envenoming, 68% had coagulopathy (half with bleeding manifestations), and 53% had neuromuscular paralysis [6]. Therefore, the mild myotoxic effects in Sri Lankan Russell's viper envenoming are most likely due to the weak myotoxicity of the venom.

In patients with Australian Mulga snake (*Pseudechis australis*) envenoming, a delay in antivenom treatment has led to an increase in the severity of the myotoxicity [20]. Since there was no control group of patients who did not receive antivenom in this study, it could be argued that the weak myotoxicity observed in Sri Lankan Russell's viper envenoming is due to an effect of antivenom. However, there was no association between the delay in antivenom treatment and the number of patients having CK >300 U/l, indicating that the weak myotoxicity observed in the patients is not due to an effect of the antivenom.

Indian Polyvalent antivenom was used at concentrations equivalent to the tested venom as recommended by the manufacturer. At these concentrations antivenom failed to prevent the myotoxic effects of Sri Lankan Russell's viper venom *in-vitro*. Although this may be due to the low efficacy of the antivenom against the myotoxins [39], testing higher antivenom concentrations was not possible due to the practical limitations of increasing antivenom concentration in the tissue organ bath environment without affecting the osmolarity of the physiological salt solution. It was therefore not possible to determine the efficacy of the Indian polyvalent antivenom to neutralise the myotoxic effects of Sri Lankan Russell's viper venom, because such a large amount of venom was required to cause myotoxicity requiring very high concentrations of antivenom.

Of the several 's' type PLA_2 toxins isolated from Sri Lankan Russell's viper venom, VRV-PL-VIIIa [40] has 100% match with the aligned trypsin digested peptide fragments of U1-viperitoxin-Dr1b. The N-terminal sequence of the first 50 amino acids of the toxin 'P1' [31] and the N-terminal sequence of first 21 amino acids of the toxin PLA_2 4 [32] are a 100% match for the sequence of U1-viperitoxin-Dr1b. Following the suggested rational nomenclature for toxins [41], and given that the 94% sequence homology of the toxin with U1-viperitoxin-Dr1a[33], we have named the above toxin as U1-viperitoxin-Dr1b (Fig 6).

The recent study on the venom proteome of the Sri Lankan Russell's viper [11] reported that five PLA_2 toxins make up a relative abundance of 35% of the whole venom, with VRV-PL-VIIIa (U1-viperitoxin-Dr1b) making 13.9% (as opposed to 22.2% in our study) of the whole venom. However, the same study did not match a PLA_2 with VRV-PL-V (U1-

```
U1-viperitoxin-Dr1b   SP|P59071|PA2B8_DABRR SLLEFGKMILEETGKLAIPSYSSYGCYCGWGGKGTPKDATDRCCFVHDCCYGNLPDCNPK 60
U1-viperitoxin-Dr1a   SP|P86368|PA2B3_DABRR SLLEFGMMILEETGKLAVPFYSSYGCYCGWGGKATPKDATDRCCFVHDCCYGNLPDCNPK 60
                                            ******.**********:** ***********.*************************

                      SP|P59071|PA2B8_DABRR SDRYKYKRVNGAIVCEKGTSCENRICECDKAAAICFRQNLNTYSKKYMLYPDFLCKGELK 120
                      SP|P86368|PA2B3_DABRR SDRYKYKRVNGAIVCEQGTSCENRICECDKAAAICFRRNLNTYSKIYMLYPDFLCKGELK 120
                                            ****************:*******************:*******.**************

                      SP|P59071|PA2B8_DABRR C 121
                      SP|P86368|PA2B3_DABRR C 121
                                            .
```

Fig 6. Alignment of U1-viperitoxin-Dr1b amino acid sequence with U1-viperitoxin-Dr1a. Sequences were obtained from UniProt database and are presented with unique identification numbers and entry names. In residues marked as '*' are single fully conserved residues. At the positions highlighted in yellow, ':' and '.' denote positions with conservation between groups of strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix) and, conservation between groups of weakly similar properties (scoring = < 0.5 in the Gonnet PAM 250 matrix) respectively.

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viperitoxin-Dr1a), which makes 19.2% of the venom in our analysis as previously published [33].

In conclusion, myotoxicity in Sri Lankan Russell's viper envenoming is mild and non-life threatening, as evident from the low frequency of generalized myotoxicity and low concentrations of serum creatine kinase in envenomed patients. The whole venom of Sri Lankan Russell's viper has weak myotoxic properties *in-vitro*. Two PLA_2 toxins, U1-viperitoxin-Dr1a and U1-viperitoxin-Dr1b that make 42% of the whole venom, are the major myotoxins in the venom, but display weak myotoxicity. There is a small possibility that myotoxicity may occur in patients with severe Sri Lankan Russell's viper envenoming in which antivenom is delayed.

Supporting Information

S1 Checklist. checklist of the guidelines for Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).
(DOC)

S1 Table. Evidence of muscle damage, renal injury and haematuria in six patients who had dark coloured urine.
(DOCX)

S2 Table. LC-MS/MS based identification of the amino acid sequence of U1-viperitoxin-Dr1b: List of all the identified proteins and the matched peptides of each proteins with the identification-related information.
(XLSX)

S1 Fig. Plasma CK concentrations in anaesthetised rats (n = 4) intramuscularly injected with 250 μg/kg Sri Lankan Russell's viper venom compared to the saline injected rats (n = 4). Note: there is no difference in the CK concentrations of the two groups. The shaded area indicates the baseline CK concentration observed in 15 rats.
(TIF)

S2 Fig. U1-viperitoxin-Dr1b protein coverage map based on MS/MS identification. All U1 Dr1b tryptic peptides that were identified by MS/MS ion search are marked as green lines and aligned according to their start and end position along U1 Drb1 sequence (shown on top). Modified residues are shown in red.
(JPG)

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Author Contributions

Conceptualization: AS GKI WCH NAB SS.

Formal analysis: AS GKI WCH SK OK.

Funding acquisition: GKI NAB.

Investigation: AS SS KM CJ SK OK DK.

Methodology: AS GKI SS WCH SK IS.

Supervision: GKI WCH.

Writing – original draft: AS.

Writing – review & editing: GKI WCH SK SS CJ NAB KM IS OK DK.

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Errata

Paper 1

The legend to Figure 2 should read: “Intact protein analysis chromatogram of MALDI-TOF: the intact mass of U1- viperitoxin-Dr1a is 13641.0 m/z.”

Paper 2

Reference 12 should read:
Gutierrez JM, Ownby CL. Skeletal muscle degeneration induced by venom phospholipases A2: insights into the mechanisms of local and systemic myotoxicity. Toxicon. 2003; 42: 915–31.

CHAPTER FIVE

Cross neutralisation of neurotoxic venoms and their pre- and post-synaptic neurotoxins by different antivenoms.

Overview of the Chapter

The ability of heterologous antivenoms to cross-neutralise the neurotoxic effects of snake venoms from closely related snake species has been previously reported for a number of venom-antivenom combinations. However, this cross-neutralisation has not been investigated on a range of different antivenoms and neurotoxic venoms from across different geographical regions. In addition, such a pharmacological investigation should include an emphasis on the neutralisation of pre- and post-synaptic neurotoxicity. This Chapter includes a study of the *in vitro* cross neutralisation of venoms from four Australian elapids, four Asian elapids, four α-neurotoxins and one β-neurotoxin by five commercially available antivenoms from South Asia, Southeast Asia and Australia which were raised against neurotoxic snake venoms. Overall, all tested venoms, except Australian brown snake venom, were cross neutralized by the heterologous antivenoms tested, indicating a remarkable cross neutraliation of snake venom neurotoxins by heterologous antibodies. Post-synaptic neurotoxicity of the toxins and venoms was neutralized by antivenoms raised against venoms containing post-synaptic neurotoxins. Similarly, pre-synaptic neurotoxicity of the toxins and venoms was neutralized by antivenoms raised against venoms containing pre-synaptic neurotoxins. Australian brown snake venom, which contains unique type III α-neurotoxins which are absent in Asian snake venoms, was not cross neutralized by Asian antivenoms. This study provides evidence on the potential for developing a universal neurotoxic antivenom by selecting representative toxins/venoms in the immunization mixture. The study was published as a research article in the journal, Toxins (Basel).

Silva A, Hodgson WC, Isbister GK. Cross-neutralisation of in vitro neurotoxicity of Asian and Australian Snake neurotoxins and venoms by different antivenoms. *Toxins (Basel)*. 2016;8(10). pii: E302.

toxins

MDPI

Article

Cross-Neutralisation of In Vitro Neurotoxicity of Asian and Australian Snake Neurotoxins and Venoms by Different Antivenoms

Anjana Silva [1,2,*], Wayne C. Hodgson [1] and Geoffrey K. Isbister [1,3]

1 Monash Venom Group, Department of Pharmacology, Biomedicine Discovery Institute, Monash University, Clayton, VIC 3800, Australia; [redacted]
2 Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura 50008, Sri Lanka
3 Clinical Toxicology Research Group, University of Newcastle, Newcastle, NSW 2298, Australia
* Correspondence: [redacted]

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Abstract: There is limited information on the cross-neutralisation of neurotoxic venoms with antivenoms. Cross-neutralisation of the in vitro neurotoxicity of four Asian and four Australian snake venoms, four post-synaptic neurotoxins (α-bungarotoxin, α-elapitoxin-Nk2a, α-elapitoxin-Ppr1 and α-scutoxin; 100 nM) and one pre-synaptic neurotoxin (taipoxin; 100 nM) was studied with five antivenoms: Thai cobra antivenom (TCAV), death adder antivenom (DAAV), Thai neuro polyvalent antivenom (TNPAV), Indian Polyvalent antivenom (IPAV) and Australian polyvalent antivenom (APAV). The chick biventer cervicis nerve-muscle preparation was used for this study. Antivenom was added to the organ bath 20 min prior to venom. Pre- and post-synaptic neurotoxicity of *Bungarus caeruleus* and *Bungarus fasciatus* venoms was neutralised by all antivenoms except TCAV, which did not neutralise pre-synaptic activity. Post-synaptic neurotoxicity of *Ophiophagus hannah* was neutralised by all antivenoms, and *Naja kaouthia* by all antivenoms except IPAV. Pre- and post-synaptic neurotoxicity of *Notechis scutatus* was neutralised by all antivenoms, except TCAV, which only partially neutralised pre-synaptic activity. Pre- and post-synaptic neurotoxicity of *Oxyuranus scutellatus* was neutralised by TNPAV and APAV, but TCAV and IPAV only neutralised post-synaptic neurotoxicity. Post-synaptic neurotoxicity of *Acanthophis antarcticus* was neutralised by all antivenoms except IPAV. *Pseudonaja textillis* post-synaptic neurotoxicity was only neutralised by APAV. The α-neurotoxins were neutralised by TNPAV and APAV, and taipoxin by all antivenoms except IPAV. Antivenoms raised against venoms with post-synaptic neurotoxic activity (TCAV) cross-neutralised the post-synaptic activity of multiple snake venoms. Antivenoms raised against pre- and post-synaptic neurotoxic venoms (TNPAV, IPAV, APAV) cross-neutralised both activities of Asian and Australian venoms. While acknowledging the limitations of adding antivenom prior to venom in an in vitro preparation, cross-neutralization of neurotoxicity means that antivenoms from one region may be effective in other regions which do not have effective antivenoms. TCAV only neutralized post-synaptic neurotoxicity and is potentially useful in distinguishing pre-synaptic and post-synaptic effects in the chick biventer cervicis preparation.

Keywords: antivenom; cross-neutralisation; venom; neurotoxicity; snake

1. Introduction

Snakebites impose a considerable health and socioeconomic burden on many nations in southern and south-eastern Asian. This is due to the large number of cases of envenomings that cause acute, life-threatening, and debilitating long-term consequences [1–4]. Neuromuscular paralysis due to snake envenoming is a common important complication because of the rapid development of bulbar and

respiratory muscle paralysis that requires early and often prolonged intervention with intubation and mechanical ventilation [5]. Envenoming by snakes such as kraits (genus *Bungarus*), some species of cobras (genera *Naja* and *Ophiophagus*), taipans (genus *Oxyuranus*), death adders (genus *Acanthophis*), tiger snakes (genus *Notechis*) and coral snakes (genus *Micrurus*) commonly leads to life threatening neuromuscular paralysis [5].

In vitro and in vivo characterisation of neurotoxic venoms and isolated neurotoxins has been useful for understanding the pathophysiology of neuromuscular dysfunction in snake envenoming [6]. Neurotoxins from medically important snakes act either pre-synaptically or post-synaptically at the neuromuscular junction. Snake venom pre-synaptic neurotoxins (i.e., β-neurotoxins) are usually phospholipase A_2 (PLA_2) toxins. They enter the motor nerve terminal and lead to a depletion of synaptic vesicles by facilitating exocytosis and inhibiting synaptic vesicle recycling. This is followed by the rapid degeneration of the motor nerve terminal which is not reversible with treatment [7,8]. Snake venom post-synaptic toxins (i.e., α-neurotoxins) possess a three finger toxin structure and have either 61–62 amino acids with four disulphide bonds (i.e., short-chain toxins) or 64–71 amino acids with five disulphide bonds (i.e., long-chain toxins). The primary amino acid sequence of these toxins shows significant homology across diverse species [9,10]. Post-synaptic toxins bind to the two agonist binding sites of the nicotinic acetylcholine receptors (nAChR) at the motor end plate with high affinity, and prevent the opening of the associated ion channel, blocking neurotransmission. Venoms of many medically important neurotoxic snakes such as kraits and Australasian elapids have both pre- and post-synaptic toxins in their venoms [6–9,11]. Although the clinical importance of the pre-synaptic toxins in causing neuromuscular paralysis in snake envenomed humans is well recognised, the importance of post-synaptic toxins in human envenoming remains unclear [8,12].

Antivenoms have been used to treat snake envenomings for more than a century, and include monovalent and polyvalent antivenoms developed to treat neurotoxic envenoming. Irrespective of the wide diversity of snakes, snake venoms share many common toxin groups, including neurotoxic PLA_2 and three finger toxins [7,9]. Despite the structural and functional variations of the toxins within these toxin groups, similarities in their immunogenicity lead to cross-neutralisation by antivenoms raised against different snakes. Cross-neutralisation has been previously reported for some venom and antivenom combinations [13–19]. In most previous studies examining neurotoxic elapid venoms and antivenoms, cross-neutralisation has been measured by means of the prevention of rodent lethality (ED_{50}) [15,16,20]. However, death in these 'envenomed' animals is almost certainly a summative effect of the many toxins. Therefore, lethality tests are not always useful in understanding the ability of the antivenoms to specifically cross-neutralise the most clinically important effect—neurotoxicity [21]. Furthermore, in investigating the cross-neutralisation of neurotoxicity it is essential to understand the type of neurotoxicity (i.e., pre-synaptic or post-synaptic) that is being cross-neutralised by the antivenom, given that many snake venoms contain both types of neurotoxins. It is therefore necessary to further investigate this phenomenon using venoms from a range of neurotoxic snakes, neurotoxins and antivenoms.

The aim of this study was to test the ability of antivenoms raised against neurotoxic venoms to cross-neutralise individual neurotoxins and whole venoms of other neurotoxic snakes in vitro.

2. Results

The neurotoxicity of the venoms ranged from a t_{90} (i.e., time to 90% inhibition of twitch height) of 15.0 ± 2.1 min (*Pseudonaja textillis*), which is highly potent, to a t_{90} of 128.0 ± 17.2 min (*O. scutellatus*; Supplementary Table S1).

2.1. Cross-Neutralisation of Asian Neurotoxic Snake Venoms by Different Antivenoms

2.1.1. *Bungarus caeruleus*

B. caeruleus venom (5 μg/mL) inhibited indirect twitches (Figures 1a and 2) and abolished the response of the chick biventer preparation to exogenous nicotinic receptor agonists—acetylcholine

(ACh) and carbachol (CCh)—indicating the venom had post-synaptic neurotoxicity (Figures 1b and 2). Thai cobra antivenom (TCAV) did not prevent the inhibition of indirect twitches but prevented the abolition of the response of the chick biventer preparation to exogenous nicotinic agonists, indicating the neutralisation of post-synaptic effects, but not the pre-synaptic neurotoxicity of the venom. Indian polyvalent antivenom (IPAV), Thai neuro polyvalent antivenom (TNPAV), death adder antivenom (DAAV), and Australian polyvalent antivenom (APAV) all prevented the inhibition of indirect twitches, and the abolition of the response of the chick biventer preparation to exogenous nicotinic receptor agonists, indicating the neutralisation of both pre- and post-synaptic effects of the venom (Figures 1b and 2).

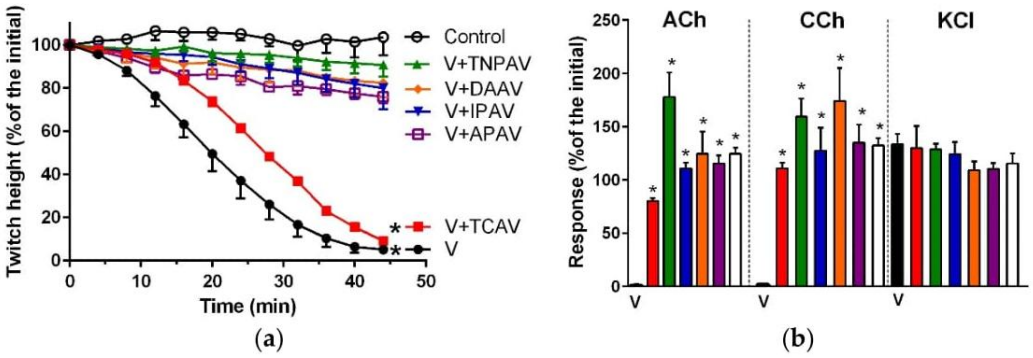

Figure 1. Cross-neutralisation of the neurotoxicity of *Bungarus caeruleus* venom (5 μg/mL, V, black) by Thai cobra antivenom (TCAV; red), death adder antivenom (DAAV; orange), Thai neuro polyvalent antivenom (TNPAV; green), Indian polyvalent antivenom (IPAV; blue), and Australian polyvalent antivenom (APAV; purple) in the chick biventer nerve-muscle preparation compared to control (white): (**a**) Prevention of the inhibition of indirect twitches caused by venom (V). Note: all antivenoms except TCAV prevented neurotoxicity (* significantly different from control at 44 min, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of venom on response to exogenous agonists (ACh, CCh and KCl) in the absence of antivenom and the presence of antivenoms compared to control. Note: all antivenoms prevented the abolition of agonist responses by the venom (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

2.1.2. *Bungarus fasciatus*

B. fasciatus venom (7.5 μg/mL) inhibited indirect twitches (Figure 3a) and abolished the response of the chick biventer preparation to exogenous nicotinic receptor agonists, indicating post-synaptic neurotoxic activity of the venom (Figure 3b). All antivenoms prevented the inhibition of indirect twitches (Figure 3a) and the abolition of the response of chick biventer preparation towards exogenous agonists (Figure 3b).

2.1.3. *Ophiophagus hannah*

O. hannah venom (5 μg/mL) inhibited indirect twitches (Figure 4a) and abolished the response of the chick biventer preparation to exogenous nicotinic receptor agonists, indicating post-synaptic neurotoxic activity of the venom (Figure 4b). All tested antivenoms prevented the inhibition of indirect twitches (Figure 4a) and the abolition of the response of the chick biventer preparation to exogenous agonists (Figure 4b).

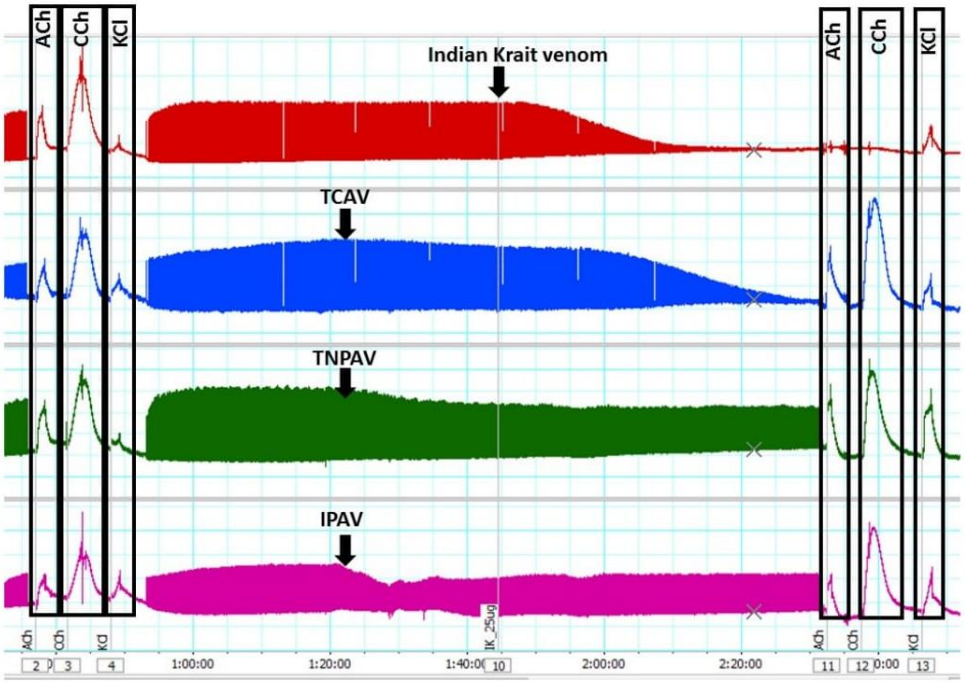

Figure 2. Traces showing indirect twitch and agonist contractile responses of the chick-biventer nerve-muscle preparation for *B. caeruleus* venom (5 μg/mL) in the absence and the presence of Thai cobra antivenom (TCAV), Thai neuro polyvalent antivenom (TNPAV) and Indian polyvalent antivenom (IPAV). Note: the venom (red trace) inhibits indirect twitches and abolishes ACh and CCh responses indicating post-synaptic neurotoxicity. In the presence of TCAV (blue trace), the venom inhibits indirect twitches but does not abolish ACh and CCh responses indicating that the pre-synaptic neurotoxicity is not neutralised. In the presence of TNPAV (green trace) and IPAV (purple trace), venom fails to inhibit indirect twitches and fails to abolish ACh and CCh responses indicating neutralisation of both pre- and post-synaptic neurotoxicity of the venom.

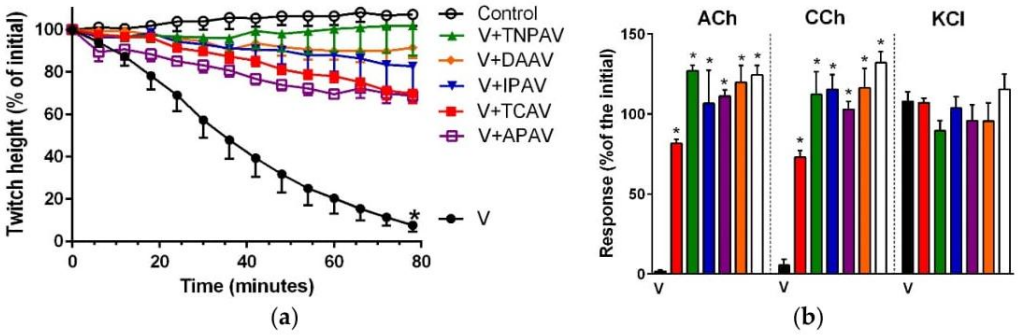

Figure 3. Cross-neutralisation of the neurotoxicity of *Bungarus fasciatus* venom (7.5 μg/mL, V, black) by Thai cobra antivenom (TCAV; red), death adder antivenom (DAAV; orange), Thai neuro polyvalent antivenom (TNPAV; green), Indian polyvalent antivenom (IPAV; blue), and Australian polyvalent antivenom (APAV; purple) in the chick biventer nerve-muscle preparation compared to control (white): (**a**) Prevention of the inhibition of indirect twitches caused by venom. Note: all antivenoms prevented neurotoxicity (twitch height as a percentage of the initial at 78 min not different from the control, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of venom on response to exogenous agonists (ACh, CCh and KCl) in the absence of antivenom and the presence of antivenoms compared to control. Note: all antivenoms prevented the abolition of agonist responses by the venom (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

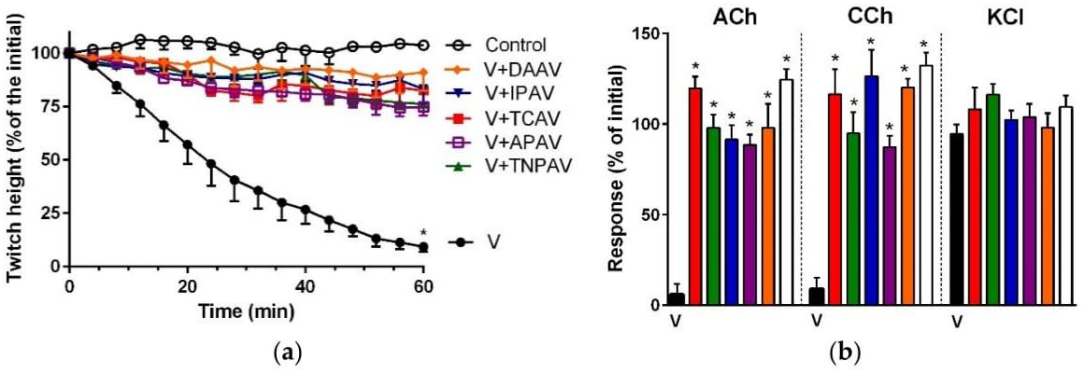

Figure 4. Cross-neutralisation of the neurotoxicity of *Ophiophagus hannah* venom (5 µg/mL, V, black) by, Thai cobra antivenom (TCAV; red), death adder antivenom (DAAV; orange), Thai neuro polyvalent antivenom (TNPAV; green), Indian polyvalent antivenom (IPVA), and Australian polyvalent antivenom (APAV) in chick biventer nerve-muscle preparation compared to control (white): (**a**) Prevention of the inhibition of indirect twitches caused by venom (V). Note: all antivenoms prevented neurotoxicity (twitch height as a percentage of the initial at 60 min not different from the control, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of venom on the response to exogenous agonists (ACh, CCh and KCl) in the absence of antivenom and the presence of antivenoms compared to control. Note: all antivenoms prevented the abolition of agonist responses by the venom (* significantly different from venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

2.1.4. *Naja kaouthia*

N. kaouthia venom (5 µg/mL) inhibited indirect twitches (Figure 5a) and abolished the response of the chick biventer preparation to exogenous nicotinic receptor agonists, indicating post-synaptic neurotoxic activity of the venom (Figure 5b). All tested antivenoms, except IPAV, prevented the inhibition of indirect twitches (Figure 5a) and abolition of the response of the chick biventer preparation towards exogenous agonists (Figure 5b). IPAV partially prevented the twitch inhibition, with the twitch force dropping by 50% over 60 min in the presence of antivenom (Figure 5a). However, the abolition of the response of chick biventer preparation towards exogenous agonists by the venom was not prevented by IPAV (Figure 5b).

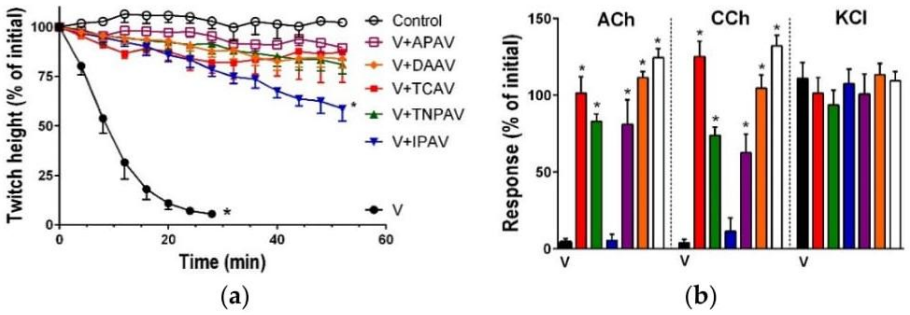

Figure 5. Cross-neutralisation of the neurotoxicity of *Naja kaouthia* venom (5 µg/mL, V, black) by Thai cobra antivenom (TCAV; red), death adder antivenom (DAAV; orange), Thai neuro polyvalent antivenom (TNPAV; green), Indian polyvalent antivenom (IPVA), and Australian polyvalent antivenom (APAV) in chick biventer nerve-muscle preparation compared to control (white): (**a**) Prevention of the inhibition of indirect twitches caused by venom (V). Note: all antivenoms except IPAV prevented neurotoxicity (* significantly lower from the control at 44 min, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of venom on the response to exogenous agonists (ACh, CCh and KCl) in the absence of antivenom and presence of antivenoms compared to control. Note: all antivenoms except IPAV prevented the abolition of agonist responses by the venom (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

2.2. Cross-Neutralisation of the Neurotoxicity of Australasian Elapid Venoms by Different Antivenoms

2.2.1. *Notechis scutatus*

N. scutatus venom (5 µg/mL) inhibited indirect twitches (Figure 6a) and abolished the response of the chick biventer preparation to exogenous nicotinic receptor agonists, indicating post-synaptic neurotoxicity of the venom (Figure 6b). TNPV, IPAV and APAV prevented venom-mediated inhibition of indirect twitches (Figure 6a) and abolition of the agonist responses in the chick biventer preparation (Figure 6b). TCAV only partially prevented the indirect twitch inhibition with the twitch inhibition being <45% after 48min (Figure 6a) but completely prevented abolishment of the response of chick biventer preparation to exogenous agonists (Figure 6b). This suggests that TCAV only blocks the post-synaptic effect of *N. scutatus* venom.

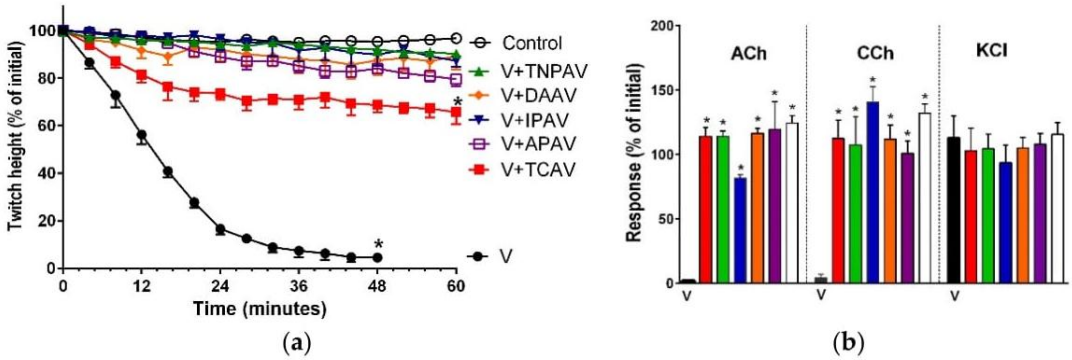

Figure 6. Cross-neutralisation of the neurotoxicity of *Notechis scutatus* venom (5 µg/mL, V, black) by Thai cobra antivenom (TCAV; red), Thai neuro polyvalent antivenom (TNPAV; green), Indian polyvalent antiveom (IPAV; blue), death adder antivenom (DAAV; orange), and Australian polyvalent antivenom (APAV; purple) in chick biventer nerve-muscle preparation compared to control: (**a**) Prevention of the inhibition of indirect twitches caused by venom (V). Note: all antivenoms except TCAV prevented neurotoxicity (* significantly lower from the control at 48 min, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of *N. scutatus* venom on the response to exogenous agonists (ACh, CCh and KCl) in the absence and the presence of antivenoms compared to controls. Note: all antivenoms prevented the abolition of agonist responses by the venom (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

2.2.2. *Oxyuranus scutellatus*

O. scutellatus venom (5 µg/mL) inhibited indirect twitches (Figure 7a), but only partially inhibited the response of the chick biventer preparation to exogenous nicotinic receptor agonists (Figure 7b) suggesting that the neurotoxicity of *O. scutellatus* venom is mainly due to pre-synaptic effects [1]. TNPAV and APAV prevented venom-mediated inhibition of indirect twitches (Figure 7a), and prevented the abolition of the exogenous nicotinic agonist responses of the chick biventer preparation (Figure 7b). TCAV and IPAV were unable to prevent the inhibition of indirect twitches, although IPAV appeared to partially prevent inhibition of indirect twitches by 70% (Figure 7a). However, both TCAV and IPAV prevented abolishment of the response of chick biventer preparation to exogenous nicotinic agonists (Figure 7b), indicating that the two antivenoms appeared to be able to neutralize post-synaptic but not pre-synaptic effects of the venom.

2.2.3. *Acanthophis antarcticus*

A. antarcticus venom (5 µg/mL) inhibited indirect twitches (Figure 8a), and abolished the response of the chick biventer preparation to exogenous nicotinic receptor agonists (Figure 8b) indicating post-synaptic neurotoxicity of the venom. All antivenoms except IPAV prevented the venom-mediated

abolition of indirect twitches (Figure 8a) and the response of the chick biventer preparation towards exogenous nicotinic receptor agonists (Figure 8b). IPAV failed to prevent the inhibition of indirect twitches, with 85% of the twitch height still inhibited at 48 min (Figure 8a), and also did not prevent abolishment of the response of the chick biventer preparation to exogenous agonists (Figure 8b). This suggests that IPAV was unable to neutralise the post-synaptic neurotoxic effects of *A. antarcticus* venom.

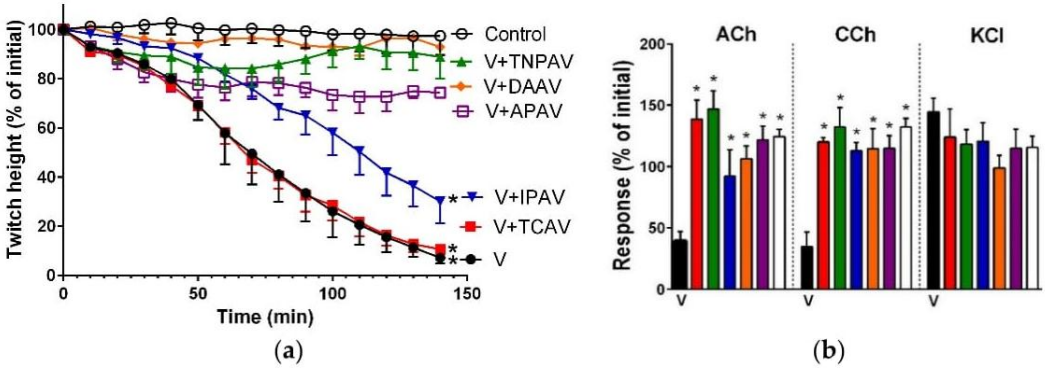

Figure 7. Cross-neutralisation of the neurotoxicity of *Oxyuranus scutellatus* venom (5 µg/mL, V, black) by Thai cobra antivenom (TCAV; red), Thai neuro polyvalent antivenom (TNPAV; green), Indian polyvalent antivenom (IPAV; blue), death adder antivenom (DAAV; orange), and Australian polyvalent antivenom (APAV; purple) in the chick biventer nerve-muscle preparation compared to control: (**a**) Prevention of the inhibition of indirect twitches caused by venom (V). Note: TCAV and IPAV failed to prevent neurotoxicity (twitch height as a percentage of the initial at 140 min is different from the control, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of venom on the response to exogenous agonists (ACh, CCh and KCl) in the absence and the presence of antivenoms compared to controls. Note: all antivenoms prevented the inhibition of agonist responses by the venom (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

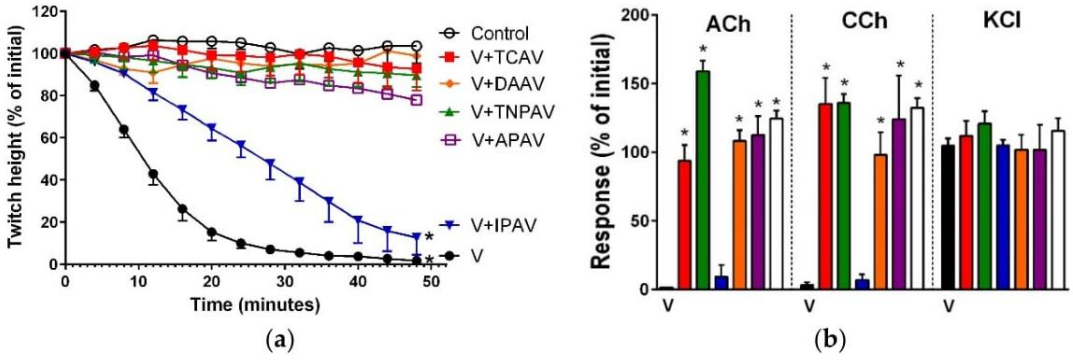

Figure 8. Cross-neutralisation of the neurotoxicity of *Acanthophis antarcticus* venom (5 µg/mL, V, black) by Thai cobra antivenom (TCAV; red), Thai neuro polyvalent antivenom (TNPAV; green), Indian polyvalent antivenom (IPAV; blue), death adder antivenom (DAAV; orange), Australian polyvalent antivenom (APAV; purple) in chick biventer nerve-muscle preparation compared to control: (**a**) Prevention of the inhibition of indirect twitches caused by venom (V). Note: TCAV and IPAV failed to prevent neurotoxicity (twitch height as a percentage of the initial at 48 min is different from the control, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of venom on the response to exogenous agonists (ACh, CCh and KCl) in the absence and the presence of antivenoms compared to controls. Note: all antivenoms prevented the inhibition of agonist responses by the venom (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

2.2.4. *Pseudonaja textillis*

P. textillis venom (5 μg/mL) abolished indirect twitches (Figure 9a) and abolished the response of the chick biventer preparation towards exogenous nicotinic receptor agonists (Figure 9b) indicating post-synaptic neurotoxicity of the venom. APAV prevented the venom-mediated inhibition of indirect twitches (Figure 9a) and abolishment of the response of nerve-muscle preparation to exogenous nicotinic receptor agonists (Figure 9b). TCAV, TNPAV and IPAV all failed to prevent the inhibition of the venom-mediated indirect twitches (Figure 9a). TCAV and IPAV did not prevent the abolishment of the response of the chick biventer preparation to exogenous agonists, (Figure 9b) and TNPAV partially prevented the abolishment of the response of the chick biventer preparation to exogenous agonists (Figure 9b).

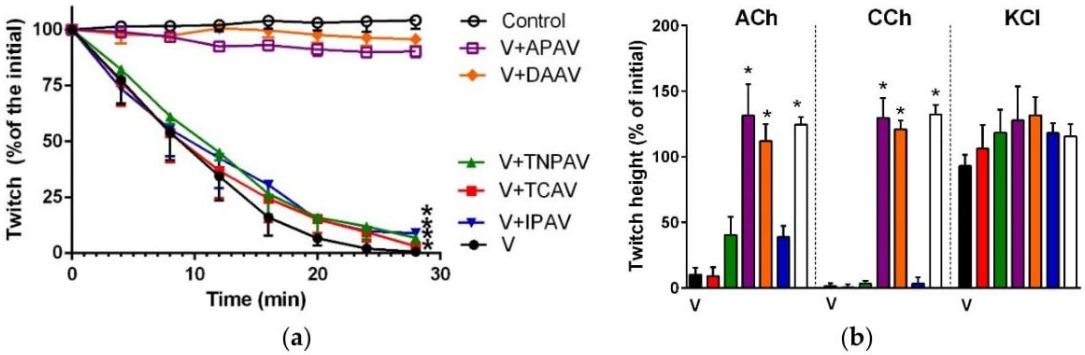

Figure 9. Cross-neutralisation of the neurotoxicity of *Pseudonaja textillis* venom (5 μg/mL, V, black) by Thai cobra antivenom (TCAV; red), Thai neuro polyvalent antivenom (TNPAV; green), Indian polyvalent antivenom (IPAV; blue), death adder antivenom (DAAV; orange), and Australian polyvalent antivenom (APAV; purple) in the chick biventer nerve-muscle preparation compared to control (white): (**a**) Prevention of the inhibition of indirect twitches caused by venom (V). Note: only APAV prevented neurotoxicity (* twitch height as a percentage of the initial at 28 min significantly different from the control, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of *P. textillis* venom on the response to exogenous nicotinic receptor agonists (ACh, CCh and KCl) in the absence and the presence of antivenoms. Note: only APAV prevented the abolition of ACh and CCh responses by the venom (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

At a lower dose of *P. textillis* venom (1 μg/mL), the inhibition of indirect twitches was not prevented by TCAV (i.e., twitch height not different from the venom group at any time point; $p < 0.05$, unpaired t test; Figure 10a). Furthermore, the venom (1 μg/mL) mediated abolition of the response of the chick biventer preparation towards exogenous nicotinic receptor agonists was not prevented by TCAV ($p < 0.05$, one-way ANOVA followed by Bonferroni's post hoc test; Figure 10b).

This suggests that there was little cross-neutralisation between *P. textillis* neurotoxicity and Asian antivenoms.

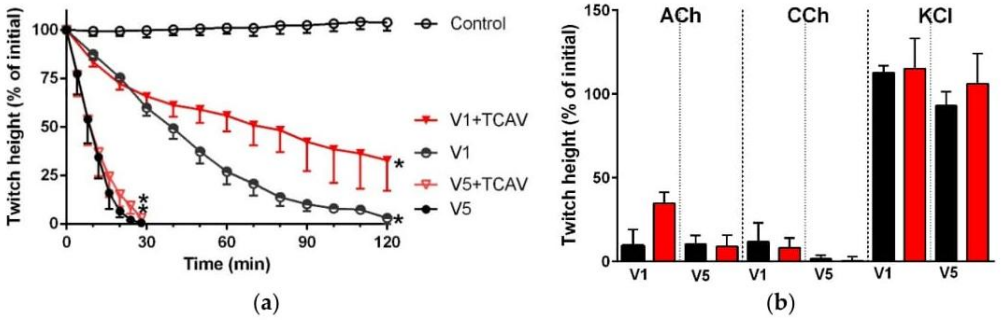

Figure 10. Cross-neutralisation of the neurotoxicity of 1 μg/mL vs. 5 μg/mL *P. textillis* venom by Thai cobra antivenom (TCAV; red): (**a**) Inhibition of indirect twitches caused by 1 μg/mL (V1) vs. 5 μg/mL (V5) *P. textillis* venom in the presence of TCAV. Note: TCAV does not prevent the twitch abolition caused by 1 μg/mL *P. textillis* venom (* significantly lower from the control at 120 min, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of 1 μg/mL (V1) vs. 5 μg/mL (V5) *P. textillis* venom on the response to exogenous nicotinic agonists (ACh, CCh and KCl) in the absence and presence of TCAV. Note: TCAV failed to prevent the abolition of agonist responses caused by 1 μg/mL venom (no significant difference from the venom, one-way ANOVA followed by Bonferroni's post-hoc test).

2.3. Cross-Neutralisation of Snake Venom Neurotoxins by Antivenoms

2.3.1. α-Neurotoxins: α-Bungarotoxin, α-Elapitoxin-Nk2a, α-Elapitoxin-Ppr1 and α-Scutoxin

Indirect twitches and the response to exogenous nicotinic receptor agonists of the chick biventer nerve-muscle preparation were abolished by the two long-chain α-neurotoxins, α-bungarotoxin (Figure 11: panels a and b) and α-elapitoxin-Nk2a (Figure 11: panels c and d), and the two short-chain α-neurotoxins, α-elapitoxin-Ppr1 (Figure 12: panels a and b) and α-scutoxin (Figure 12: panels c and d) at 100 nM. APAV and TCAV both fully neutralised the effects of all the toxins except α-scutoxin (Figure 11, panels a and c; Figure 12, panel c), which was only partially neutralised, in terms of the prevention of twitch inhibition by the two antivenoms (Figure 12, panel a).

2.3.2. β-Neurotoxin: Taipoxin

Taipoxin (100 nM) abolished indirect twitches of the chick biventer nerve-muscle preparation (Figure 13a), but did not abolish the response of the preparation to exogenous nicotinic receptor agonists (Figure 13b). IPAV failed to prevent the toxin-mediated twitch inhibition, but both APAV and TNPAV were able to neutralise this effect (Figure 13a).

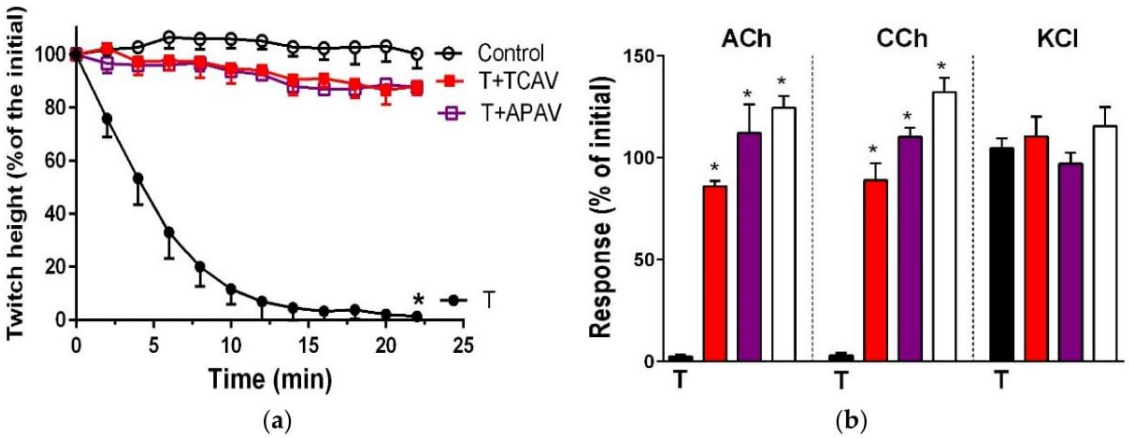

Figure 11. *Cont.*

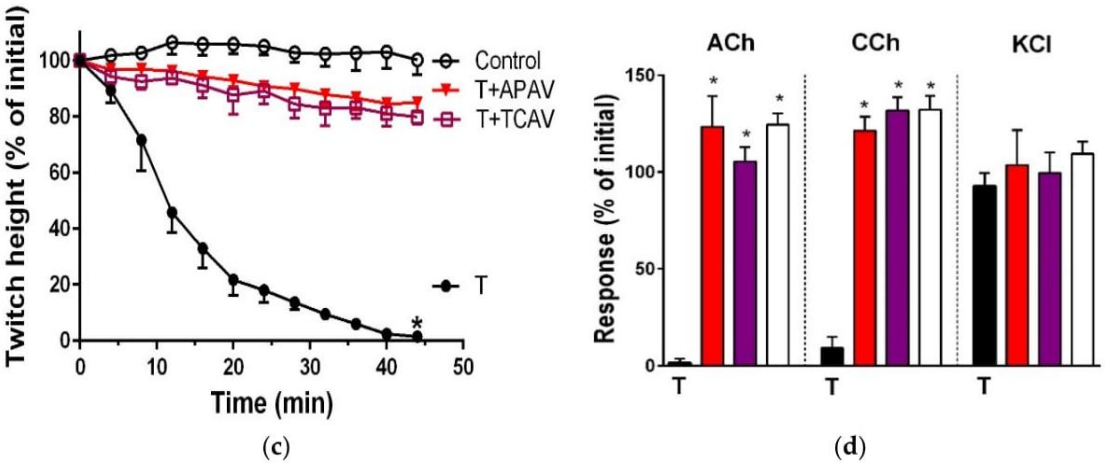

Figure 11. Cross-neutralisation of the neurotoxicity of the long-chain α-neurotoxins (α-bungarotoxin and α-elapitoxin-Nk2a; black) by Thai cobra antivenom (TCAV; red) and Australian polyvalent antivenom (APAV; purple) in the chick biventer nerve-muscle preparation compared to control (white): (**a**) Prevention of the inhibition of indirect twitches caused by 100 nM α-bungarotoxin (T). Note: APAV and TCAV effectively prevented neurotoxicity (no difference from the control at 22 min, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of α-bungarotoxin on response to exogenous nicotinic receptor agonists (ACh, CCh and KCl) in the absence and the presence of antivenoms. Note: APAV and TCAV prevented the abolition of agonist responses caused by the toxin (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test); (**c**) Prevention of the inhibition of indirect twitches caused by 100 nM α-elapitoxin-Nk2a (T). Note: APAV and TCAV prevented neurotoxicity (twitch height as a percentage of the initial at 44 min not different from the control, one-way ANOVA followed by Bonferroni's post-hoc test); (**d**) The effect of 100 nM α-elapitoxin-Nk2a on response to exogenous agonists (ACh, CCh and KCl) in the presence of antivenoms. Note: APAV and TCAV prevented the abolition of agonist responses by the venom (* significantly different from the toxin, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

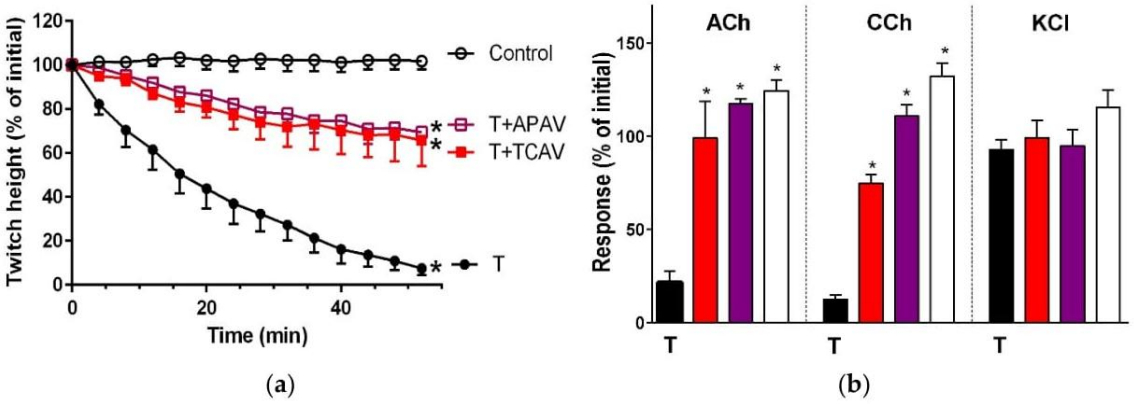

Figure 12. *Cont.*

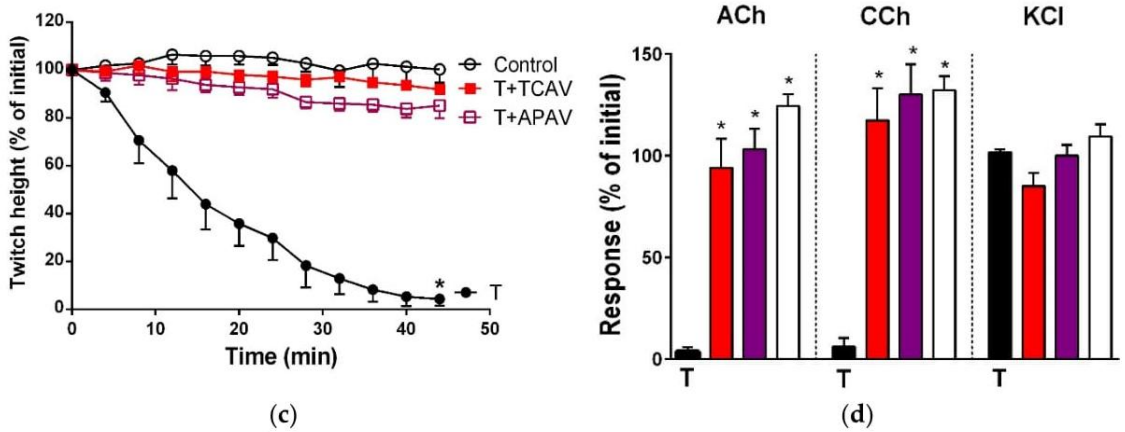

Figure 12. Cross-neutralisation of the neurotoxicity of the short-chain α-neurotoxins (α-scutoxin and α-elapitoxin-Ppr-1; T, black) by Thai cobra antivenom (TCAV; red) and Australian polyvalent antivenom (APAV; purple) in chick biventer nerve-muscle preparation compared to control (white); (**a**) Prevention of the inhibition of indirect twitches caused by α-scutoxin (T). Note: APAV and TCAV partially prevented neurotoxicity (* significantly lower from the control and the toxin at 54 min, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of 100 nM α-scutoxin on the response to exogenous nicotinic receptor agonists (ACh, CCh and KCl) in the absence and the presence of antivenoms compared to control. Note: APAV and TCAV prevented the abolition of agonist responses caused by the toxin (* significantly different from the venom, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test); (**c**) Prevention of the inhibition of indirect twitches caused by 100 nM α-elapitoxin-Ppr-1 (T). Note: APAV and TCAV prevented neurotoxicity (twitch height as a percentage of the initial at 44 min not different from the control, one-way ANOVA followed by Bonferroni's post-hoc test); (**d**) The effect of 100 nM α-elapitoxin-Ppr1 on the response to exogenous nicotinic receptor agonists (ACh, CCh and KCl) in the absence and the presence of antivenoms compared to control. Note: APAV and TCAV prevented the abolition of agonist responses by the venom (* significantly different from the toxin, $p < 0.05$, one-way ANOVA followed by Bonferroni's post-hoc test).

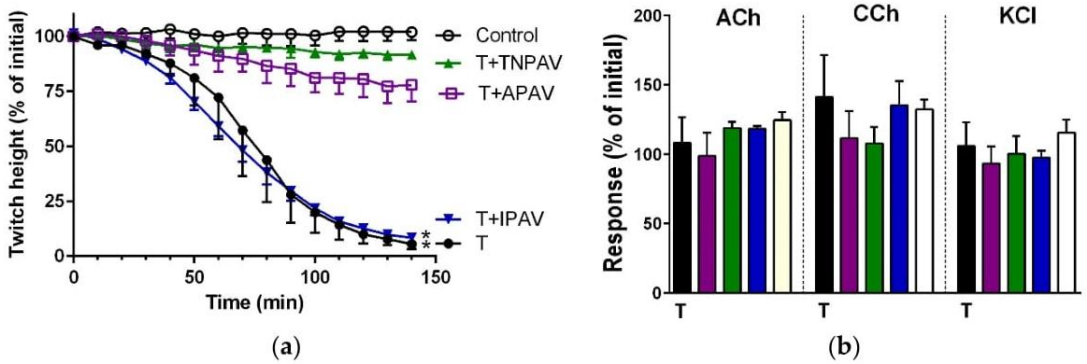

Figure 13. Cross-neutralisation of the neurotoxicity of the pre-synaptic neurotoxin from *Oxyuranus scutellatus*, taipoxin (100 nM, T, black), by Thai neuro polyvalent antivenom (TNPAV, green), Indian polyvalent antivenom (IPAV, blue) and Australian polyvalent antivenom (APAV, orange) in the chick biventer nerve-muscle preparation: (**a**) Prevention of the inhibition of indirect twitches caused by 100 nM taipoxin (T). Note: All antivenoms except IPAV effectively prevented the neurotoxicity (* significant difference from the control at 140 min, One-way ANOVA followed by Bonferroni's post-hoc test); (**b**) The effect of taipoxin on the response to exogenous agonists (ACh, CCh and KCl) in the absence and the presence of antivenoms compared to control. Note: neither the toxin alone nor the toxin in the presence of antivenoms was able to abolish the agonist responses (no significant difference among groups, one-way ANOVA followed by Bonferroni's post-hoc test).

3. Discussion

This study has shown cross-neutralisation of the in vitro neurotoxicity of eight elapid venoms from Asia and Australia, four α-neurotoxins and one β-neurotoxin, using five Asian and Australian antivenoms. This demonstrates remarkable cross-neutralisation of snake venom neurotoxicity, both at a pre-synaptic and post-synaptic level, by a range of antivenoms raised against geographically distinct elapids. With the exception of *P. textillis* venom, the post-synaptic neurotoxicity of all tested venoms and toxins was neutralised by TCAV. Both pre- and post-synaptic neurotoxicity of all the tested venoms and toxins was neutralised by TNPAV, APAV and DAAV. IPAV was able to neutralise some of the pre- and post-synaptic neurotoxic effects of the tested venoms.

Although the effectiveness of antivenoms in treating established neurotoxic envenoming in humans has been questioned for many snakes, these antivenoms are clearly efficacious in preventing in vitro neurotoxicity and have been shown to bind with circulating venom [22–25]. Snake antivenoms contain polyclonal antibodies—Fab or $F(ab')_2$ fragments—purified from serum of animals immunised against snake venoms [26]. Depending on the antigenicity of the different constituent toxins and their relative abundance in the venom, or the venoms used in the immunogen mixture, the antibodies against each toxin type can be found within the antivenoms [27]. The presence of common antigenic regions leads to cross-neutralisation of the snake venom toxins by antibodies raised against different toxins [28]. This occurs most commonly with toxins of the same type (e.g., phospholipases, snake venom metalloproteinases). α-Neurotoxins display a high degree of homology at some key regions in their primary structure, especially related to the residues essential for their post-synaptic activity [29,30]. Of the α-neurotoxins, both short-chain (type I) and long-chain (type II) toxins, which are present in Australasian and Asian elapid venoms, share some key structural characteristics. However, Type III toxins, which are structurally distinct from the former two groups, are only present in the venom of brown snakes (*Pseudonaja* spp.) [9,29]. Investigation of the structural determinants of the common antigenic regions in α-neurotoxins is currently not available. However, it is not unreasonable to assume that the remarkable cross-neutralisation of post-synaptic neurotoxicity seen in this study is due to the sharing of common antigenic regions across a range of α-neurotoxins. The inability of all tested antivenoms, except APAV and DAAV, (which contain antibodies against *P. textillis* venom) [31], to neutralise the post-synaptic neurotoxicity of *P. textillis* venom is most likely due to the inability of Asian antivenoms to cross-neutralise the type III α-neurotoxins only present in *P. textillis* venom. This is further supported by the observation that, when the concentration of *P. textillis* venom was lowered by a factor of five, while maintaining the same concentration of TCAV, the antivenom was still unable to prevent post-synaptic neurotoxicity.

PLA_2 neurotoxins in their monomeric form are generally 13 to 15 kDa molecules and display high homology in their primary sequences [7]. Presynaptic neurotoxic proteins with PLA_2 activity, together with other proteins with or without PLA_2 activity, are most commonly multi-subunit toxins. Notexin is a monomer from *N. scutatus* venom; taipoxin is a trimer from *O. scutellatus* venom; β-bungarotoxin is a heterodimer from Chinese banded krait (*Bungarus multicinctus*) venom, and textilotoxin is a pentamer from eastern *P. textillis* venom; all are examples of biochemically well characterized pre-synaptic toxins [7,32]. Irrespective of the structural differences in toxins, the effective cross-neutralisation of the pre-synaptic neurotoxicity of all venoms and toxins by TNPAV, APAV, DAAV and IPAV is most likely due to the sharing of common antigenic regions among different pre-synaptic toxins, similar to what is speculated for the α-neurotoxins.

IPAV was able to effectively neutralise both pre- and post-synaptic neurotoxicity of *N. scutatus* venom, *B. fasciatus* and the post-synaptic activity of *O. hannah* venom. Previously, it has been reported that 8.3 μL of IPAV effectively neutralised the in vivo neuromuscular blocking effects (in anaesthetised rats) of 1 μg *N. kaouthia* venom [15]. However in the current study, 2.8 μL of 5 times concentrated IPAV (equivalent to 14 μL of the standard solution) failed to effectively neutralise the post-synaptic neurotoxicity of 1 μg *N. kaouthia* venom. Further, IPAV failed to neutralise the neurotoxicity of *A. antarcticus* and *O. scutellatus* venom as well as taipoxin. This is most likely because the tested antivenom amounts were small in proportion to the venoms being used and therefore insufficient to

fully cross-neutralise neurotoxins. This may have been due to the poor efficacy of the antivenom batch used. IPAV has been reported to have poor efficacy and there is considerable inter-batch variation of the actual protein content in each vial [21].

Of the in vitro nerve-muscle preparations, the chick-biventer nerve-muscle preparation has advantages compared to other preparations due to the uncomplicated dissection and, most importantly, the ability of the muscle to contract in response to exogenous nicotinic receptor agonists. This enables the preparation to be used to differentiate between pre- and post-synaptic neurotoxicity [6,33]. The chick biventer preparation is also beneficial for testing the efficacy of antivenoms against neurotoxins in venoms [18,33]. To elicit contractile responses to ACh and CCh, which is required to be able to demonstrate a pre-synaptic site of action, nAChR at the motor end plate must be functioning. So, when both pre- and post-synaptic toxins are present in a venom, the post-synaptic neurotoxins block the nAChR, making it impossible to detect pre-synaptic neurotoxicity. Further, post-synaptic toxins act faster than pre-synaptic toxins, making the pre-synaptic neurotoxicity even harder to identify [6]. Interestingly, in the current study, TCAV effectively neutralised the post-synaptic neurotoxicity of all the venoms but failed to neutralise the pre-synaptic effects of *B. caeruleus*, *B. fasciatus*, *O. scutellatus* and *N. scutatus* venoms. These venoms all cause paralysis in humans and are known to contain pre-synaptic toxins [11,18,34–36].

The recently published comparative venom proteome of *N. kaouthia* found that 78% of the venom consists of three finger toxins, including long-chain α-neurotoxins, that make up 33% of the venom. However, no toxins similar to pre-synaptic neurotoxins, such as basic PLA_2s, were identified [37]. Therefore, it could be assumed that TCAV possesses high relative abundance of antibodies against post-synaptic neurotoxins, and may contain very few or no antibodies against toxins with pre-synaptic neurotoxicity. This means that by pre-incubating venoms with TCAV, the antibodies raised against α-neurotoxins in the antivenom would bind any α-neurotoxins, allowing the pre-synaptic neurotoxicity of the venom to be studied in the chick-biventer preparation.

Pre-synaptic neurotoxins similar to β-bungarotoxin are present in *B. fasciatus* venom [35]. However, such activity was not prominent in this study, even with the presence of TCAV. This is probably because of a combination of the low relative abundance of the pre-synaptic toxins and the venom concentration used being insufficient for eliciting such activity.

An important limitation of any in vitro experiment on antivenom efficacy is that it cannot be directly translated to the clinical effectiveness of antivenom. In these studies the antivenom was added to the organ bath 20 min prior to the venom, similar to pre-incubation studies. This is the ideal situation for testing antivenom and may not reflect the reality in treating patients with antivenom.

4. Conclusions

Using an in vitro nerve muscle preparation we have shown that the neurotoxicity of several clinically important Asian and Australian snake venoms is effectively cross-neutralised by a range of different antivenoms. There was a consistent pattern of cross-neutralisation of pre- and post-synaptic neurotoxicity suggesting that monovalent antivenoms raised against venoms with post-synaptic activity can cross-neutralise post-synaptic activity of other snake venoms and polyvalent antivenoms raised against both pre- and post-synaptic venoms can cross-neutralise both pre-and post-synaptic activity of different venoms. While acknowledging the limitation of adding antivenom prior to venom in an in vitro preparation, our study suggests that region-specific or universal neuro polyvalent antivenoms could be developed by raising antibodies against several carefully selected representative pre- and post-synaptic neurotoxins from snake venoms.

5. Materials and Methods

5.1. Venoms

The following snake venoms were used for the study: *Bungarus caeruleus* venom from Sri Lanka, *Bungarus fasciatus* venom from Thailand (Queen Saovabha Memorial Institute, Bangkok),

Ophiophagus hannah venom from Indonesia (Venom Supplies, Tanunda, South Australia), *Naja kaouthia* (Venom Supplies, Tanunda, South Australia), and *Pseudonaja textillis*, *Oxyuranus scutellatus*, *Notechis scutatus*, *Acanthophis antarcticus* and *Pseudechis porphyriacus* (Venom Supplies, Tanunda, South Australia). Venom was dissolved in MilliQ water and stored at −20 °C until required. Protein quantification of the venom, fractions and toxins was carried out using a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA), as per the manufacturer's instructions.

5.2. Neurotoxins

Using a single step reverse-phase high performance liquid chromatography (RP-HPLC) (LC-10ATVP pump and SPD-10AVP detector, Shimadzu, Kyoto, Japan, the short chain α-neurotoxins, α-scutoxin and α-elapitoxin-Ppr1 were isolated from *O. scutellatus* and *Pseudechis porphyriacus* venoms, respectively, as previously described in our laboratory [38,39]. Briefly, venoms were dissolved in solvent A (0.1% trifluoroacetic acid [TFA], Auspep, Tullamarine, VIC, Australia) and 2 mg of total protein was injected into a Phenomenex Jupiter semi-preparative C18 column (250 mm × 10 mm; 5 µm; 300 A°, Phenomenex, Lane Cove, NSW, Australia), equilibrated with solvent A. Fractions were eluted using the following gradient of solvent B (90% acetronitrile [ACN, Merck KGaA, Darmstadt, Germany] in 0.1% TFA): 0%–20% over 5 min (4% gradient), 20%–60% over 5–45 min (1% gradient), and 60%–80% for 45–50 min (4% gradient) at a flow rate of 2.0 mL/min, monitoring at 214 nm. The long-chain α-neurotoxin, α-elapitoxin-Nk2a, was isolated and purified from *Naja kaouthia* venom using RP-HPLC following the same methodology described above. α-Bungarotoxin was purchased from Invitrogen (OR, USA; Batch no: 1601). The β-neurotoxin, taipoxin, was isolated and purified using size-exclusion chromatography, following the method described in Barber et al. [11]. Briefly, *O. scutellatus* venom (1 mg) was run through a Superdex G-75 column (13 µm; 10 mm × 300 mm; GE Healthcare, Uppsala, Sweden) equilibrated with ammonium acetate buffer (0.1 M, pH 6.8). The sample was run at a flow rate of 0.5 mL/min and was monitored at 280 nm.

The identity and the purity of the isolated toxins were further verified using matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometry(4700 Proteomics Analyser MALDI TOF/TOF, AB Sciex, Foster City, CA, USA) as described previously [40,41]. HPLC and MALDI-TOF chromatograms are included as Supplementary Figure S1.

5.3. Antivenoms

Thai cobra (*Naja kaouthia*) monovalent antivenom (TCAV; Thai Red Cross Society, Bangkok, Thailand; Batch No: NK00111, date of expiry: 04.01.2016), death adder antivenom (DAAV; CSL, Parkville, VIC, Australia; Batch No: 07301, date of expiry: 09.2006), Indian polyvalent antivenom (IPAV; raised against *Bungarus caeruleus*, *Naja naja*, *Daboia russelii* and *Echis carinatus*; VINS Bioproducts, Andra Pradesh, India; Batch No: 01AS14001; date of expiry: 12.2017), Australian polyvalent antivenom (APAV; raised against *Psudechis australis*, *Notechis scutatus*, *Pseudonaja textillis*, *Acanthophis antarcticus* and *Oxyuranus scutellatus*; CSL, Parkville, VIC, Australia; Batch No: 18501, date of expiry: 02.2016) and Thai neuro polyvalent antivenom (TNPAV; raised against *Bungarus candidus*, *B. fasciatus*, *O. hannah* and *N. kaouthia*; Thai Red Cross Society, Bangkok, Thailand; Batch No: NP00113, date of expiry: 27.02.2018) were used for this study. According to the manufacturers of IPAV, TCAV and TNPAV, each antivenom vial should be dissolved in 10 mL of sterile water. The neutralization potential of the antivenom as stated on the labels are as follows: 1 mL IPAV for 0.45 mg *B. caeruleus* venom, 0.6 mg Indian cobra venom, 0.6 mg Russell's viper venom, 0.45 mg Saw-scaled viper venom; 1 mL TCAV for 0.6 mg Thai cobra (*N. kaouthia*) venom; 1 mL TNPAV for 0.8 mg *O. hannah* venom, 0.6 mg *N. kaouthia* venom, 0.4 mg Malayan krait venom, 0.6 mg *B. fasciatus* venom. For Australian antivenoms which come in liquid form, 1 vial of APAV (34.4 mL) contains 1000 units of *P. textillis*, 6000 units of *A. antarcticus*, 18,000 units of *Pseudechis australis*, 12,000 units of *O. scutellatus* and 3000 units of *N. scutatus* antivenoms. One vial of DAAV (31.72 mL) contains 6000 units of *A. antarcticus* antivenom (1 unit neutralizes 1 µg venom).

5.4. Chick Biventer Cervicis Nerve-Muscle Preparation

Male chickens (aged 4–10 days) were humanely killed by exsanguination following CO_2 inhalation. Biventer cervicis nerve-muscle preparations were dissected and then mounted on wire tissue holders under 1 g resting tension in 5 mL organ baths. Tissues were maintained at 34 °C, bubbled with 95% O_2 and 5% CO_2, in physiological salt solution of the following composition (mM); 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 11.1 glucose. Indirect twitches were evoked by stimulating the motor nerve (rate: 0.1 Hz; pulse duration: 0.2 ms) at supramaximal voltage (7–15 V), using a Grass S88 stimulator (Grass Instruments, Quincy, MA, USA). Selective stimulation of the nerve was confirmed by the abolishment of twitches with d-tubocurarine (10 μM, Sigma-Aldrich, St. Louis, MO, USA). Tissues were then repeatedly washed with physiological salt solution to restore twitch response to nerve stimulation. Contractile responses of the tissues to exogenous acetylcholine (ACh; Sigma-Aldrich, St. Louis, MO, USA; 1 mM for 30 s), carbachol (CCh; Sigma-Aldrich, St. Louis, MO, USA; 20 μM for 60 s) and KCl (40 mM for 30 s) were obtained in the absence of nerve stimulation. The preparations were then stimulated for 30 min, before the addition of antivenom. After the tissues were equilibrated with antivenom for 20 min, the venom or toxin was added. For each venom and toxin, observations were made for all antivenom experiments for at least the time at which the venom or toxin causes full twitch inhibition. At the conclusion of the experiment, ACh, CCh and KCl were re-added as above.

Initial experiments were carried out to select individual venom concentrations that fully abolish indirect twitches consistently, and based on those, all venoms, apart from *B. fasciatus* and *P. textillis* venoms were tested at 5 μg/mL. *B. fasciatus* venom was tested at 7.5 μg/mL and *P. textillis* venom was tested at both 1 μg/mL and 5 μg/mL. All toxins were tested at 100 nM. Each vial of TCAV and TNPAV antivenom was reconstituted with 10 mL of sterile water as instructed by the manufacturer. One vial of IPAV was reconstituted with 2 mL of sterile water, which is 5 times more concentrated than the manufacturer's instructions. In order to achieve a sufficiently high concentration of antivenom for the venom, all antivenoms were tested at 40 μL/mL bath concentrations except IPAV which was tested at 15 μL/mL bath concentrations. Because the addition of large amounts of antivenom can alter the osmolarity of the physiological salt solution, antivenom control experiments (i.e., antivenom only) were performed to ensure the antivenom was not affecting the tissue viability.

5.5. Data Analysis and Statistics

Indirect twitch responses and responses to exogenous agonists (ACh, CCh and KCl) were measured via a Grass FTO3 force displacement transducer (Grass Instruments, Quincey, MA, USA) and recorded on a PowerLab system (ADInstruments Pty Ltd., Bella Vista, NSW, Australia). The t_{90} values (i.e., time taken for 90% inhibition of the maximum twitch response to occur) were determined for each of the venoms and the five toxins. All twitch and agonist responses were expressed as percentages of their pre-venom/toxin values. A one-way ANOVA was used to compare the responses to exogenous agonists following the administration of venom. All ANOVAs were followed by Bonferroni's multiple comparison post-tests. Data are presented in the form of mean ± standard error of the mean (S.E.M.) of three to five experiments. All statistical analyses and presentation of data were generated using GraphPad Prism 6.07 software (GraphPad software Inc., La Jolla, CA, USA). For all statistical tests $p < 0.05$ was considered statistically significant.

5.6. Animal Ethics

All animal experiments used in this study were approved by the Monash University Animal Ethics Committee (Approval no: MARP/2014/097).

Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/8/10/302/s1, Figure S1: Chromatograms of HPLC and MALDI-TOF Mass spectrometry of the neurotoxins, Table S1: Comparison of the neurotoxicity of the venoms and neurotoxins in the absence of and presence of different antivenoms.

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Author Contributions: A.S., W.C.H. and G.K.I. conceived and designed the experiments; A.S. performed the experiments and analyzed the data; W.C.H. and G.K.I. contributed reagents, materials and analysis tools; A.S., W.C.H. and G.K.I. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER SIX

Differential susceptibility of human and rat nicotinic acetylcholine receptors (nAChR) to snake venom short-chain post-synaptic neurotoxins.

Overview of the Chapter

It has long been considered that snake venom α-neurotoxins cause paralysis in envenomed humans by antagonising nAChR at the neuromuscular junction. Short-chain (type I) α-neurotoxins show some differences to long-chain (Type II) α-neurotoxins structurally and in pharmacological properties. Snake α-neurotoxins have evolved to maximise prey specific toxicity of the venoms hence show differential potency on nAChR of different animals. However, a key question regarding snake α-neurotoxins that remains unanswered is whether they are able to cause paralysis in humans at the circulating toxin concentrations which occur after snake envenoming. Further, the ability of commercially available antivenoms to reverse the paralysis caused by α-neurotoxins has not been pharmacologically investigated. This Chapter answers the above questions using a variety of experimental techniques.

Using electrophysiological techniques on frog oocytes, we demonstrate the lower potency and rapid reversibility of short-chain α-neurotoxins, in comparison to long-chain α-neurotoxins, on human nAChR, which suggests that short-chain α-neurotoxins are unlikely to cause paralysis in humans. In addition, we demonstrate the comparatively high susceptibility of rat nAChR to snake short-chain α-neurotoxins, compared to human nAChR indicating that rats may not be an appropriate model for examination of neurotoxicity, and the efficacy of antivenoms, in humans. Further, using rodent and avian skeletal muscle preparations, we demonstrate that antivenoms accelerate the reversibility of α-neurotoxin mediated inhibition. Based on these findings, we hypothesise even greater reversibility could be expected on the comparatively less susceptible human nAChR.

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Defining the role of post-synaptic α-neurotoxins in neuromuscular paralysis due to snake envenoming in humans.

Anjana Silva[1,2], Ben Christofori-Armstrong[3], Lachlan D. Rash[3], Wayne C. Hodgson[1], Geoffrey K. Isbister[1,4*]

[1]Monash Venom Group, Department of Pharmacology, Biomedicine Discovery Institute, Monash University, Victoria 3800, Australia

[2]Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura 50008, Sri Lanka.

[3]Institute of Molecular Bioscience, University of Queensland, St. Lucia 4072, Australia.

[4]Clinical Toxicology Research Group, University of Newcastle, New South Wales, Australia.

*corresponding [redacted]

Abstract

Snake venom α-neurotoxins inhibit nicotinic acetylcholine receptors (nAChRs), however their role in human paralysis has remained unclear. Here we demonstrate, with two short chain α-neurotoxins (SαNTx) and two long chain α-neurotoxins (LαNTx), that SαNTx are markedly less potent and more reversible at human muscle-type nAChR compared to rat. In contrast, LαNTx show no species differences in pharmacology. A review of venom proteome and clinical data indicates that paralysis in humans following snakebite is likely to be due to LαNTx, but not SαNTx. SαNTx and LαNTx mediated neuromuscular block in rat and chick in vitro nerve-muscle preparations was reversed to differing extents by commercially available antivenoms supporting the benefit of antivenom reversing post-synaptic mediated paralysis in humans. The differential susceptibility of rat and human nAChR towards SαNTx challenges the usefulness of rodent lethality prevention assays in assessing efficacy of antivenoms for clinical use.

Introduction

Neuromuscular paralysis is a common clinical effect of snake envenoming and is often life-threatening when the paralysis involves bulbar and respiratory muscles[1,2]. Snake neuromuscular paralysis is a lower-motor neuron type, flaccid paralysis due to the blockade of neurotransmission at the neuromuscular junction (NMJ)[2–5]. Of the snake venom neurotoxins acting on the NMJ, the vast majority are either phospholipase A_2 toxins which act pre-synaptically (i.e. β-neurotoxins) or curare mimetic three-finger toxins which act post-synaptically (i.e. α-neurotoxins). β-neurotoxins result in a depletion of synaptic vesicles and subsequent irreversible destruction of the motor nerve terminal[6]. Whereas, α-neurotoxins are competitive antagonists that bind with high affinity to the two agonist binding sites of the adult (α1-δ and α-ε in $(\alpha1)_2\beta\delta\varepsilon$) and foetal (α1-δ and α1-γ in $(\alpha1)_2\beta\delta\gamma$) nicotinic acetylcholine receptors (nAChR) on the motor end plate, leading to neuromuscular blockade[7]. The α-neurotoxins belong to the three-finger toxin structural family and two major groups have been pharmacologically and structurally characterised: short-chain α-neurotoxins (SαNTx), which consist of 61–62 amino acids and four disulfide bridges; and long-chain α-neurotoxins (LαNTx), which consist of 66–75 amino acids with five disulfide bridges[8]. Although both types of α-neurotoxins bind to the same sites on the nAChR with high affinity, SαNTxs dissociate more rapidly, so are generally more reversible in their antagonism[8]. Despite recent advances in our knowledge of the molecular basis of the interaction of α-neurotoxins with the nAChR, several key questions remain in regards to their reversibility, and role in paralysis in human envenoming[1].

Our current knowledge of the relevance of snake α-neurotoxins in human envenoming and paralysis is largely derived from assumptions based on rodent, avian and amphibian pharmacological models[8–10]. Indeed, the World Health Organisation recommended standard assay for testing the efficacy of snake antivenoms in clinical use is the rodent lethality prevention assay[11]. The clinical applicability of such animal models assumes similar susceptibility of the nAChRs to snake α-neurotoxins between experimental animals and humans. However, it is well documented that nAChRs from different animals show substantially different susceptibility to α-neurotoxins[12–14]. A further complicating factor

in our understanding of neurotoxicity in human snakebite is the variation in content and relative concentrations of toxins that interfere with neuromuscular transmission.

Neurotoxic envenoming is common in Asia and Australia. In these geographical regions, the venoms of kraits (*Bungarus spp.*), taipans (*Oxyuranus spp.*), tiger snakes (*Notechis scutatus*), death adders (*Acanthophis spp.*) and brown snakes (*Pseudonaja textillis*) in Australia possess both β and α-neurotoxins. Neuromuscular paralysis in humans envenomed by these snakes is attributed to β-neurotoxins[6,15–17]. Asiatic cobras (Genera *Naja* and *Ophiophagus*) contain large amounts of α-neurotoxins in their venoms and lack β-neurotoxins and frequently cause paralysis in humans indicating that α-neurotoxins are capable of causing neuromuscular paralysis in humans[18–20]. In contrast, some Australian snakes, such as black snakes (*Pseudechis* spp), possess only α-neurotoxins in their venoms, but do not cause paralysis in humans[21]. The reason for this discrepancy in the ability of the two major types of α-neurotoxins in the above venoms to cause human paralysis is unknown. The present study was undertaken to investigate the molecular pharmacology of SαNTx and LαNTx on muscle type nAChRs and how this translates to toxicity in rodents and envenoming in humans. These data will elucidate: (1) the clinical relevance of snake α-neurotoxins in human envenoming, (2) the relevance of rodent lethality tests and *in vitro* nerve-muscle preparations to the pathophysiology of paralysis in snake envenoming in humans; and (3) the potential effectiveness of antivenom in α-neurotoxin mediated neurotoxicity and paralysis in humans.

We studied the binding and the reversibility of two SαNTx (α-elapitoxin-Ppr-1 from red-bellied black snake, *P. porphyriacus* and α-scutoxin-1 from coastal taipan, *O. scutellatus*) and two LαNTx (α-bungarotoxin from Chinese banded krait, *B. multicinctus* and α-elapitoxin-Nk2a from Thai cobra, *N. kaouthia*) on human and rat muscle-type nAChRs [$(\alpha1)_2\beta\delta\varepsilon$; i.e., adult] expressed on *Xenopus* oocytes using two-electrode voltage clamping (TEVC). We compared this to the effects of atracurium besylate and rocuronium bromide, two short acting non-depolarising muscle relaxants used for paralysis in humans for rapid sequence intubation and surgery. We show that the SαNTxs are less potent and rapidly reversible, in comparison to LαNTxs, on human nAChR suggesting that they are unlikely to

cause medically important paralysis in humans. In addition, the high potency and comparatively slow reversibility of SαNTx on rat nAChRs indicate that they are responsible for neurotoxicity in rodents and therefore that the rat may be an inappropriate model of neurotoxicity for testing the efficacy of antivenoms for humans. Finally, we compare the venom concentrations reported in human envenoming with the known abundance and IC_{50} of neurotoxins for the respective snakes to estimate the likelihood that a given toxin may be clinically relevant for each type of snake. Based on our data, we hypothesise that LαNTx cause paralysis in envenomed humans only if present in high relative abundance and/or if the snake, such as some cobra species (gen. *Naja* and *Ophiophagus*), is capable of injecting large amounts of venom. Further, using the skeletal muscle preparations chick biventer (CBVNM) and rat phrenic nerve-hemidiaphragm (RPNHD), we demonstrate that the antivenoms accelerate the reversibility of α-neurotoxin mediated inhibition of rat and chicken nAChR hence hypothesise an even greater reversibility can be expected on the comparatively less susceptible human nAChR.

Results

SαNTx are less potent and rapidly reversible on human nAChR but not rat nAChR.

To investigate the species specificity of the α-neurotoxins on adult muscle type nAChRs, we studied their ability to inhibit ACh-induced (3μM) membrane currents in *Xenopus* oocytes expressing either rat or human $(\alpha1)_2\beta\delta\varepsilon$ nAChR subunits. Two representative α-neurotoxins from each of the SαNTx and LαNTx groups were chosen representing Australian and Asian snakes with known clinical effects of envenoming. Two α-neurotoxins from snake venoms that contain no β-neurotoxins were selected, one from a snake species that does not cause paralysis in humans (SαNTx, α-elapitoxin-Ppr-1 from the red-bellied black snake *P. porphyriacus*) and one from a species that causes paralysis in humans (LαNTx, α-elapitoxin-Nk2a from Thai cobra *N. kaouthia*). The other two toxins, i.e. α-bungarotoxin (LαNTx) and α-scutoxin-1 (SαNTx), were selected from snakes that commonly cause paralysis in humans with their venoms having both α- and β-neurotoxins (Figure 1).

All four toxins potently inhibited both human and rat nAChR in a concentration-dependent manner with IC_{50}s below 100 nM (Figure 2). The two SαNTxs exhibited marked species dependence on inhibition with 5 and 17-fold higher IC_{50}s at the human channel compared to that of rat nAChR (Fig. 2e & f). In contrast, the LαNTx mediated inhibition was not different on rat and human nAChR (Fig. 2g & h; Table 1). The commonly used muscle relaxants atracurium and rocuronium also completely and concentration-dependently inhibited both rat and human muscle nAChR, with IC_{50}s comparable to those previously reported using *Xenopus* oocytes[22]. Rocuronium and atracurium were 1.5 and 7-fold more potent on the human channel (Fig. 2i & j).

We then studied the reversibility of the α-neurotoxin-mediated inhibition of the rat and human nAChR and compared this to the reversibility of the two muscle relaxants, because of their known rapid dissociation from the channel required in human use. The reversibility of the two SαNTxs was both species- and peptide-dependent (Figure 3b & c). While α-elapitoxin-Ppr-1 and α-Scutoxin-1-induced inhibition of the human channel was rapidly and completely reversible (full recovery in ~6 min), the effect of α-elapitoxin-Ppr-1 on the rat channel was very slowly reversible and the inhibitory effect of Scutoxin-1 on the rat nAChR was moderately reversible (~50% after 6 min washout). Consistent with previous studies, the LαNTxs were slowly reversible from both rat and human muscle nAChR, still showing >60% inhibition after 15 min of thorough washing. As expected, the recovery of human nAChR from the two muscle relaxants was extremely rapid (<2 min) compared to the recovery from the snake neurotoxins. There was little difference observed in the reversal between the rat and human nAChR for the LαNTx and the two muscle relaxants (Figure 3 d-i).

Together, these results show that the two SαNTxs are less potent and more rapidly reversible on the human nAChR compared to rat nAChR, while the LαNTxs show no such species difference. Our results using heterologously expressed channels are in agreement with previous studies that suggest the human receptor is relatively resistant to snake short-chain neurotoxins compared to the rat receptor [23]. The high potency and poor reversibility of the LαNTx mediated inhibition of the human muscle nAChR suggests that LαNTx are substantially more likely to cause paralysis in envenomed humans compared to SαNTxs.

Role of SαNTx and LαNTx in clinically detectable paralysis in envenomed humans

To further investigate the association between post-synaptic α-neurotoxins and paralysis in envenomed humans we need to quantify the relative exposure of the muscular nAChR to these peptides in the context of their activity at the human receptor. Thus, we collated available experimental data on the type (presynaptic, SαNTx and LαNTx), relative abundance and potency of neurotoxins in the venom of snakes that are associated with paralysis in human envenoming, and combined this with data on the maximum serum venom concentrations observed in humans envenomed by these snakes (summarised in Table 2).

We found information on the relative abundance of SαNTx and LαNTx from four major neurotoxic snake groups, i.e. kraits (genus: *Bungarus*), taipans (genus: *Oxyuranus*), tiger snakes (genus: *Notechis*) and cobras (genus: *Naja*), which cover the source of three of the four toxins used in our functional studies. All above snake groups except cobras have co-existing pre-synaptic neurotoxins in the venoms making it difficult to draw conclusions on the role of these post-synaptic neurotoxins alone in human paralysis. However, the absence of pre-synaptic neurotoxins in cobra venoms as determined by proteomics strongly supports that neurotoxicity observed following cobra envenoming is entirely due to the action of α-neurotoxins. Cobra bites result in large venom concentrations in envenomed humans hence higher concentrations of α-neurotoxins compared to most other elapids such as kraits and Australasian elapids. Based on the assumption that the average molecular weights of SαNTx and LαNTx are 6.5 and 7.5 kDa, respectively, and the pharmacokinetics and pharmacodynamics of α-neurotoxins are generally similar to other venom toxin groups, the expected maximum serum venom concentrations in envenomed humans ranges from 0.27-170.5 and 0.09-139.8 nM, respectively. The IC_{50}s of the four α-neurotoxins on human nAChR ranged from 2.3-93.3 nM hence, in severe envenomings, it is likely that the concentrations of some of the SαNTx and LαNTx at the neuromuscular junctions could exceed the IC_{50} on the human nAChR.

All cobra species possess relatively high abundances of SαNTx, but species possessing higher relative abundances of LαNTx, such as *N. kaouthia* and *N. naja*, more commonly cause paralysis in humans

(Table 2). Similar to these Asian neurotoxic cobras, African cobras (e.g. *N. haje*) that possess more LαNTx in their venom[24] cause neuromuscular paralysis in envenomed humans[25]. LαNTx are therefore clinically important in causing neuromuscular paralysis in humans, consistent with their presence in *Naja* and *Ophiophagus* venoms which are reported to cause neurotoxicity[20,26,27]. The lack of clinically detectable paralysis in patients envenomed by most African cobras having venoms rich in SαNTx, with no LαNTx, is likely due to the rapid reversibility of the antagonism by SαNTx on human nAChR.

While acknowledging the possible reporting bias as well as the heterogeneity of the studies, the available data further supports our hypothesis that LαNTx are more clinically important than SαNTx in causing paralysis in humans.

Antivenoms can reverse the SαNTx and LαNTx mediated inhibition at the nAChR.

To investigate whether antivenoms used clinically can reverse the neuromuscular block due to inhibition of nAChR by SαNTx and LαNTx, we exposed the chick biventer nerve muscle preparation and rat phrenic nerve hemi diaphragm preparations to 0.1 M SαNTx (α-Elapitoxin-Ppr-1) and SαNTx (α-Elapitoxin-Nk2a) and investigated the reversal of the toxin-mediated inhibition of twitches with the antivenom. Antivenom resulted in 80% recovery of the indirect twitches inhibited by both types of toxins in the RPNHD within 40 min compared to no recovery by regular washing of the preparation for 60 min (Figure 4). In contrast, the antivenom mediated indirect twitch recovery in the CBVNM preparation was only partial and very slow compared to the RPNHD during the observation period. There was no recovery of the twitches with regular washing of the preparation during the observation period (Figure 4). This suggests that antivenom can accelerate the recovery of nAChR from α-neurotoxin mediated inhibition, effectively reversing neuromuscular blockade. This demonstrates that antivenom therapy is 'never too late' for α-neurotoxin induced neuromuscular paralysis, if the antivenom can effectively reach the NMJ.

Discussion

Rodents have been used extensively as key *in-vivo* and *in-vitro* animal models of snake envenoming in humans. Based on the assumption that rodents and humans are similarly susceptible to medically important snake venom toxins, rodent lethality prevention tests are currently widely used for testing the efficacy of antivenoms in clinical use[11]. Here, we demonstrated that the human nAChR is resistant to snake venom SαNTx compared to rat nAChR due to the marked differences in the sensitivity of the nAChR to the toxin as well as the reversibility of toxin mediated block of the receptor. This difference in the susceptibility was not found with the LαNTx. More importantly, the study has demonstrated that commercial antivenoms can reverse the neuromuscular block caused by both SαNTx and LαNTx, on both rat and chicken nerve-muscle preparations, providing specific evidence for the potential effectiveness of antivenom in reversing post-synaptic neurotoxicity in humans.

Consistent with our results, previous binding studies have suggested that the human nAChR has low affinity towards the SαNTx erabutoxin-b compared to high affinity binding with the LαNTx α-bungarotoxin. In contrast, toxins bind with high affinity to the mouse nAChR[23]. The nAChR of snakes themselves, and their predators such as the mongoose and hedgehog, are resistant to snake α-neurotoxins. This resistance is associated with the presence of N-linked glycosylation sites within the ligand binding region, which are absent in the human and rodent receptor[13,14]. However, similar to species resistant to snake α-neurotoxins, humans lack aromatic amino acid residues at positions 187 and 189 of the α-subunit of the nAChR that are required for high-affinity binding of α-neurotoxins [28]. Therefore, the binding of snake α-neurotoxins to the human nAChR is considered to be 'partial', as opposed to the 'complete' binding which occurs for rodent and avian nAChR[14].

Our findings show that SαNTx are unlikely to be medically important and snakes with venoms that contain a larger proportion of these toxins are unlikely to cause neurotoxicity in humans. For example, the beaked sea snake venom (*Hydrophis schistosus*) contains of 68% SαNTx and only 14% LαNTx [29], consistent with neuromuscular paralysis being rarely reported in the absence of systemic myotoxicity in envenomed humans[30]. Similarly, *N. atra* venom possesses high relative abundance of SαNTx with

low abundance (< 3%) of LαNTx, and bites by this snake do not cause neuromuscular paralysis in humans[31,32]. Australian red-bellied black snake (*P. porphyriacus*) venom possesses a single SαNTx (α-elapitoxin-Ppr1), which is responsible for the neurotoxicity seen *in vitro* because no LαNTx has been isolated [21]. Neuromuscular paralysis has never been reported in red bellied black snake envenoming, again consistent with the lack of LαNTx in the venom[33].Venoms of African spitting cobras possess SαNTx, but lack LαNTx and paralysis is not reported in human envenoming [19,34]. The absence of neurotoxic features or paralysis in human envenoming by snake venoms that contain a low relative abundance of LαNTx, but a high relative abundance of SαNTx, may be simply due to the smaller amounts of venom being injected in human envenoming cases, resulting in lower serum concentrations of LαNTx. However, the competition between the slow binding and reversing LαNTx and the more abundant, fast-binding and reversible SαNTx for the agonist binding sites of the nAChR, might further reduce the net effect of the LαNTx, and cannot be excluded as a reason.

The literature search based comparison of venom proteomes of neurotoxic snakes included the most important genera which cause most neurotoxic envenomings in South Asia, South-East Asia and Pacific regions, which record over two thirds of the global snakebite burden[35]. Of the genera which were excluded in the search, apart from the genus *Ophiophagus*, the venoms of other genera possess pre-synaptic neurotoxins as major neurotoxins in their respective venoms, hence exclusion of those are unlikely to affect the generalisability of the study findings. However, the reporting bias and the heterogeneity associated with such literature search based conclusions cannot be excluded. Further, the use of emzyme-immunoassay-based venom antigen concentrations to predict the serum LαNTx and SαNTx might be less accurate unless the assay picks the two types toxins similar to how the assay picks other venom antigens.

The present study demonstrated functionally, that commercially used antivenoms are able to reverse the neuromuscular block caused by both SαNTx and LαNTx on the rat hemidiaphragm. Based on the similar susceptibility of human and rat nAChR to LαNTx, the rat diaphragm is an appropriate *in-vitro* model for testing antivenom mediated reversibility of the neuromuscular block by LαNTx in humans.

Together with previous observations[36–38], this supports the idea that antivenom is effective in potentially reversing or partially reversing established neuromuscular paralysis caused by snake long-chain α-neurotoxins. The administration of antivenom is therefore never too late for α-neurotoxin mediated neuromuscular block, although very delayed administration may only be partially effective. The above depends on the ability of the antibody fragments in commercial antivenoms to reach motor end plates, which should be tested *in-vivo*.

The use of lethality based rodent models for testing the efficacy of antivenoms for clinical use[11] is problematic with regards to translation of results to the clinical setting[39]. Venom-induced consumption coagulopathy and neuromuscular paralysis are the two most important systemic effects of envenoming. This requires that animal models used to examine antivenom efficacy reasonably represent the pathophysiology of the coagulopathy and neurotoxicity in humans. However, the resistance of rodent plasma to procoagulant snake venoms[40], along with our current findings of differential susceptibility of human and rat nAChR to snake SαNTx, suggests that rodent lethality models may misrepresent the pathophysiology of envenoming in humans. For example, rodent lethality and ED50 studies of cobra venoms that only contain SαNTx, are unhelpful because these snakes cause cytotoxic effects in humans and rats are likely to succumb to neurotoxicity due to the SαNTx in the venom, which humans are unaffected by.

In our study, we did not have a direct comparison of the binding and reversibility of the snake α-neurotoxins with the chicken vs human and rat nAChR. However, the reversibility of the neuromuscular block caused by both SαNTx and LαNTx was slow and partial on chicken tissues compared to rat tissues. This might indicate that α-neurotoxins bind more strongly to chicken nAChR, compared to rat nAChR, which is in agreement with previous observations[41]. Therefore, the appropriateness of the chick biventer preparations as a model for α-neurotoxin action on human nAChR requires further evaluation.

Methods

Toxins and drugs

The LαNTx α-Bungarotoxin from *Bungarus multicinctus* was purchased from Invitrogen (OR, USA; Batch no: 1601). LαNTx α-elapitoxin-Nk2a was purified from Thai Monocellate cobra venom (*Naja kaouthia*; Venom Supplies, Tanunda, South Australia). The two SαNTx, α-Elapitoxin-Ppr1 and α-scutoxin-1 were purified from Australian Rred-bellied black snake (*Pseudechis porphyriacus*; Venom Supplies, Tanunda, South Australia) and Australian coastal taipan (*Oxyuranus scutellatus*; Venom Supplies, Tanunda, South Australia). Toxins were purified using a single step reverse-phase high performance liquid chromatography (RP-HPLC) (LC-10ATVP pump and SPD-10AVP detector, Shimadzu, Kyoto, Japan) as described previously[21,42,43]. Briefly, venoms were dissolved in solvent A (0.1% trifluoroacetic acid [TFA], Auspep, Tullamarine, VIC, Australia) and 2 mg of total protein was injected into a Phenomenex Jupiter semi-preparative C18 column (250mm×10 mm; 5 µm; 300 A, Phenomenex, Lane Cove, NSW, Australia), equilibrated with solvent A. Fractions were eluted using the following gradient of solvent B (90% acetronitrile [ACN, Merck KGaA, Darmstadt, Germany] in 0.1% TFA): 0%–20% over 5 min (4% gradient), 20%–60% over 5–45 min (1% gradient), and 60%–80% for 45–50 min (4% gradient) at a flow rate of 2.0 mL/min, monitoring at 214 nm. The identity and the purity of the isolated toxins were further verified using matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometry(4700 Proteomics Analyser MALDI TOF/TOF, AB Sciex, Foster City, CA, USA) as described previously[16,44]. Protein quantification of the toxin samples were performed using NanoDrop™ 2000c Spectrophotometer.

Atracurium Besylate (Aspen Pharmacare, Australia; Batch: T738) and Rocuronium Bromide (Esmeron, UK; Batch: 685003) 10mg/ml injection solutions were a gift from the Calvary Mater Newcastle pharmacy department.

Antivenoms

Thai neuro polyvalent antivenom (raised against *B. candidus*, *B. fasciatus*, *Ophiophagus hannah* and *N. kaouthia*; Thai Red Cross Society, Bangkok, Thailand; Batch No: NP00113, date of expiry:

27.02.2018) and Australian polyvalent antivenom (raised against *P. australis, N. scutatus, P. textillis, A. antarcticus* and *O. scutellatus*; CSL, Parkville, VIC, Australia; Batch No: 18501, date of expiry: 02.2016) were used for this study

Two-electrode voltage clamp of* Xenopus *oocytes

Oocytes were surgically removed from anaesthetised (Tricaine methanesulfonate, MS-222) female *X. laevis* frogs and defolliculated by treating with collagenase (1 mg/ml; Sigma type I). Plasmids containing cloned rat and human muscle type nAChR subunits were linearised, then capped cRNAs were synthesised using either a T7 or SP6 mMESSAGE mMACHINE transcription kit (Ambion Inc., Austin, TX, USA). Stage V–VI oocytes were injected with 8.2 ng of cRNA (Nanoject 2000; WPI, Sarasota, FL, USA) and stored at 17 °C in ND96 solution (96 mM NaCl, 1.8 mM CaCl2, 2 mM KCl, 2 mM MgCl2, 5 mM HEPES, pH 7.4) supplemented with 2.5 mM pyruvic acid, 50 μg/mL gentamicin, and 2.5% horse serum. Membrane currents were recorded 1–3 days after injection under voltage-clamp at –60 mV (Axoclamp 900A, Molecular Devices, CA, USA) using two standard glass microelectrodes of 0.5–2 MΩ resistance when filled with 3 M KCl solution. Data acquisition (5000 Hz and filtered at 0.01 Hz) and analysis were performed using pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed at room temperature (18–21 °C) in ND96 solution. Following the Initial experiments to identify the appropriate ACh concentration, currents were elicited by application of 3 μM ACh for 5 s every 60 s using a flow rate of ~1.5 mL/min (bath volume = 25 μL) to allow rapid solution exchange. Toxins were prepared by serial dilutions in ND96 solution containing 0.1% fatty acid free-bovine serum albumin (Sigma), and administered post-stimulation at pH 7.4 and oocytes were bathed in the compound solution for ~ 3 min. Muscle relaxants were prepared by serial dilutions in ND96 solution containing 1 uM ACh, and administered with ACh stimulus to allow access to the open state. The concentration response curves were fitted to the Hill equation (GraphPad Prism 7). IC50 values determined for individual curves were compared using an extra sum-of-squares F test assuming a Gaussian distribution and equal standard deviation.

All data points are shown as mean ± S.E.M., and replicates (n) represent separate experimental oocytes. All work using *X. laevis* was carried out in strict accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes. The protocol was approved by the Anatomical Biosciences group of the Animal Ethics Committee at The University of Queensland (Approval Number QBI/059/13/ARC/NHMRC).

Chick biventer nerve-muscle preparation (CBVNM)

CBVNM experiments were conducted as described previously[43,44]. Briefly, male chickens (aged 4–10 days) were killed by exsanguination following CO_2 inhalation. Biventer cervicis nerve-muscle preparations were dissected and mounted under 1 g resting tension in 5 mL organ baths. Tissues were maintained at 34°C, bubbled with 95% O_2 and 5% CO_2, in physiological salt solution of the following composition (mM); 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 11.1 glucose. Indirect twitches were evoked by stimulating the motor nerve (rate: 0.1 Hz; pulse duration: 0.2 ms) at supramaximal voltage (7–15 V), using a Grass S88 stimulator (Grass Instruments, Quincy, MA, USA). Selective stimulation of the nerve was confirmed by the abolishment of twitches with d-tubocurarine. Tissues were then repeatedly washed with physiological salt solution to restore twitch response to nerve stimulation. Contractile responses of the tissues to 1mM exogenous acetylcholine (ACh) for 30 s, 20 µM carbachol (CCh) for 60 s and 40 mM KCl for 30 s were obtained in the absence of nerve stimulation. The preparations were then stimulated for 20 min, before the addition of 0.1 µM α-elapitoxin-Ppr1 or α-elapitoxin-Nk2a. Once the twitch height reduced to 10% of the pre-toxin twitch height, the preparation was washed once and then, either 200µl of antivenom was added to the organ bath or washed the preparation every 5 min for 5 s. Then, the recovery of the twitches was observed for approximately 60 and 120 min for α-elapitoxin-Ppr1 and α-elapitoxin-Nk2a treated preparations, respectively. At the conclusion of the experiment, ACh, CCh and KCl were re-added as above. All in-vitro nerve-muscle experiments in this study were approved by the Monash University Animal Ethics Committee (Approval No: MARP/2014/097).

Rat phrenic nerve-hemi-diaphragm preparation (RPNHD)

RPNHD were conducted as described previously[44]. Briefly, adult rats (males; 300–350 g) were killed by CO_2 inhalation. The hemi-diaphragms with intact phrenic nerves were carefully dissected and mounted at a resting tension of 1 g in 15 ml organ baths, under the same bath conditions used for the chick preparation. The electrical stimulation of the phrenic nerve and the recording was carried out as described for the chick preparation, except the supramaximal voltage used for stimulation ranged from 2 to 5 V. Selective stimulation of the motor nerve was confirmed by the abolition of twitches by d-tubocurarine (10 μM). Tissues were then washed, and once the twitches were fully recovered, the stimulation was carried out for another 20 min before the toxins were added to the organ bath. Once the twitch height reduced to 10% of the pre-toxin twitch height, the preparation was washed once and then, either 200μl of antivenom was added to the organ bath or washed the preparation every 5 min for 5 s. All in-vitro nerve-muscle experiments in this study were approved by the Monash University Animal Ethics Committee (Approval No: MARP/2014/097).

Comparison of snakes containing different proportions of SαNTx and LαNTx and human neurotoxicity

A MEDLINE search was carried out using the search terms ‘venome’ and / or the combination of ‘venom’ and ‘proteome’ in order to find any articles describing venomic data of the major snake genera that belong neurotoxic species, namely, *Bungarus*, *Naja*, *Oxyuranus*, *Notechis*, *Micrurus*, *Ophiophagus*, *Acanthophis*, *Vipera*, *Crotalus and Daboia*. Only the articles specifying the relative abundance of SαNTx and LαNTx were selected and such snake genera and the species were identified. Species without α-neurotoxins in the venom proteome were excluded. Then, another MEDLINE search was carried out in order to identify the clinical reports of envenoming caused by the selected genera. Based on the above search, the presence or absence of neuromuscular paralysis in humans envenomed by different species of snakes belonging to each genera was authenticated and the maximum venom concentrations in envenomed humans were identified. The species on which both venomic and clinical data are available, belonging to snake genera with venom concentration data in

envenomed humans were identified and included into the Table 2. Genera excluded were *Daboia*, *Acanthophis*, *Micrurus*, *Vipera*, *Crotalus* and *Ophiophagus* due to one or several of the following reasons (1) lack of α-neurotoxins in the venoms, (2) lack of venomic data that specify SαNTx and LαNTx, (3) lack of venom concentration data in humans.

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Author Contributions

G.K.I., W.C.H., L.D.R. and A.S. conceived the paper; A.S., L.D.R., B C-A and W.C.H. conducted experiments and analysed data; A.S wrote the paper which was improved by all authors. All authors read and approved the final version of the manuscript.

Competing Financial Interests statement

The authors declare no conflict of interest.

FIGURES

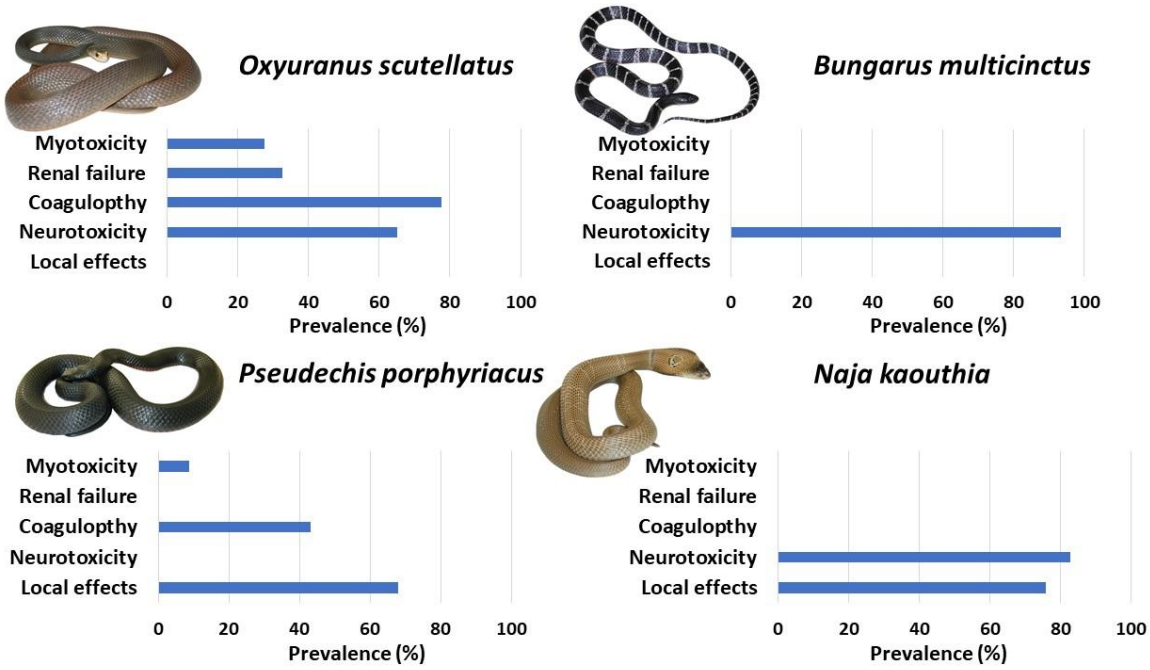

Figure 1: Clinical effects reported in human envenoming by the four snake species from which the four α-neurotoxins were isolated; *Oxyuranus scutellatus*[58], *Bungarus multicinctus*[49], *Pseudechis porphyriacus*[33] and *Naja kaouthia*[63]. *Note: All except P. porphyriacus cause paralysis in humans.*

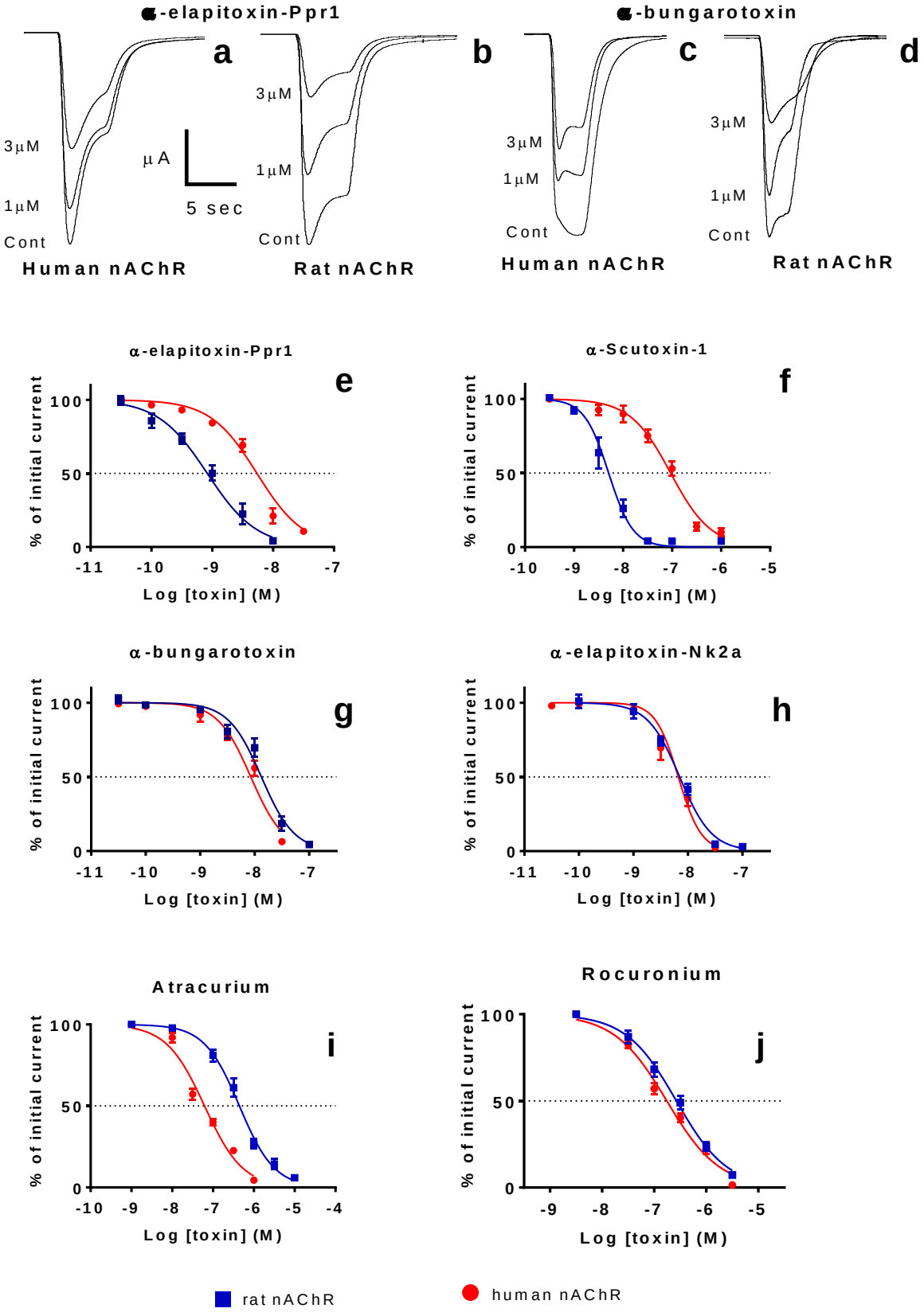
α-elapitoxin-Ppr1
a
3μM
1μM
Cont
μA
5 sec
Human nAChR
3μM
1μM
Cont
Rat nAChR
α-bungarotoxin
b
3μM
1μM
Cont
Human nAChR
c
3μM
1μM
Cont
Rat nAChR
d
α-elapitoxin-Ppr1
e
% of initial current
Log [toxin] (M)
α-Scutoxin-1
f
% of initial current
Log [toxin] (M)
α-bungarotoxin
g
% of initial current
Log [toxin] (M)
α-elapitoxin-Nk2a
h
% of initial current
Log [toxin] (M)
Atracurium
i
% of initial current
Log [toxin] (M)
Rocuronium
j
% of initial current
Log [toxin] (M)
rat nAChR
human nAChR

Figure 2: Species selectivity of α-neurotoxins and muscle relaxants on muscle-type nAChRs: Current traces were evoked by ACh-induced (3μM, 5 s) currents in oocytes expressing the rat and human $(\alpha1)_2\beta\delta\varepsilon$ nAChR. Toxins were applied for 3 min prior to subsequent ACh application. Atracurium and rocuronium were co-applied with the ACh stimulus due to their rapid off rates. Example traces of the inhibition of the human and rat $(\alpha1)_2\beta\delta\varepsilon$ nAChR by 3 μM α-elapitoxin-Ppr-1 **(a & b)** and α-bungarotoxin **(c & d)** compared to the control currents. Concentration-effect curves comparing the inhibitory activity of α-neurotoxins and clinically used muscle relaxants on ACh-induced currents in oocytes expressing the rat (blue curve) or human (red curve) $(\alpha1)_2\beta\delta\varepsilon$ nAChR. by the two SαNTx, **(e)**, α-elapitoxin-Ppr-1 and **(f)**, α-scutoxin-1, the two LαNTx, **(g)**, α-bungarotoxin and **(h)** α-Elapitoxin-Nk2a and the two muscle relaxants, **(i)** atracurium and **(j)** rocuronium. Data are expressed as a percentage of control current and fitted to the best-fit curves in GraphPad 7.0. (n = 5–6; Error bars: SEM).

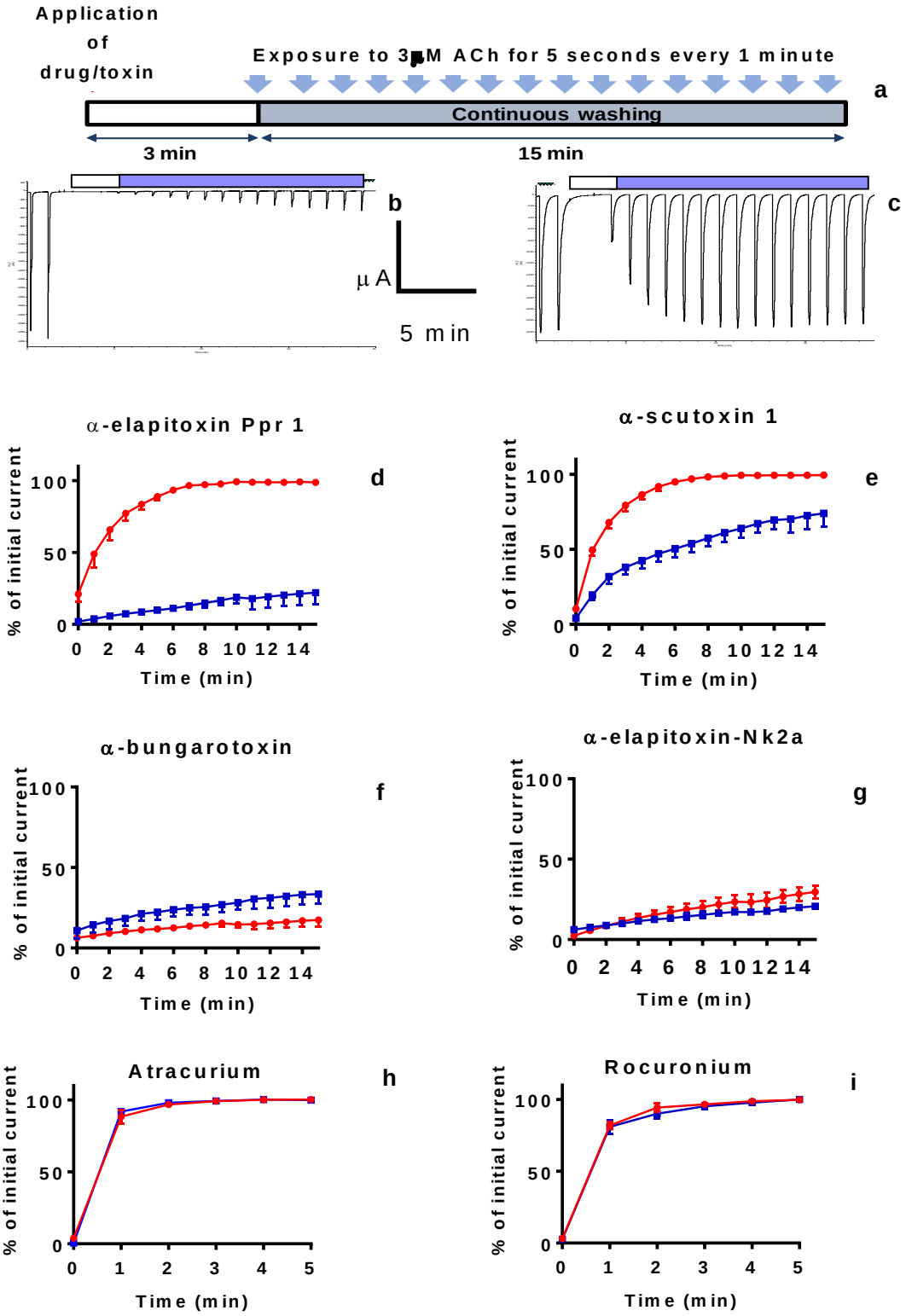

Figure 3: Reversibility of the four α-neurotoxins and muscle relaxant-induced inhibition of the rat and human muscle-type nAChR: (a) Schematic diagram illustrating the protocol used to determine the reversibility of the inhibition of rat and human nAChR by the toxins. Atracurium and

rocuronium were co-applied with the ACh stimulus due to their rapid off rates. Example traces of the recovery of rat **(b)** and human **(c)** $(\alpha1)_2\beta\delta\varepsilon$ nAChR from the 10nM α-elapitoxin-Ppr-1 mediated inhibition, indicating two control currents, application of the toxin for three minutes followed by the recovery of the currents. The recovery of 10μM ACh induced currents in oocytes that expressed rat (blue curve) vs human (red curve) $(\alpha1)_2\beta\delta\varepsilon$ nAChR from the inhibition caused by two SαNTx, **(d)**, 10nM α-Elapitoxin-Ppr-1 and **(e)**, 1μM α-scutoxin-1, the two LαNTx, **(f)**, 10nM α-bungarotoxin and **(g)** 10nM α-Elapitoxin-Nk2a and the two muscle relaxants, **(h)** 1μM atracurium and **(i)** 1μM rocuronium. Note: the human nAChR fully recovered from the SαNTx mediated inhibition, within 8 min while the rat nAChR was not fully recovered after 15 min. The human nAChR showed 80% recovery from snake SαNTx and the two muscle relaxants in 4 and 1 minutes respectively. The post-toxin current was expressed as a percentage of the initial current. (n = 5–8; Error bars: SEM).

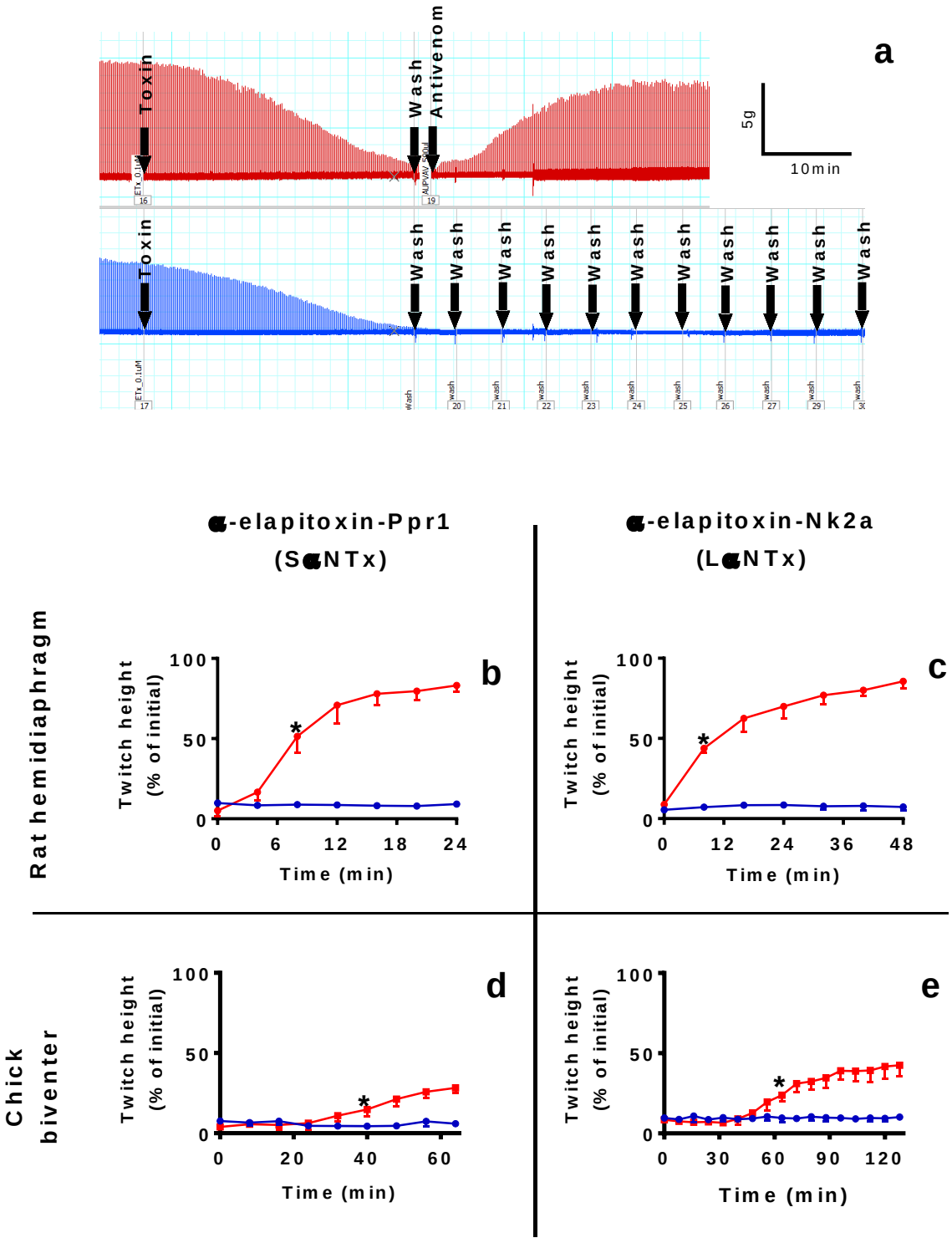

Figure 2: Reversal of 0.1µM α-neurotoxin mediated neuromuscular block in rodent and avian neuromuscular preparations by antivenoms in-vitro: (a) CBNMP and RPNHD were exposed to 0.1µM α-neurotoxin and after the nerve-mediated twitches reached 10% of their original height, the

preparation was washed once and the respective antivenoms added at 40µl/ml (red trace) as opposed to washing of the preparation for 5 s every 5 min (blue trace). The recovery of α-Elapitoxin-Ppr-1 (SαNTx) and α-Elapitoxin-Nk2a (LαNTx) mediated inhibition of the indirect twitches in rat hemidiaphragm (b & c) and in chick biventer nerve-muscle preparation (d & e) in the presence of antivenom (red line) was significant as opposed to washing the preparation (blue line). Australian polyvalent antivenom was used for experiments with α-Elapitoxin-Ppr-1 and Thai neuro-polyvalent antivenom was used for experiments with α-Elapitoxin-NK2a (* indicates that the twitch height of the antivenom added preparations is different to the regular washing on and beyond that time point, $p<0.05$ unpaired t-test; error bars: SEM; n=3).

TABLES

Table 1: Half-maximal inhibitory concentrations (IC_{50}) with 95% confidence intervals (CI) and the hill slopes based on the concentration-response curves of α-Elapitoxin-Ppr-1, α-scutoxin-1, α-bungarotoxin, α-Elapitoxin-Nk2a, atracurium and rocuronium on human and rat nAChR.

	α-Elapitoxin-Ppr-1			**α-scutoxin-1**			**α-bungarotoxin**			**α-Elapitoxin-Nk2a**			**atracurium**			**rocuronium**		
Species	IC_{50} (nM)	95% CI of IC50	Hill slope	IC_{50} (nM)	95% CI of IC50	Hill slope	IC_{50} (nM)	95% CI of IC50	Hill slope	IC_{50} (nM)	95% CI of IC50	Hill slope	IC_{50} (nM)	95% CI of IC50	Hill slope	IC_{50} (nM)	95% CI of IC50	Hill slope
Human	4.5*	3.9-5.4	-1.4	93.3*	75.6-	-1.0	3.9	2.8-6.8	-1.2	2.3	1.7-3.1	-1.3	61.9	52.7-	-0.92	174.5	149.1-	-0.9

					114.8									72.7			204.2	
Rat	0.9	0.7-1.1	-1.0	5.3	4.5-6.3	-1.5	5.4	4.1-7.5	-1.3	2.3	1.9-2.9	-1.3	443*	376.2-521.6	-1.01	266.3*	223.5-317.3	-0.9

Significantly different from the other species * $p<0.05$; extra sum-of-squares F test

Table 2: Maximum venom concentrations recorded in envenomed patients, relative abundance of SαNTx, LαNTx and pre-synaptic neurotoxins and the neurological outcome of envenoming of the snakes of four major neurotoxic genera.

Genus	Maximum venom concentration reported in humans (ng/ml)	Species	Relative abundance of SαNTx %	Maximum SαNTx concentration expected in blood (nM)	Relative abundance of LαNTx %	Maximum LαNTx concentration expected in blood (nM)	Relative abundance of pre-synaptic neurotoxins / basic PLA_2s(%)	Paralysis commonly reported in envenomings
Bungarus	52.2[2]	*B. fasciatus*[45] (Malaysia)	3.4	0.273	1.33	0.092	Not specified	Data deficient
		B. candidus[45] (Malaysia)	-	-	14.51	1.009	Not specified	Yes (in Viet Nam[46] and Thailand[47])
		B.multicinctus[48] (Vietnam)	Not stated	-	19.9	1.385	41	Yes[49]
		B. caeruleus[50] (Sri Lanka)	-	-	-	-	8.25	Yes[2]
Naja	3168[51]	*N. naja* [52] (Sri Lanka)	1.47	7.16	4.73	19.97	None	Yes[5]
		N. kaouthia [18] (Thailand)	7.7	37.5	33.1	139.8	None	Yes[53]
		N. atra[54] (Taiwan)	5-35	170.5	<3	9.5	None	No [31]
		N. sputatrix [55] (Java)	7.89	38.45	0.49	2.06	None	The only case report available describes severe local effects without paralysis.[56]
		N. nigricollis [19] (Nigeria)	0.4	1.94	-	-	None	No[34,57]
		N.katinensis [19] (Burkina-	4.4	21.44	-	-	None	No[34]

		Faso)						
		N.pallida [19] (Kenya)	2.8	13.64	-	-	None	No[34]
		N. nubiae [19] (North Africa)	12.6	61.41	-	-	None	No[34]
		N.mossambica [19] (Tanzania)	1.6	7.79	-	-	None	No[34]
Oxyuranus	3212 [58]	*O.scutellatus* [59] (Australia)	1.5	7.41	-	-	20	Yes[58]
		O.scutellatus [59] (Papua New-Guinea)	2.8	13.82	1.4	6.0	56	Yes[60]
Notechis	152 [61]	*N. scutatus* [62] (Australia)	1.7	0.39	4.0	0.81	32.4	Yes[61]

CHAPTER SEVEN: GENERAL DISCUSSION AND FUTURE DIRECTIONS

This thesis explored the neuromuscular dysfunction in snake envenoming in humans and the role of antivenom as a treatment, using a multi-pronged approach that included clinical, neurophysiological, pharmacological studies and reviews. The literature review highlights the absence of good evidence to support the use of antivenom in the treatment of neuromuscular paralysis in snake envenoming. The clinical and neurophysiological studies suggested that pre-synaptic neuromuscular paralysis has a rapid progression and slow recovery, and highlighted the inability of early antivenom to prevent or reverse the paralysis despite antivenom efficaciously clearing the circulating venom promptly. The pharmacological studies suggested that snake short-chain α-neurotoxins are unlikely to cause paralysis in humans whereas paralysis in humans may occur if the snake can inject large amounts of venom containing long-chain α-neurotoxins in high relative abundance.

The critical review in Chapter two followed a systematic search strategy for publications in MEDLINE and EMBASE, similar to a systematic review (Silva, Hodgson and Isbister, 2017). The search yielded no high-quality evidence from randomized controlled trials for antivenom as a treatment for paralysis in snake envenoming. Our search was limited to two databases and the study was not designed as a systematic review according to PRISMA guidelines. Therefore, a Cochrane systematic review is currently underway to exclusively searching for randomized controlled trials (Appendix 1) across a wide range of databases and the protocol has already been published (Silva *et al.*, 2017). A lack of high quality clinical evidence to support the use of antivenom in treating venom-induced consumption coagulopathy has been recently published (Maduwage *et al.*, 2015). Due to ethical concerns, randomized controlled trials are likely to be impossible to conduct in the future given that antivenom has been accepted as the standard treatment for snake envenoming globally. Therefore, observational studies with better snake authentication, serial measures of venom concentrations, serial objective clinical parameters and serial sensitive biomarkers are important in understanding the role of antivenom in treating snake envenoming (Silva, Maduwage, Sedgwick, Pilapitiya, Weerawansa, Dahanayaka, *et al.*, 2016; Silva, Maduwage, Sedgwick,

Pilapitiya, Weerawansa, Dahanayake, *et al.*, 2016).

The search yielded few observational studies that provide evidence for the effectiveness of antivenom therapy for neuromuscular dysfunction in snake envenoming. A recent study on Taipan envenoming showed that early antivenom therapy can reduce the incidence and severity of paralysis which is predominantly due to pre-synaptic neurotoxicity (Johnston *et al.*, 2017). In contrast, the two clinical studies included in Chapter three showed that antivenom is unable to prevent the occurrence or to reverse the course of already initiated predominantly pre-synaptic neuromuscular dysfunction, despite rapidly clearing circulating venom (Silva *et al.*, 2016a; 2016b). This indicates that by the time the patients receive antivenom, pre-synaptic toxins have initiated their damage at a significant number of motor nerve terminals which then cannot be reversed (Dixon and Harris, 1999; Prasarnpun, Walsh and Harris, 2004; Prasarnpun *et al.*, 2005). Identification of the pre-synaptic neurotoxin, U1-viperitoxin-Dr1a as the major neurotoxin in Sri Lankan Russell's viper venom further supported the previous observations on the role of pre-synaptic neurotoxins in treatment resistant paralysis. However, it is unclear about the 'window period' during which antivenom can be effective in preventing pre-synaptic neurotoxicity, and it could be affected by various factors such as the degree of envenoming and the pharmacological characteristics of the individual pre-synaptic toxins. Further clinical and pharmacological studies are required to identify the beneficial period of antivenom therapy in pre-synaptic neurotoxicity in snake envenoming. However, the study included in Chapter six experimentally showed the ability of antivenoms to reverse neuromuscular dysfunction due to both long- and short-chain neurotoxins. This indicates that antivenom therapy could be 'never too late' for post-synaptic type neuromuscular paralysis, provided that the antivenom molecules can reach the neuromuscular junctions effectively.

The high significance of snake α-neurotoxins in potentiating prey specific toxicity has previously been recognized (Jackson *et al.*, 2013). Therefore, it is likely that α-neurotoxins of venoms from snake species also show differential potency on nAChR of different animal tissues such as rodent,

avian and amphibian, which are prey species of different snake species. Although this does not necessarily limit the usefulness of the above animal tissues as pharmacological models in investigating neurotoxins and neurotoxic venoms, projecting the results from such models directly to envenoming in humans may be misleading. The standard measure of antivenom efficacy is the median effective dose (ED_{50}), which is calculated based on the lethality of venoms in rodents (World Health Organisation, 2010). The study included in Chapter six demonstrated that human nAChR are more resistant to snake short-chain α-neurotoxins compared to rat nAChR. Many snake venoms contain short-chain α-neurotoxins (Barber, Isbister and Hodgson, 2013), and during lethality tests, rodents can die due to toxins that are clinically less relevant to humans. A recent study demonstrated that rodent plasma is highly resistant to the clinically relevant snake procoagulant toxins, compared to human plasma (Maduwage *et al.*, 2016). Collectively, this challenges the validity of the currently established rodent lethality based assays such as LD_{50} and ED_{50} in the study of envenoming in humans, and emphasizes the need for clinically focussed *in-vitro* antivenom efficacy tests to replace the lethality tests (Maduwage *et al.*, 2016).

The study included in Chapter four demonstrated that the rate of indirect twitch inhibition in rat and chicken tissues by U1-viperitoxin-Dr1a is similar. The lack of a straightforward *in-vitro* human neuromuscular model to examine snake pre-synaptic neurotoxins has led to the need to directly project information obtained from *in-vitro* rodent, avian and amphibian nerve-muscle preparations to neurotoxic envenoming in humans (Hodgson and Wickramaratna, 2002; Prasarnpun *et al.*, 2005). Unlike α-neurotoxins, the comparative susceptibility of different animal tissues to snake venom pre-synaptic neurotoxins has not previously been studied in detail. Therefore, testing the susceptibility of different animal tissues to different snake pre-synaptic neurotoxins would be an important future direction in understanding the validity of nerve-muscle preparations as pharmacological models for neuromuscular dysfunction caused by snake pre-synaptic neurotoxins in humans.

In the study included in Chapter five, the remarkable ability of the commercially used antivenoms

from Asia and Australasia to cross neutralize a range of pre- and post-synaptic neurotoxins and venoms from the two regions was observed (Silva, Hodgson and Isbister, 2016). These observations, together with some previous reports (Kornhauser *et al.*, 2013; Leong *et al.*, 2014, 2015) clearly indicate the potential for the development of universal or regional specific antivenoms for neurotoxic envenoming. This development would require the careful identification of a few neurotoxins from pre- and post-synaptic neurotoxin groups, which are antigenically representative of a wide range of snake neurotoxins from their groups to be included in the immunization mixture.

The assessment of neuromuscular dysfunction in snake envenomed patients relies heavily on clinical examination, hence may be less objective. The two clinical studies included in Chapter three used sfEMG for the first time as a biomarker to study the evolution and resolution of neuromuscular dysfunction in snake envenoming (Silva *et al.*, 2016; 2016b). The severity of the sfEMG abnormalities correlated well with the clinical severity of paralysis and the test detected the subclinical neuromuscular dysfunction before paralysis became clinically detectable. Therefore, sfEMG is a promising research tool that can be used in further investigation of neuromuscular paralysis in snake envenoming. However, the technique is expensive and demands time and a high level of operator skills, hence is unlikely to be used as a diagnostic test for neuromuscular paralysis in snake envenoming, even in high resource settings.

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APPENDIX 1

Antivenom for snake venom-induced neuromuscular paralysis – Protocol for Cochrane review.

[Intervention Protocol]

Antivenom for snake venom-induced neuromuscular paralysis

Anjana Silva[1], Kalana Maduwage[2], Nick A Buckley[3], David G Lalloo[4], H Janaka de Silva[5], Geoffrey K Isbister[6]

[1]Department of Pharmacology, Monash University, Victoria, Australia. [2]Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Peradeniya, Sri Lanka. [3]Department of Pharmacology, University of Sydney, Camperdown, Australia. [4]Clinical Research Group, Liverpool School of Tropical Medicine, Liverpool, UK. [5]Department of Medicine, University of Kelaniya, Ragama, Sri Lanka. [6]School of Medicine and Public Health, University of Newcastle, Waratah, Australia

Contact address: Anjana Silva, Department of Pharmacology, Monash University, Victoria, Australia. [redacted]

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ABSTRACT

This is a protocol for a Cochrane Review (Intervention). The objectives are as follows:

To assess the effects of antivenom on neuromuscular paralysis in people with neurotoxic snake envenoming.

BACKGROUND

Description of the condition

Snakebite leads to significant morbidity and mortality globally, with an estimated burden of 421,000 to 1,841,000 envenomings and 20,000 to 94,000 deaths per year. Of this, more than 90% of envenomings are reported from tropical Asia, sub-Saharan Africa, and Latin America (Kasturiratne 2008). Venom-induced neuromuscular paralysis is one of the major clinical manifestations of envenoming, predominately by elapid snakes. In some neurotoxic snakebites, such as by kraits (genus *Bungarus*) in Asia, life-threatening paralysis occurs in more than 50% of patients (Kularatne 2002; Hung 2009).

Neurotoxic snake venoms primarily affect the neuromuscular junction causing a disruption of neurotransmission, resulting in paralysis of the skeletal muscles (Harris 2009; Ranawaka 2013). Snake venom neurotoxins target multiple sites in the neuromuscular junction. The majority of the snake venom neurotoxins either act on the motor nerve terminals (presynaptic) or the nicotinic acetylcholine receptor on the motor end-plate (postsynaptic).

Presynaptic toxins initially lead to a depletion of the synaptic vesicles and ultimately cause structural damage to the motor nerve terminals (Logonder 2008; Prasarnpun 2005). This type of insult is most likely to be treatment resistant, and recovery depends on the natural regeneration of the nerve terminal, as shown from experimental studies using presynaptic toxins isolated from krait and viper venoms (Dixon 1999; Logonder 2008; Prasarnpun 2004; Prasarnpun 2005). Snake venom postsynaptic neurotoxins competitively bind to the agonist-binding sites of the nicotinic acetylcholine receptors on the motor end-plate with high affinity and poor reversibility, blocking neuromuscular transmission (Ishikawa 1985; Vincent 1998). Some neurotoxic snake venoms, as in kraits, contain both types of toxins (Rusmili 2014). Several snake venom toxins act on specific ion channels or affect acetylcholinesterase activity in the neuromuscular junction (Harris 2009).

Whatever the mechanism, all of these toxins result in the same clinical effect: neuromuscular weakness, which can range from a

mild weakness of the eyelid and facial muscles to fatal paralysis of bulbar and respiratory muscles (Connolly 1995; Isbister 2012; Johnston 2012; Kularatne 2000; Kularatne 2002; Silva 2016). In extreme cases, complete neuromuscular paralysis involving all skeletal muscles of the body can occur (Silva 2016). To sustain life, mechanical ventilation is essential in people with respiratory paralysis. Depending on the snake species involved, neuromuscular paralysis can co-exist with other clinical manifestations of envenoming, such as local tissue necrosis seen in cobras, Kularatne 2009, and venom-induced consumptive coagulopathy in vipers, Sano-Martins 2001, and some Australasian elapids (Isbister 2012). The detection and monitoring of the neuromuscular paralysis in people with snakebite in the clinical setting as well as for research purposes are almost entirely dependent on the clinical examination. For this, patients are constantly monitored for clinical features of neurotoxicity such as ptosis, ophthalmoplegia, and facial, neck, bulbar, respiratory, and limb weakness (Isbister 2012; Johnston 2012; Kularatne 2000; Kularatne 2002). Neurophysiological tests such as single-fibre electromyography have also been used for this purpose (Silva 2016). However, such tests require equipment and skills beyond the reach of rural settings, where snakebites are mostly prevalent.

Description of the intervention

Antivenoms have been used for the treatment of snakebite for more than a century (Gutiérrez 2011; WHO 2010). They are polyclonal whole immunoglobulin (IgG) or immunoglobulin fractions (Fab or $F(ab')_2$) raised against venom from one (monovalent) or several (polyvalent) snake species in other animals, most commonly horses. The immunised animals are periodically bled and the immunoglobulins are separated from the blood using ammonium sulphate or caprylic acid to produce whole IgG antivenom. During the production of many commercial antivenoms, the whole immunoglobulins are fractionated by papain or pepsin digestion to make Fab or $F(ab')_2$, respectively (Chippaux 2006; Gutiérrez 2011; WHO 2010). Depending on the production protocol, the immunoglobulins or fractions may be subject to further purification involving chromatographic steps and pasteurisation (León 2013). Antivenoms are available in freeze-dried powdered form (where the powder is reconstituted with sterile water prior to use) or liquid form. Snake antivenoms are almost always delivered to the patients via the intravenous route. Antivenom therapy is associated with adverse reactions, and frequent life-threatening reactions are a major problem associated with some antivenoms (de Silva 2011; de Silva 2015; León 2013).

How the intervention might work

In doses used in the clinical setting, antivenom molecules (polyclonal antibodies) likely outnumber the venom molecules (toxins) in the circulation (Allen 2012; Isbister 2015). The polyclonal nature of the antivenoms means that they contain a range of antibodies or antibody fractions against a range of neurotoxins (both pre- and postsynaptic), relevant to this review, as well as non-neurotoxic toxins. These antivenom molecules bind with circulating toxins, forming large venom-antivenom complexes, trapping the venom molecules in the circulation (O'Leary 2006; O'Leary 2014). The antibodies likely act via a number of mechanisms, including blocking the active site of the neurotoxin molecules, preventing the toxins from interacting with the target site (neuromuscular junction) by restricting the movement of the neurotoxins to the extravascular target sites, and also increasing the elimination of the toxins (Maduwage 2015). In addition, if the antivenom molecules are able to distribute from the circulation, they might be able to reach the neuromuscular junctions and neutralise the neurotoxins at their target site. However, it is unclear how effectively the whole IgG, $F(ab')_2$, or Fab molecules in the antivenoms can distribute to the neuromuscular junctions.

Presynaptic neurotoxins result in structural damage to the motor nerve terminals that is irreversible (in the short term). Antivenom is therefore unlikely to be able to reverse already established presynaptic neurotoxic injury (Harris 2013; Logonder 2008; Prasarnpun 2005). In contrast, postsynaptic neurotoxins act in a similar way to reversible non-depolarising type neuromuscular blockers or muscle relaxants. The reversibility of the binding of postsynaptic toxins to the nicotinic acetylcholine receptor varies based on the structural properties of the individual toxins (Barber 2013). Experimental evidence suggests that specific immunoglobulins are able to increase the recovery of the neuromuscular junctions from postsynaptic toxin-mediated neuromuscular block (Gatineau 1988).

Why it is important to do this review

Although antivenom therapy is commonly utilised for neurotoxic snake envenoming, its effectiveness in preventing or reversing neurotoxicity is less clear and has been questioned in several studies conducted in different regions (Johnston 2012; Richardson 2007; Theakston 1990; Silva 2016). Recovery of the neurotoxicity in snake envenoming without antivenom has also been reported (Hung 2009; Pochanugool 1997). In practice, it is doubtful whether the antivenom could be delivered early enough to prevent the neurotoxins from reaching neuromuscular junctions. Furthermore, it is unclear whether the antivenoms can speed recovery of already established neurotoxicity. A recent study of common krait envenoming demonstrated that even in the patients who received early antivenom (median 3.5 hours postbite) in an adequate dose to bind with all circulating venom antigens, antivenom was unable to prevent the subsequent development of life-threatening paralysis (Silva 2016). In contrast, a study of taipan bites in Papua New Guinea found that early administering of antivenom prevented intubation in a proportion of patients (Connolly 1995).

OBJECTIVES

To assess the effects of antivenom on neuromuscular paralysis in people with neurotoxic snake envenoming.

METHODS

Criteria for considering studies for this review

Types of studies

We will consider randomised controlled trials in humans for inclusion in this review. Of the randomised controlled trials published after 2010, we will exclude those without an accessible, registered protocol. We will not consider cluster trials due to issues related to the unit of analysis.

Types of participants

People of any age, who were bitten or envenomed by neurotoxic snakes, and have either developed venom-induced neuromuscular paralysis or have not yet developed venom-induced neuromuscular paralysis. We will base diagnosis of venom-induced neuromuscular paralysis on clinical features of neuromuscular paralysis such as ptosis, ophthalmoplegia, and facial, neck, bulbar, respiratory, and limb weakness.

Types of interventions

Intravenous administration of snake antivenom regardless of the type or dose of antivenom. The comparison group will be people who were not treated with antivenom.

Types of outcome measures

Primary outcomes

1. Mortality as a direct result of neuromuscular paralysis within 14 days of the snakebite.

Secondary outcomes

1. Incidence of life-threatening paralysis that requires intubation or mechanical ventilation, or both within 24 hours of the snakebite.
2. Duration of mechanical ventilation.
3. Incidence of any of the following clinical effects of neuromuscular paralysis*: ptosis, ophthalmoplegia, weakness of facial, neck, bulbar, or limb muscles within 48 hours of the snakebite.
4. Incidence of immediate systemic hypersensitivity reactions within four hours of antivenom administration.
5. Incidence of serum sickness within 14 days of the administration of antivenom.

*Neuromuscular paralysis is defined here as presence of at least a single clinical feature of clinically detectable paralysis (e.g. ptosis, ophthalmoplegia, facial muscle weakness, neck muscle weakness, bulbar palsy, respiratory muscle weakness, weakness in upper and lower limbs).

Information size calculation

To our knowledge, an estimate of the mortality rates due to venom-induced neuromuscular paralysis is unavailable. However, the prevalence of life-threatening paralysis (that required intubation and mechanical ventilation) in krait and taipan envenomings is 49% to 51% (Trevett 1995; Silva 2016; Kularatne 2002). In a previous study, antivenom therapy improved the outcome of 3 out of 6 people with paralysis due to Papuan death adder envenoming (Lalloo 1996). Based on this, we can assume that the mortality rate for untreated patients (i.e. no antivenom given) due to venom-induced neuromuscular paralysis in neurotoxic snake envenoming is 50%. Conservatively we expect the antivenom will lower the mortality by 25% (i.e. half the mortality). Based on the above assumptions, with a statistical power of 90% and alpha of 0.05, the information size required is a total of 168 participants (84 participants with antivenom treatment and 84 participants without antivenom treatment).

Search methods for identification of studies

In order to reduce publication and retrieval bias, we will not restrict our search by language, date, or publication status.

Electronic searches

The Cochrane Injuries Group's Information Specialist will search the following databases:

1. Cochrane Injuries Group Specialised Register (present version);
2. The Cochrane Library (www.cochranelibrary.com) (latest issue);
3. Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R) (1946 to present);
4. Embase Classic + Embase (OvidSP) (1947 to present);
5. ISI Web of Science: Science Citation Index Expanded (SCI-EXPANDED) (1970 to present);
6. ISI Web of Science: Conference Proceedings Citation Index-Science (CPCI-S) (1990 to present);
7. ISI BIOSIS Citation Index (1969 to present);
8. KoreaMed (www.koreamed.org) (all available dates);

9. IndMed (indmed.nic.in) (all available dates);
10. LILACS (Latin American and Caribbean Center on Health Sciences Database) (lilacs.bvsalud.org/en/) (all available dates);
11. ClinicalTrials.gov (www.clinicaltrials.gov);
12. World Health Organization International Clinical Trials Registry Platform (WHO ICTRP) (apps.who.int/trialsearch/).

We will adapt the MEDLINE search strategy provided in Appendix 1 as required for the other databases.

Searching other resources

We will search the reference lists of all relevant studies and contact experts in the field in order to identify ongoing and completed studies. We will also run a search on regional databases and journals from South and Southeast Asia, sub-Saharan Africa, and Latin America, and search the guidelines, conference proceedings, theses, and other sources of grey literature.

Data collection and analysis

We will perform a systematic review following the instructions in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011).

Selection of studies

Two review authors (AS and GKI) will independently scan the titles and abstracts of all articles identified by the search strategy. If either or both review authors identify an article as possibly meeting the inclusion criteria, we will obtain the full text of the published article. Both review authors will review the full text of each article to determine if it meets the inclusion criteria. Disagreements between the two review authors will be resolved by a third review author (NB). We will provide details of the included studies in the appropriate tables within the review. We will report studies not meeting the inclusion criteria in the 'Characteristics of excluded studies' section of the review and the reasons for exclusion in the 'Characteristics of excluded studies' table. In the event of disagreement between the review authors, we will seek the opinion of a third review author (NB). For ambiguous studies and where there are insufficient data, we will attempt to contact the authors of the articles for further clarification and more information. We will grade the studies for quality, using the instructions in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011).

Data extraction and management

Two review authors will independently review each article that meets inclusion criteria, and extract data from the article onto a standard data extraction form for the following items.

- General information about the article: title of the article, source, publication year, years the study was conducted, language of publication.
- Clinical trial characteristics: design, diagnostic ascertainment, standard care provided, randomisation, allocation concealment, interventions, dropouts and lost to follow-up, definitions of outcomes, and methods of outcome assessment.
- Participants: inclusion and exclusion criteria, sample size, baseline characteristics (participant age, past history of neuromuscular disorders, clinical severity on enrolment).
- Interventions: type of antivenom (polyvalent or monovalent), manufacturer, dose of antivenom (number of vials or milligrams), time administered postbite and duration of administration.
- Outcomes: mortality*, duration of clinical features of neuromuscular paralysis including ptosis, ophthalmoplegia, facial, neck, bulbar, respiratory, and limb weakness, duration of mechanical ventilation, length of hospital stay, immediate systemic hypersensitivity reactions, serum sickness.

*In instances where other clinical manifestations of envenoming, such as coagulopathy and local effects, coexisted and are a likely cause of mortality rather than neurotoxicity, we will exclude such cases from the analysis.

Assessment of risk of bias in included studies

Two review authors (AS and GKI) will independently assess the included studies for risk of bias using the suggested domains and guidance provided in the Cochrane 'Risk of bias' tool as detailed in section 8.5 of the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011). We will assess random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attribution bias), selective reporting (reporting bias), and other sources of bias (in particular funding source). If information to make a judgement is insufficient, we will initially assess domains as 'unclear risk' and will attempt to clarify the risk of bias by contacting the study authors. We plan to include all studies irrespective of the risk of bias. However, we plan to perform a sensitivity analysis; if the sensitivity analysis shows substantial differences, we will present alternative estimates that exclude studies with high or unclear risk of bias.

We will categorise the overall risk of bias of individual studies as follows:

- low risk of bias (plausible bias unlikely to seriously alter the results) if all domains were at low risk of bias;
- unclear risk of bias (plausible bias that raises some doubt about the results) if one or more domains had an unclear risk of bias; or
- high risk of bias (plausible bias that seriously weakens confidence in the results) if one or more domains were at high

risk of bias.

Measures of treatment effect

We will define measures of treatment effects as follows.

Dichotomous data

We will present dichotomous data outcomes as risk ratios (RR) with 95% confidence intervals (CI) for individual trials.

Continuous data

We will present continuous data outcomes with mean difference (MD) and 95% CI. As mean differences are easier for clinicians and readers to interpret, we will calculate mean difference where possible; we will use standardised mean difference when different scales are used in the trials.

Ordinal data

We will report ordinal data outcomes such as types of adverse events and complications depending on the length of the scales used. If the scale is longer (> 5), we will treat the data as continuous; if the scale is short (5 or less), we will combine adjacent categories to produce dichotomous data.

Unit of analysis issues

The unit of analysis will be the individual participant. To answer our primary question (does antivenom change mortality due to neuromuscular paralysis compared to no antivenom treatment), we will in the first instance simply combine all active intervention groups of the study into a single group and compare their outcomes to the control groups(s) not receiving antivenom, whilst acknowledging the limitations related to the heterogeneity of the data.

We have excluded cluster randomised trials from the review. We do not expect to identify cross-over trials as they are an inappropriate study design for this type of treatment.

Dealing with missing data

We will contact the authors of the original studies if essential data are missing from their trial reports. If we receive no reply after eight weeks, we will extract the available data from the published reports. We will assess the missing data and attrition rates for each of the included studies and report the number of participants who are included in the final analysis as a proportion of all participants in the study.

Assessment of heterogeneity

We will evaluate statistical heterogeneity using the Chi^2 test, and the I^2 statistics for quantifying heterogeneity across studies. The importance of the observed value of I^2 depends on (i) magnitude and direction of effects and (ii) strength of evidence for heterogeneity (e.g. P value from the Chi^2 test, or a confidence interval for I^2 as outlined in Higgins 2011. We expect high levels of heterogeneity due to considerable variation across trials in setting, snake, intervention, and outcomes; we will consider I^2 values of more than 85% as considerable heterogeneity. The possible elements of heterogeneity will be included for exploration in a subgroup analysis, as mentioned in Subgroup analysis and investigation of heterogeneity. We intend to use the random-effects model to account for this heterogeneity in any summary estimates of effect. We will discuss the implications of heterogeneity and how they relate to external validity in the Discussion.

Assessment of reporting biases

We will refer to systematic differences between reported and unreported findings as reporting bias. We will include selective-outcome reporting assessment as part of the 'Risk of bias' table and also under intention-to-treat analysis.

We will assess publication biases by using funnel plots when at least 10 studies are included in the meta-analysis.

Data synthesis

We will pool dichotomous outcomes such as mortality, risk of immediate-type hypersensitivity reactions, and risk of serum sickness, and report the RR with 95% CIs. We will use a Mantel-Haenszel random-effects model for dichotomous data meta-analysis. For continuous outcomes (duration of mechanical ventilation), we will use an inverse-variance, random-effects model for the analysis and the mean difference, or the standardised mean difference if outcomes were measured using different scales. We will perform meta-analysis if we find two or more studies assessing the same outcome. If a meta-analysis is not possible, we will write a narrative summary of the study findings and follow alternative methods as described in the *Cochrane Handbook* (Higgins 2011).

Subgroup analysis and investigation of heterogeneity

Where possible (if sufficient data and information are available) we will perform subgroup analysis based on the following factors, which are thought to affect outcomes after neuromuscular paralysis.

- Presynaptic versus postsynaptic versus mixed mechanism of neurotoxic snake envenoming
- Each specific species of snake envenoming
- Type of snake antivenom
- Dose of antivenom

The immediate hypersensitivity reactions and serum sickness (secondary outcome measures) are likely to be strongly affected by the type of snake antivenom, hence we will analyse these based on the different types of antivenom.

Sensitivity analysis

We will restrict sensitivity analyses to include studies with both (1) allocation concealment carrying low risk of bias and (2) having blinded outcome assessment. Different batches of the same antivenom used for the same study may show interbatch variation of efficacy (leading to variation in effectiveness). Strict implementation of the random allocation is therefore important in minimising bias. Some secondary outcomes such as the duration of the clinical features of paralysis and the duration of mechanical ventilation are purely based on the clinical decision-making of the treating staff, hence implementing the blinded outcome assessment is important in minimising bias. Furthermore, since immediate hypersensitivity reactions may be affected by pretreatment with epinephrine, we will carry out sensitivity analysis excluding those participants treated with epinephrine.

Summarising findings and assessing the quality of the evidence

We will generate a 'Summary of findings' table for comparing antivenom versus no antivenom. We will report the following outcomes in the 'Summary of findings' table.

1. Mortality as a direct result of neuromuscular paralysis within 14 days of the snakebite.
2. Incidence of life-threatening paralysis that requires intubation or mechanical ventilation, or both within 24 hours of the snakebite.
3. Duration of mechanical ventilation.
4. Incidence of immediate systemic hypersensitivity reactions within four hours of antivenom administration.
5. Incidence of serum sickness within 14 days of the administration of antivenom.

We will grade the quality of the evidence in the studies as high, moderate, low, or very low according to the section 11.5 of Higgins 2011 using GRADE methods and GRADEpro software (GRADE 2004; GRADEpro 2015). We will assess the body of evidence based on the risk of bias of the included studies, directness of the evidence, inconsistency in results, imprecision of the measure of effects, and publication bias.

We will provide citations and a rationale for the figures on which the calculation of assumed and corresponding risks in the 'Summary of findings' table are based.

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* *Indicates the major publication for the study*

APPENDICES

Appendix 1. Search strategy

Ovid MEDLINE (databases) will be searched using the following terms:

1. Snake Bites/

2. (snakebit* or ((snake* or rattlesnake* or viper* or cobra* or asp or asps or mamba* or krait* or adder* or Vipirid* or Vipirin* or Elapid* or Colubrid* or Hydrophiin* or Laticaudin* or Crotalid* or Crotalin* or Bitis* or Vipera* or Ophiophagus* or Bungarus* or Crotalus* or Daboia* or Micrurus* or Micruroides* or Adenorhinos* or Atheris* or Cerastes* or Echis* or Eristicophis* or Macrovipera* or Montatheris* or Proatheris* or Pseudocerastes*) adj3 bit*)).ti,ab,kf.

3. 1 or 2

4. exp Snakes/

5. (snake* or rattlesnake* or viper* or cobra* or asp or asps or mamba* or krait* or adder* or Vipirid* or Vipirin* or Elapid* or Colubrid* or Hydrophiin* or Laticaudin* or Crotalid* or Crotalin* or Bitis* or Vipera* or Ophiophagus* or Bungarus* or Crotalus* or Daboia* or Micrurus* or Micruroides* Adenorhinos* or Atheris* or Cerastes* or Echis* or Eristicophis* or Macrovipera* or Montatheris* or Proatheris* or Pseudocerastes*).ti,ab,kf,hw.
6. Poisoning/
7. (poison* or toxin* or venom* or envenom* or antivenom*).ti,ab,kf,hw.
8. (neurotoxin* or phospholipase A2*).ti,ab,kf,hw.
9. (4 or 5) and (6 or 7 or 8)
10. exp Snake Venoms/
11. (fasciculin* or dendrotoxin* or alpha-neurotoxin* or crotamine* or bungarotoxin*).ti,ab,kf,hw.
12. Antivenins/
13. or/9-12
14. (neuro* or paralys*).ti,ab,kf.
15. (ptosis* or weakness* or flaccid* or palsy*).ti,ab,kf.
16. exp Paralysis/
17. or/14-16
18. 13 and 17
19. 3 or 18
20. randomized controlled trial.pt.
21. controlled clinical trial.pt.
22. placebo.ti,ab,kf.
23. trial.ti,ab,kf.
24. (RCT or random*).ti,ab,kf.
25. clinical trials as topic.sh.
26. (animals not (humans and animals)).sh.
27. (or/20-25) not 26
28. 19 and 27

CONTRIBUTIONS OF AUTHORS

GKI and AS conceived the idea. AS, KM, and GKI developed the initial draft of the protocol, which was improved by HJdeS, NAB, and DGL.

DECLARATIONS OF INTEREST

Anjana Silva: No conflicts of interest

Kalana Maduwage: No conflicts of interest

Nick A Buckley: No conflicts of interest

David G Lalloo: No conflicts of interest

H Janaka de Silva: No conflicts of interest

Geoffrey K Isbister: No conflicts of interest

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APPENDIX II

List of other publications during PhD candidature (Not included as a part of the thesis)

1. Kularatne SA, Weerakoon K, **Silva A**, Maduwage K, Walathara C, Rathnayake I, Medagedara S, Paranagama R, Mendis S, Kumarasiri PV. Efficacy of intravenous hydrocortisone administered 2-4 h prior to antivenom as prophylaxis against adverse drug reactions to snake antivenom in Sri Lanka: An open labelled randomized controlled trial. *Toxicon* 2016; 120:159-65.

2. Maduwage K, **Silva A**, O'Leary M, Hodgson WC, Isbister GK. Efficacy of Indian polyvalent snake antivenoms against Sri Lankan snake venoms: lethality studies or clinically focussed *in-vitro* studies. *Sci. Rep.* 2016, 6: 26778; doi: 10.1038/srep26778 (2016).

3. Maduwage KP, O'Leary MA, **Silva A**, Isbister GK. Detection of Snake Venom in Post-Antivenom Samples by Dissociation Treatment Followed by Enzyme Immunoassay. *Toxins (Basel)* 2016; 8: 130.

4. **Silva A**, Samarasinghe R, Pilapitiya S, Dahanayake N, Siribaddana S. Viper bites complicate chronic agrochemical nephropathy in rural Sri Lanka. *J Venom Anim Toxins Incl Trop Dis* 2014;20:33.

5. Giles ML, Vincent FB, Volkenstein S, Shefin SM, **Silva A**, Fredrick F; Young Physician Leadership Group 2013. What has the Young Physician Leaders Programme achieved? *Lancet* 2014;384(9937):26-7.

6. **Silva A**, Marikar F, Murugananthan A, Agampodi S. Awareness and perceptions on prevention, first aid and treatment of Snake bites among Sri Lankan farmers: A knowledge practice mismatch? *J Occuational Med Toxicol* 2014; 9: 20.

7. Kularatne SAM, **Silva A**, Weerakoon KGAD, Maduwage K, Walathara C, Mendis S, Paranagama R. Revisiting Russell's viper (*Daboia russelii*) bite in Sri Lanka: Is abdominal pain an early feature of systemic envenoming? *PLoS ONE* 2014; 9 (2):e90198.

8. Kularatne SAM, **Silva A**, Maduwage K, Ratnayake I, Walathara C, Mendis S, Pranagama R. Victims' response to snakebite and socio-epidemiological factors of 1018 snakebites in a tertiary care hospital in Sri Lanka. *Wilderness and Environmental Medicine* 2014; 25: 35-40