Investigating cardiovascular activities of animal venom

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ABSTRACT

The current study examined the cardiovascular effects of animal venoms. This included eight snake species (i.e. *Daboia russelii, Daboia siamensis, Bitis arietans, Crotalus vegrandis, Bitis gabonica, Echis ocellatus, Pseudonaja textilis* and *Oxyuranus temporalis*) that are of medical importance and one bat species (*Desmodus rotundus*). In previous animal studies, including many from ourlaboratory, the characteristics of hypotension/cardiovascular collapse due to snake envenoming wereunclearly defined. In this study, we show that *B. gabonica, C. vegrandis* and *B. arietans* venoms (allat 200 µg/kg, i.v.) cause mild transient hypotension in anaesthetised rats, while *D. russellii* (100 µg/kg, i.v.) and *D. siamensis* (100 µg/kg, i.v.) venoms caused a slow, prolonged decrease in blood pressure. In contrast, *P. textilis* (5 µg/kg, i.v.) and *E. ocellatus* (50 µg/kg, i.v.) venoms cause a rapiddrop in blood pressure without recovery, which we have classified as 'collapse'. Interestingly, priming with small doses of venom, prior to a dose which caused 'collapse' when administered alone,prevented collapse but not the prolonged hypotension while the use of artificial respiration prevented prolonged hypotension but not collapse. These two key findings enabled us to distinguish the two different patterns of effects as **rapid cardiovascular collapse;** defined as a sudden drop (i.e. with 2 min) in blood pressure that is usually unrecoverable and **prolonged hypotension;** characterised as apersistent slow decrease in blood pressure with recovery. Prevention of collapse via 'priming' dosesof venom suggests that depletable endogenous mediators may play a role in this phenomenon.

Further *in vitro* studies revealed that *D. russelii* venom is a potent vasodilator of small blood vessels, providing a possible mechanism for the prolonged hypotension observed *in vivo*. *D. russelli* venom (1 ng/mL - 1 µg/mL) caused concentration-dependent relaxation of rat small mesenteric arteries pre-contracted with U46619 and mounted in a myograph. The potency of the venom was not changed in the presence of the nitric oxide synthase inhibitor, L-NAME (100µM), or removal of the endothelium, indicating that the vasorelaxation occurred via an endothelium-independent mechanism. Blocking voltage-dependent K_V or calcium activated K_{Ca}, but not ATP- sensitive K^+ channels, markedly attenuated the vasodilatory effects of the venom indicating that the venom was acting via both K_{Ca} and K_V channels. Similarly blocking transient receptor potential cation channel subfamily V member 4 (TRVP4) also markedly decreased venom-induced vasodilation. However, blocking histamine receptors did not affect vasodilatation. Further investigation is required to identify the toxin(s) responsible for this effect.

D. rotundus venom is known to contain strong anticoagulant and proteolytic components, which interfere with the blood coagulation cascade. In this study, we identified and examined a compound

from the venom similar in size and amino acid sequence to human calcitonin gene-related peptide (CGRP). The *in vitro* effects of this compound (termed vCGRP) were examined in a myograph, vCGRP displayed vasodilatory properties, similar to rat CGRP (rCGRP) in both potency and efficacy. However, a key difference between rCGRP and vCGRP is that vCGRP acts selectively on CGRP1 receptors present on the smooth muscle while rCGRP activates receptors both on the endothelium and smooth muscle. Interestingly, human CGRP also selectively acts endothelium-independently. The similarity in mechanism between human CGRP and vCGRP suggests that vCGRP could be used as a potential candidate for future therapeutic studies.

Overall, these studies highlight that animal venoms contain many compounds that can affect the cardiovascular system via different mechanisms. Exploring these mechanisms is invaluable for future therapeutic interventions as well as gaining a better understanding the effects of envenoming and potential treatment strategies.

DECLARATION

In accordance with the Doctor of Philosophy Regulations of Monash University, the following declarations are made:

I hereby declare that this thesis contains no materials 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.

All of the experiments described were performed in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) code of practice for the care and use of animals for experimental purposes in Australia. All animal experiments were approved by the Animal Ethics Committee (Department of Pharmacology, Monash University).

In accordance with the Doctorate Regulation 17 of Monash University, this thesis is a combination of original papers published in peer reviewed journals and unpublished chapters. The ideas, development and writing up of all the papers in the main body of the thesis were the principle responsibility of myself, the candidate, working within the Department of Pharmacology under the supervision of Professor Wayne C Hodgson.

The inclusion of co-authors in published papers reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research and provision of venoms or access to specialised equipment.

Signature: 

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Date: 28.06.2021

THESIS INCLUDING PUBLISHED WORK DECLARATION

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 3 original papers published in peer reviewed journals. The core theme of the thesis is investigating the cardiovascular effects of animal venoms, in particular snakes and vampire bats. 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, Faculty of Medicine, Nursing and Health Sciences, Monash University under the supervision of Professor Wayne C. Hodgson, Professor Geoffrey K. Isbister, Associate Professor Barbara K. Kemp-Harper and Dr 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, and 4, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
2	An *in vivo* examination of the differences between rapid cardiovascular collapse and prolonged hypotension induced by snake venom	Published	80%: Study design, conducting laboratory experiments, analysis of data, preparation of figures and writing of the first version of the manuscript	Barbara Kemp-Harper (S) 5% Anjana Silva (C) 2.5% Sanjaya Kuruppu (S) 2.5% Geoffrey K. Isbister (S) 5% Wayne C. Hodgson (C,S) 5%	No No No No No
3	*D. russelii* venom mediates vasodilatation of resistance like arteries via activation of Kv and K_{Ca} channels	Published	70%: Study design, conducting laboratory experiments, analysis of data, preparation of figures and writing of the first version of the manuscript	Barbara K. Kemp-Harper (C,S) 10% Sanjaya Kuruppu (S) 5% Lachlan D. Rash (C) 5% Geoffrey K. Isbister (S) 5% Wayne C. Hodgson (C,S) 5%	No No No No No
4	Vampire venom: vasodilatory mechanisms of vampire bat	Published	70%: Study design, conducting laboratory experiments, analysis of data, preparation	Wayne C. Hodgson (S) 2.5% Ravina Ravi (L) 2.5%	No No

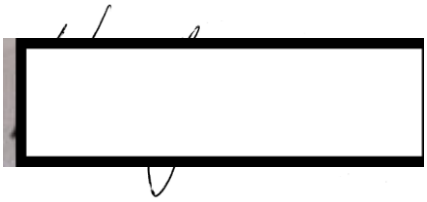
	(*Desmodus rotundus*) blood feeding		of figures and writing of the first version of the manuscript	Alejandro Alagon (L) 2.5% Richard J. Harris (L) 2.5% Andreas Brust (L) 2.5% Paul F. Alewood (L) 2.5% Barbara K.Kemp-Harper (C, S) 10% Bryan G. Fry (C) 5%	Yes No No No No

C, concept; S, supervision; L, laboratory work

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

Student signature:  **Date: 28.06.2021**

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:  **Date: 28.06.2021**

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LIST OF PUBLICATIONS INCLUDED AS PART OF THIS THESIS

1. **Kakumanu, R**.; Hodgson, W.; Ravi, R.; Alagon, A.; Harris, R.; Brust, A.; Alewood, P.; Kemp-Harper, B.; Fry, B. Vampire venom: vasodilatory mechanisms of vampire bat (*Desmodus rotundus*) blood feeding. *Toxins* **2019**, 11, 26-35.

2. **Kakumanu, R.;** Kuruppu, S.; Rash, L.D.; Isbister, G.K.; Hodgson, W.C.; Kemp-Harper, B.K. *D. russelii* venom mediates vasodilatation of resistance like arteries via activation of K_v and K_{Ca} channels. *Toxins* **2019**, 11, 197-206.

3. **Kakumanu, R.**, Kemp-Harper, B.K., Silva, A., Kuruppu, S., Isbister, G.K., Hodgson, W.C. An *in vivo* examination of the differences between rapid cardiovascular collapse and prolonged hypotension induced by snake venom. *Scientific Reports*. **2019**, 9, 1-9.

CONFERENCE PRESENTATIONS DURING PhD CANDIDATURE

Rahini Ragavan, Barbara K. Kemp-Harper, Geoffrey K. Isbister, Sanjaya Kuruppu and Wayne C. Hodgson. ***In vivo* and *in vitro* cardiovascular effects of Sri Lankan Russell's viper venom.** *APSA-ASCEPT Joint Scientific Meeting* 2015, Hobart, Australia – Poster presentation

Rahini Ragavan, Barbara K. Kemp-Harper, Geoffrey K. Isbister, Sanjaya Kuruppu and Wayne C. Hodgson. **Venom-induced cardiovascular collapse; are potassium channels the key to understanding the mechanism?** *The 12th Congress of the Pan-American Section of the International Society on Toxinology* 2016, Miami Beach, USA – Poster presentation (and selected Rapid Fire speaker)

Rahini Ragavan, Barbara Kemp-Harper, Anjana Silva, Geoffrey K Isbister, Sanjaya Kuruppu and Wayne C Hodgson. **Cardiovascular collapse induced by *Echis ocellatus* venom: an *in vivo* and *in vitro* examination.** *ASCEPT-MPGPCR Joint Scientific Meeting* 2016, Melbourne, Australia – Poster presentation

Rahini Ragavan, Barbara K. Kemp-Harper, Wayne C. Hodgson and Bryan G. Fry. **Investigating vasodilatory effects of *D. rotundus* venom.** *ASCEPT Cardiovascular Special Interest Group Student Symposia* 2017, Melbourne, Australia – Oral presentation

Rahini Ragavan, Barbara K. Kemp-Harper, Wayne C. Hodgson and Bryan G. Fry. **Bat venom mediates relaxation of rat small mesenteric arteries in part via activation of the CGRP8-37 receptor and Kv channels.** *19th World Congress of the International Society on Toxinology* 2017, Haikou, China – Oral presentation **(Awarded Best Student Oral Presentation award)**

Rahini Ragavan, Barbara K. Kemp-Harper, Geoffrey K. Isbister and Wayne C. Hodgson. **The *in vivo* and *in vitro* cardiovascular activity of Malayan pit viper venom.** *Venom to Drugs Meeting* 2017 Queensland, Australia – Poster presentation

Rahini Ragavan, Barbara Kemp-Harper, Anjana Silva, Geoffrey K Isbister, Sanjaya Kuruppu and Wayne C Hodgson. **Cardiovascular collapse induced by *Echis ocellatus* venom: an *in vivo* and *in vitro* examination.** *The 19th EU-IST Congress* 2018, Yerevan, Armania – Oral and poster presentation **(Awarded 2nd Best Outstanding Oral Presentation award)**

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LIST OF ABBRIVATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
AC	Adenylate cyclase
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
ADH	Anti-diuretic hormone
ANP	Atrial natriuretic peptides
BK_{Ca}	Large (Big) conductance calcium activated K^+ Channel
BP	Blood Pressure
BSA	Bovine Serum Albumin
BV	Bat venom
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin Gene-Related Peptide
CO	Cardiac Output
DMSO	Dimethylsulfoxide
e.g.	Exempli gratia (for example)
EC_{50}	Agonist concentration at 50% of maximal efficacy
$-logEC_{50}$	Agonist concentration at 50% of the maximal efficacy in molar
eNOS	Endothelial Nitric Oxide Synthase
et al.	Et alii (and other)
g	Grams
GFR	Glomerular filteration rate
H_20	Water

hCGRP	human Calcitonin Gene-Related Peptide
HPLC	High performance liquid chromatography
HR	Heart Rate
ICPP	Increasing capillary permeability protein
iKca	Intermediate conductance potassium channel
K^+	Potassium ions
K_{ATP}	ATP sensitive potassium channel
KCl	Potassium Chloride
K_{Ca}	Calcium activated potassium channel
K_{IR}	Inward rectifying Potassium channel
KPSS	Potassium Physiological Salt Solution
K_V	Voltage gated potassium channel
M	Molar
MAP	Mean Arterial Pressure
min	Minute(s)
N.D.	Not done
nM	Nanomolar
N.S. (n.s.)	Not significant
NA	Noradrenaline
NHMRC	National Health and Medical Research Council
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
ODQ	Guanylyl cyclase inhibitor
PBS	Phosphate buffered Saline
PGI2	Prostacyclin
PLA_2	Phospholipase A_2

RAAS	Renin-Angiotensin-Aldosterone System
rCGRP	rat Calcitonin Gene-Related Peptide
SEC-HPLC	Size exclusion high performance liquid chromatography
S.E.M	Standard Error of the Mean
sGC	Soluble Guanylate Cyclase
SK_{Ca}	Small conductance calcium activated potassium Channel
SNP	Sodium Nitroprusside
SV	Stroke Volume
TBS	Tris Buffered Saline
TPR	Total Peripheral Resistance
TRPV4	Transient receptor potential cation channel subfamily V member 4
vCGRP	venom Calcitonin Gene-Related Peptide
VEGF	Vascular endothelial growth factor
VICC	Venom-induced consumption coagulopathy
WHO	World Health Organisation

CHAPTER 1- INTRODUCTION

1.1 The role of venom

Venom is a complex mixture of proteins and peptides that are produced by highly specialised glands found in certain animal species (Casewell et al., 2013, Low et al., 2013). Venom is delivered via a wound and is generally used for three purposes – (i) as a defensive mechanism again predators, (ii) as an aid to capture and digest prey and/or (iii) as a tool to deter/challenge competitors (Fry et al., 2009). There are large numbers of terrestrial and marine animals that are classified as venomous, including different species of snakes, spiders, scorpions, sea snails, sea anemones, jellyfish, fish, cephalopods, centipedes, bats, insects, lizards, platypus and shrews (Fry et al., 2009). Similarly, the mode of venom delivery is quite diverse and includes the use of fangs or modified teeth, spines, sprays, harpoons, spurs, barbs, stingers and beaks (Fry et al., 2006, Fry et al., 2009).

Venoms consist of a highly complex cocktail of toxins, enzymes, proteins, salts, and organic molecules such as polyamines, amino acids and neurotransmitters (Casewell et al., 2013, Fox and Serrano, 2008, Fry, 2005, Fry et al., 2009, Inceoglu et al., 2003, Olivera and Teichert, 2007). Venoms have a wide range of biological activities which target all major physiological pathways and organs accessible by the bloodstream (Fry et al., 2009).

The primary focus of this thesis is on snake venoms. Ninety to ninety-five percent of snake venom consists of proteins and peptides (Mackessy, 2009). These components often possess enzymatic activity and ligand binding abilities that, in combination and/or separately, result in clinical envenoming in other organisms, including humans (Calvete et al., 2009, Casewell et al., 2013).

1.2 Introduction to snake families

Snakes originate from the class Reptilia, order Squamata, and are found in most countries across a wide variety of geographical and environmental habitats (Warrell, 1989). There are two types of snakes, venomous and non-venomous. Venomous snakes fall under the superfamily Colubroidea (advanced snakes) or Caenophidea, and can be categorised into four subfamilies: i.e. Viperidae, Elapidae, Colubridae and Atractaspididae (O'Shea, 2008).

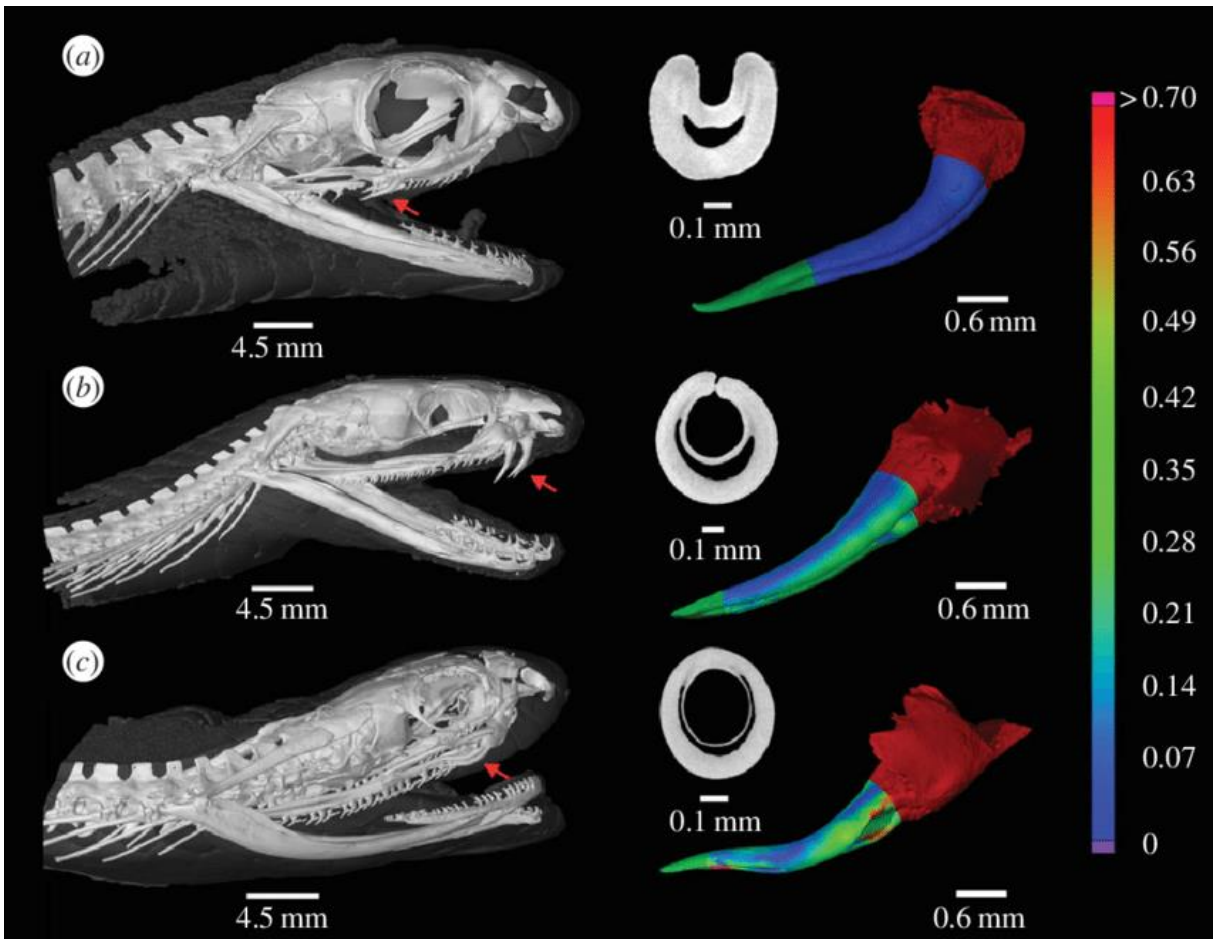

Figure 1.1: 3D mCT images showing the morphological differences in snake fang phenotypes. (a) Grooved phenotype typical of a Colubridae (*Dispholidus typus*) located at the posterior end of the maxilla; (b) non-fused phenotype of an Elapidae (*Naja nivea*) with the fangs found in an anterior fixed position in the maxilla; (c) closed, fused phenotype of a Viperidae (*Causus rhombeatus*) with fangs that are mobile in the anterior position in the maxilla. The red arrows indicate the position of the fang. The stress distributions of each fang phenotype are shown on the right with the gradient representing the maximum Von Mises stress (in GPa). A force was applied at the tip of the fang (shown in green) while the base of the fang (shown in red) is considered immobile (Broeckhoven and du Plessis, 2017).

1.2.1 Colubridae

The Colubrid family are the most dominant and diverse snake family, being found all over the world (except Antarctica), and consist of over 2,500 species (Gutiérrez et al., 2017). Colubrids are generally considered "harmless" to humans (O'Shea, 2008). This is due to their small fangs that are present at the end of the upper jaw (Figure 1.1a), making it difficult to effectively envenom humans or even their prey (O'Shea, 2008). There have been a few documented cases of human envenoming, however the clinical symptoms are much less severe than those following viperidae or elapidae envenoming

(Weinstein et al., 2013). Colubrids are characterized as venomous due to the presence of the Duvernoy's gland in some colubrid species which is homologous to a venom gland (Broeckhoven and du Plessis, 2017, O'Shea, 2008, Westeen et al., 2020). In Australia, only one venomous species of colubrid has been described, i.e. the brown tree snake (*Boiga irregularis*) (Pla et al., 2018) which is aggressive in nature but not fatal given an inability to deliver a sufficient amount of venom to envenomed humans (Mackessy, 2009). In comparison to research on elapidae and viperidae venoms, colubridae venom research is relatively sparse. However, studies do suggest the potential of this snake family to contain novel proteins and peptides that could be used in drug therapies (Mohamed Abd El-Aziz et al., 2019).

1.2.2 Elapidae

Elapidae is recognised as the most venomous and medically important family of snakes in the world, consisting of over 60 genera and 300 species (Fry et al., 2003, Keogh, 1998). Geographically, elapids are found in remote sub-tropical regions of Australia, Asia, Africa and the Americas, as well as in both terrestrial and aquatic regions (Ludington and Sanders, 2021, Martinez et al., 2021, Shine, 1995, Swindells and Schaer, 2018). Some of the common terrestrial genera in this family include tiger snakes (*Notechis* spp.), brown snakes (*Pseudonaja* spp.), taipans (*Oxyuranus* spp.), black snakes (*Pseudechis* spp.), land kraits (*Bungarus* spp.) and cobras (*Naja* spp.). Aquatic species include the yellow-bellied sea snake (*Hydrophis platurus.*), sea kraits (*Laticauda* spp.) and a semi-aquatic species of coral snake (*Micrurus surinamensis*).

Whilst all sea snakes produce live young, some terrestrial elapids produce live young while some lay eggs (Shine, 1977). Elapids are characterised by small, fixed fangs that are found angled at the front of the mouth and known as proteroglyphous (Figure 1.1b) (Cogger, 2014). Interestingly, some elapids (e.g. *Naja* spp.) have evolved their venom mechanism to spit or spray their venom though their fangs when attacking/defending (Kazandjian et al., 2021, Wüster et al., 2007).

1.2.3 Viperidae

Adders, Old World vipers and Pit-vipers are all included in the broader umbrella term 'Vipers'. There are 230 species of vipers, subdivided in 28 genera (Alencar et al., 2016, Mallow et al., 2003). Vipers are found all over the world including on non-polar land masses, except Australia and Antarctica (McDiarmid et al., 1999), and are the only family found on the furthest north (European Adder *Vipera berus*, 69°N) and furthest south (Patagonian Lancehead *Bothrops ammodytoides*, 47°S) regions of the equator. In Africa, the Puff Adder *(Bitis arietans)* is considered to be the deadliest snake as it is

responsible for the most human deaths due to envenoming (Rainer et al., 2010). Pit vipers arethe largest group of venomous snakes in the USA and cause over 99% of snake bites annually (Ruhaet al., 2017). In Asia, the Russell's viper is the most widely distributed species of the viperade family,and causes a high rate of mortality due to snake bite (Warrell, 1989).

Vipers are one of the largest vertebrate predators found in some terrestrial ecosystems. They are usually characterised as sedentary and big boned. However, arboreal (tree dwelling) vipers are slender (Wüster et al., 2008). As for elapids, vipers are characterised by their front-fangs, although vipers have larger fangs (figure 1.1c) that project in a hinge motion (Zahradnicek et al., 2008). Most vipers subdue, swallow and rapidly digest their prey, even at low temperatures (Cundall, 2002, Pough and Groves, 1983).

1.3 Snake bite

Globally, clinical manifestations caused by snake bite are an important health issue (Chippaux, 2008, Warrell, 2010). According to recorded reports of snake bites, it is estimated that 1.8-2.7 million envenomings, and 81,000– 138,000 deaths, occur annually around the world (Figure 1.2) (Gutiérrez et al., 2017). However, the extent of envenoming is likely to be substantially understated due to the inadequate reporting of snake envenoming, especially in many tropical and/or developing countries. The lack of reliable data has led to poor accessibility to treatment, and management, of snakebite. South Asia, South-East Asia, Sub-Saharan Africa and Latin America are reported to have the highest rate of envenoming and mortality, especially in rural farming communities (Kasturiratne et al., 2008). Snake bites occur so frequently in these areas that it has become an occupational and environmental health hazard (Williams et al., 2010) and considered a 'disease' in many under-developed, poverty-stricken countries (Harrison et al., 2009). In addition, many victims of snake bite in these communities use traditional treatments and remedies based on those used by their forefathers and are less likely to seek medical assistance (Fox et al., 2006). These factors contribute to the under representation of hospital statistics on the severity of snake bite as a globally important health burden. Thus, the World Health Organisation has recognised snake bite as a Neglected Tropical Disease and is establishing support and research for better treatment for victims (Chippaux, 2017, Williams et al., 2019a).

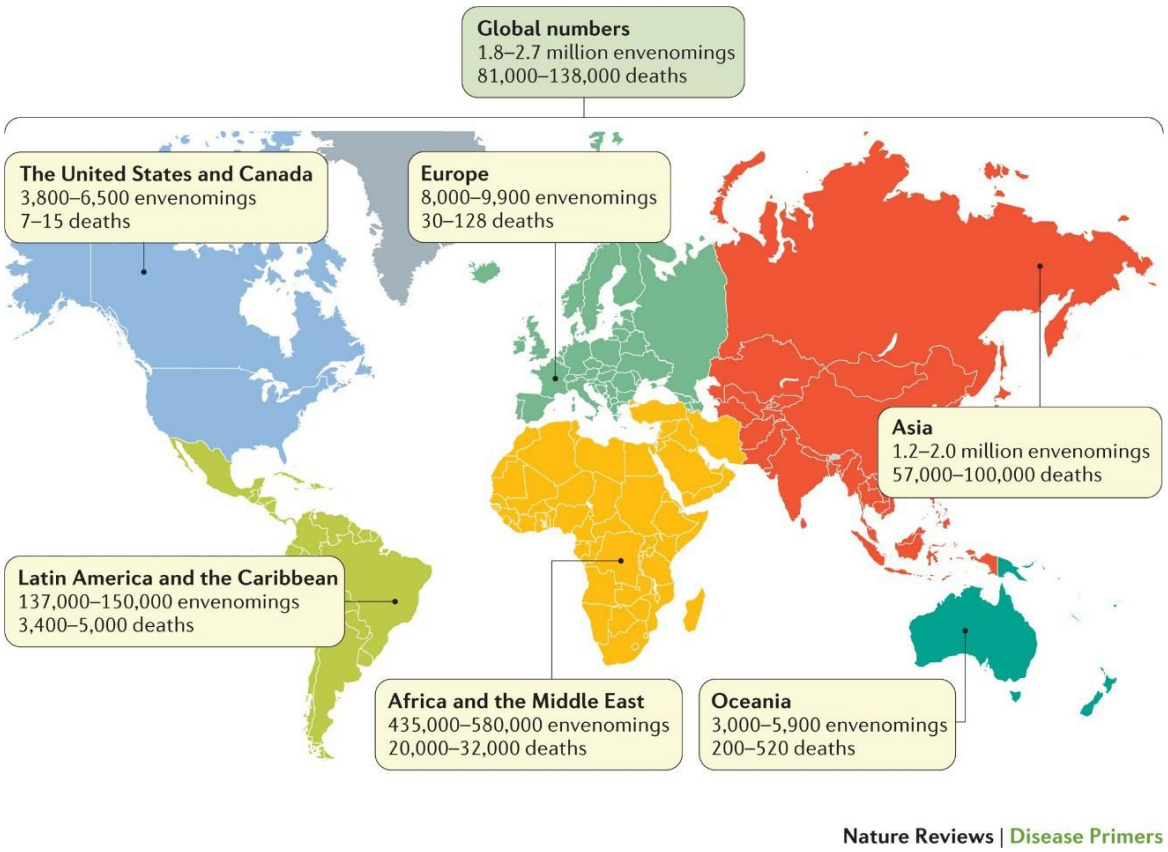

Figure 1.2: Global map showing the annual snake bite envenomings and mortalities (Gutiérrez et al., 2017).

1.4 Snake venom

The primary role of snake venom is to capture/paralyse/digest prey, as well as act as a defence mechanism against predators (Casewell et al., 2013). Snake venom is produced by venom glands located in the head of the snake. Different species have developed effective and sophisticated delivery systems to inject venom from the glands into the prey (Jackson, 2003). While the evolution of venom apparatus is believed to have originated 200 million years ago from a single ancestor, the front-fanged venom systems present in elapids, viperids and atractaspidids evolved separately at different time points (Fry et al., 2006, Jackson, 2003). This was mainly due to variations in diet (Daltry et al., 1996), resulting in different venom components being present not only in different species but also within the same species. Factors such as geographical distribution, sex and ontogeny also played a role in venom diversification (Andrade and Abe, 1999, Suzuki et al., 2010). Venom proteins vary structurally and functionally, and can be classified in numerous ways. High molecular weight proteins have a range of enzymatic activity and bind to an array of substrates in target sites that play a role in major physiological systems (Casewell et al., 2013). This includes the coagulation cascade, neuromuscular junction, skeletal and cardiac muscles, vascular endothelium, red cell membrane and extracellular space (Chippaux, 2006). Another enzymatic group of snake venom components, called

metalloproteins, rely on metal ions such as calcium or zinc for their activity. Many venom components such as phospholipases are toxic due to their enzymatic activity (Kini, 2003). Phospholipase A_2 (PLA_2) toxins are found in all snake venoms and display an array of properties such as neurotoxicity, myotoxicity, cardiotoxicity, anti-coagulation, haemolytic, hypotensive and local tissue necrotic activity (Gutiérrez and Lomonte, 2013b, Kini, 2003). Metalloproteinases and thrombin-like serine proteinases found in snake venom cause coagulopathy, fibrinolysis and haemorrhage, and are present in high concentrations in crotaline venoms (Chippaux, 2006, Markland, 1998).

Snake venoms also contain high amounts of low molecular weight polypeptide toxins. These toxins have no enzymatic properties but show high affinity to a wide range of receptors, ion channels and plasma proteins (McCleary and Kini, 2013, Fry et al., 2009). Once bound, these toxins cause an array of toxic effects that, in combination, lead to the prey becoming immobilized (McCleary and Kini, 2013). Examples of these toxin families include three finger toxins, bradykinin-potentiating peptides, proteinase inhibitors, vascular endothelial growth factors and C-type lectins. Toxins within each family share similar primary, secondary and tertiary structures, yet possess vastly different pharmacological properties (Casewell et al., 2013, McCleary and Kini, 2013, Vonk et al., 2011).

1.4.1 Phospholipases

Venom from all snake families contain phospholipase enzymes (Kudo and Murakami, 2002). The four main classes of phospholipases (PL) are A1, A2, C and D. These classes, which are further subdivided, are identified by the site at which they hydrolyse ester bonds of 3-sn-phosphoglycerides (Fry, 1999). Toxic PLA_{2S} found in snake venom catalyse the Ca^{2+}-dependant hydrolysis of acyl-ester at the sn-2 position of glycerophospholipids. There are two main groups of PLA_2.

1) PLA_{2S} present in Viperidae venom. This is similar to non-pancreatic inflammatory secretory PLA_2s (Lambeau and Lazdunski, 1999) and,
2) PLA_{2S} found in Elapidae venom. These PLAs are similar to PLA_2s secreted by the pancreas.

PLA_2s are further subdivided into five groups according to their effects on the body; i.e. myotoxic, neurotoxic, haemotoxic, non-toxic but enzymatically active and non-enzymatically active (Fry, 1999).

1.4.2 Myotoxins

Myotoxins cause direct cytotoxicity to skeletal muscles. Types of myotoxins include:

1) Low molecular mass myotoxins that act on sodium channels (Mebs and Ownby, 1990);

2) Membrane-active cardiotoxins (Duchen et al., 1974);
3) Toxic phospholipase A_2 (PLA_2s) (Gutiérrez and Ownby, 2003). These toxins cause the most damage to muscle.

Myotoxin-a and crotamine are low molecular mass myotoxins isolated from *Crotalus viridis viridis* and *Crotalus durissus* venoms. These toxins bind specifically to sodium channels and causestructural damage to skeletal muscles as well as myonecrosis (Cameron and Tu, 1978, Williams et al., 2019b). Cardiotoxins present in cobra venoms destroy plasma membrane of skeletal muscle cells via depolarization and degradation (Shiau et al., 1976). They are also known to cause cardiac arrhythmia (Virmani, 2002).

PLA_2 myotoxins, which are the largest group of myotoxins, act directly on muscle and cause muscles to degenerate, resulting in initial muscle pain and weakness (Currie, 2000, Montecucco et al., 2008). In the final stages of myotoxicity, clinical manifestations result in either local necrosis, as seen in viper species envenoming, or systemic myotoxicity, present in elapid species envenoming (Gutiérrez and Ownby, 2003).

There are two forms of myotoxic PLA_2s, i.e. Lys-49 and Asp-49. Lys-49 PLA_2s have no catalytic activity due to a lysine substitution for aspartate at position 49 of the amino acid sequence (Gutiérrez and Lomonte, 1995, Lomonte et al., 2009, Rufini et al., 1992). Asp49 PLA_2s cause hydrolysis of cell membranes due to high levels of enzymatic activity (Mora-Obando et al., 2014). Myoglobinuria is a likely result of myotoxicity which is clinically important as it is often linked to renal failure (Ponraj and Gopalakrishnakone, 1997).

1.4.3 Neurotoxins

Neurotoxins can be divided into two major classes; i.e. pre-synaptic and post-synaptic. Pre-synaptic toxins act on the pre-synaptic terminal of the neuromuscular junction and are often referred to as β-neurotoxins. β-Neurotoxins are prevalent in the venoms of Viperidae, Elapidae and Crotalidae (Barber et al., 2013b, Chang, 1985). Similar to myotoxins, pre-synaptic neurotoxins exhibit PLA_2 activity (Kini, 1997, Madhushani et al., 2020). Pre-synaptic neurotoxins interfere with neuromuscular transmission in a tri-phasic manner which starts with a weak inhibition of acetylcholine (ACh) release, followed by a prolonged phase of facilitated release and, finally, a progressive reduction of neurotransmission (Rigoni et al., 2004, Su and Chang, 1984b). Rigoni et al. (2004) have postulated that the toxins cause bulging of the pre-synaptic terminal which then leads to disruptions in distribution of actin and neurofilaments in neurons. They also suggested that membrane defects may

occur due to phospholipids being hydrolysed which leads to excess Ca^{2+} flowing into the presynaptic terminal and causing ACh release (Rigoni et al., 2004, Tonello and Rigoni, 2017). It has also been suggested that pre-synaptic toxins may lead to structural damage and depletion of synaptic vesicles (Prasarnpun et al., 2004).

Post-synaptic neurotoxins (also known as α-neurotoxins) are low molecular weight peptides that block postsynaptic nicotinic acetylcholine receptors (nAChR). α-Neurotoxins are present primarily in elapid venoms although some viper venoms also contain these toxins. α-Neurotoxins are similar in activity to d-tubocurarine, which also binds to nAChR, and thus are often referred to as "curaremimetics" (Lee, 1970) or "three-finger toxins" (Nirthanan and Gwee, 2004). There are two typesof post-synaptic neurotoxins; i.e. short-chain and long-chain. Short-chain neurotoxins consist of 60- 62 amino acids and contain four conserved disulphide bonds (Endo and Tamiya, 1991, Barber et al.,2013b). Toxin-α and erabutoxin-b are short-chain neurotoxins that have been used extensively to study the neuromuscular junction (Tsetlin and Hucho, 2004). Receptor/toxin studies have demonstrated that amino acids at positions 7, 8, 10, 27, 29, 31, 33, 38 and 47 of the toxin play a key role in the binding of the neurotoxin to nAChR (Antil et al., 1999, Mordvintsev et al., 2005, Trémeau et al., 1995). Long-chain neurotoxins consist of 66-75 amino acids and contain five disulphide bonds, with the extra disulphide bond being situated on the second loop of the toxin (Endo and Tamiya, 1991). Amino acids at positions 23, 25, 27, 29, 33, 35, 36, 49 and 65 are important in the binding of the toxins to the nAChR (Nirthanan and Gwee, 2004). However, short-chain neurotoxins bind 6-7 times faster with the receptor and dissociate 5-9 times faster than long-chain neurotoxins (Nirthanan & Gwee, 2004).

1.4.4 Coagulating Agents

Many snake venoms contain toxins that affect the coagulation cascade. This includes pro-coagulants, anti-coagulants, platelet active compounds, thrombotic effects and fibrinogen clotting toxins (Maduwage and Isbister, 2014, Isbister, 2009). Coagulopathy is defined by abnormalities present in the coagulation cascade, and is caused by all venomous snake families to some extent (Maduwage and Isbister, 2014). This leads to the inability of blood to clot properly in snake bite victims, which can lead to serious haemorrhaging (Isbister, 2010). Most viperid and Australasian elapid venoms contain potent pro-coagulant toxins that activate the clotting cascade by binding to different target sites (Isbister, 2010, Maduwage and Isbister, 2014). There are different types of pro-coagulant toxins. Thrombin-like enzymes cleave fibrinogen and cause mild coagulopathy. Other toxins cause severe coagulopathy by activating clotting factors that are present higher up in the clotting cascade and this is evident in Russell's viper venom (Maduwage and Isbister, 2014). This leads to clotting factors

being used up (i.e. ‘consumed’) at a higher rate than normal, which can result in small blood clots forming and then extensive bleeding from bite site and body orifices (Jacob, 2006). In some cases, no bleeding has been reported externally however severe gastro-intestinal and intra-cranial bleeding has been reported (Jacob, 2006). Currently research is directed at the effectiveness of anti-venom therapy and fresh frozen plasma (Holla et al., 2018, Isbister et al., 2013, Rathnayaka et al., 2020).

1.5 Clinical effects of snake envenoming

Not every bite from a venomous snake results in envenoming. In order for successful envenoming to occur, the venom needs to be delivered in sufficient amounts into the body of the prey/victim. The degree of envenoming is dependent on an array of factors including volume and concentration of venom injected, venom composition, and body mass and health of prey/victim (Chippaux, 2006, Mackessy, 2009). In the case of humans, the time and type of medical intervention (such as first aid, and anti-venom) plays a crucial role in the clinical manifestations and outcome of envenoming. Thus, this can lead to humans displaying different degrees of clinical outcomes, ranging from non-envenoming to severe envenoming, the latter leading to death soon after being bitten. Clinical effects of envenoming can be categorized into “local effects” and “systemic effects” (Figure 1.3). Depending on the combination of toxins present in the venom and degree of envenoming, snake bite in humans can result in all or some of the following clinical symptoms: local tissue necrosis, neurotoxicity, coagulopathy, haemorrhage, kidney damage, myotoxicity and cardiovascular damage (Chippaux, 2006).

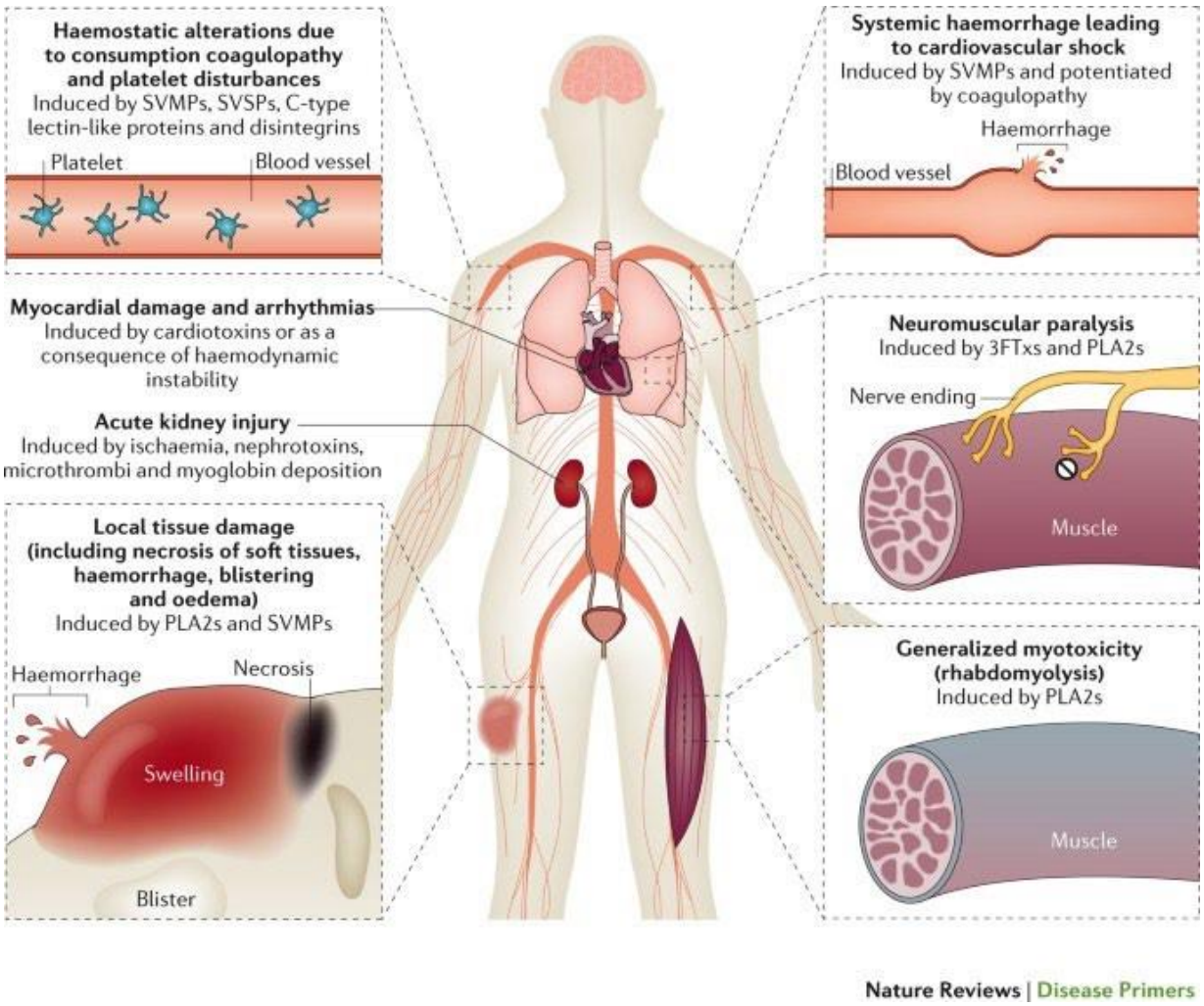

Figure 1.3: Local and systemic effects of snake toxins on different body systems (Gutiérrez et al., 2017).

1.5.1 Local effects of envenoming

The 'local' effects of envenoming refer to direct tissue damage caused by the venom at, or around, the bite site (Figure 1.3). This is quite a common outcome for viper and cobra bite victims, and can range from inflammation and minor tissue damage to severe damage requiring surgical intervention such as amputation of limbs. However, local envenoming usually results in the following characteristics during the acute stage: fang marks, pain, bleeding and bruising, haemorrhagic blistering, local tissue necrosis, regional lymphadenopathy and gangrene (Chippaux, 2006, Kularatne et al., 2009). Following some pit viper bites, local envenoming leads to increased pressure in the affected area requiring decompression via surgery. Failure to respond in a timely manner could result in amputation (Bucaretchi et al., 2010). The timely clinical management of the effects of local envenoming is still a challenge due to factors such as the time it takes for patients to reach hospital and the type of snake bite (Rojnuckarin et al., 2006, Sellahewa et al., 1995). Hence many victims of snake bite end up with varying degrees of disability due to local envenoming (Gutiérrez et al., 2006).

1.5.2 Systemic envenoming

Systemic envenoming refers to venom being distributed via the lymphatic system and blood vessels throughout the body (Warrell, 2010). This can lead to multiple organ damage, organ failure and death (Figure 1.3).

Neurotoxicity

Neurotoxicity is a common sign of envenoming by many snake species, including Elapidae such as cobras, kraits, mambas, taipans, coral snakes, death adders, tiger snakes and sea snakes, as well as some Viperidae such as pit vipers (Chippaux, 2006, Mackessy, 2009, Ranawaka et al., 2013). Neurotoxicity is due to snake venom toxins (see 1.4.3) affecting the nervous system, in particular, inhibiting transmission at the skeletal neuromuscular junction, leading to flaccid paralysis. This paralysis can present as mild weakness of eyelids to total paralysis of the body, which can lead to respiratory arrest and death. Paralysis begins from the eyes, descends to the lower extremities and is dependent on the type of snake and amount of venom delivered (Faiz et al., 2010, Isbister et al., 2012).

Kidney damage

Acute kidney damage can occur following snake envenoming due to toxins present in some viperid and elapid venoms (Sitprija and Sitprija, 2012). The pathogenesis of renal damage includes decreased renal blood flow that can occur due to haemodynamic alterations caused by systemic bleeding and vascular leakage. SVMPs can cause proteolytic degradation of the glomerular base membrane while PLA_2 toxins cause haemolysis, cytotoxicity in renal tubular cells and thrombotic microangiopathy (Pinho et al., 2005, Sitprija and Sitprija, 2012).

Rhabdomyolysis can also occur due to envenoming by sea snakes, some viperid species and some Australian terrestrial elapids (Gutiérrez et al., 2017, Warrell, 2010). This involves the action of myotoxic PLA_2s binding to receptors on muscle fibres. Myotoxins disrupt and damage the integrity of the plasma membrane of muscle cells which leads to an influx of calcium and cellular degeneration (Gutiérrez and Ownby, 2003). This leads to large amounts of creatine kinase and myoglobin being released. Deposition of myoglobin within the renal tubules can also contribute to acute kidney disease (Sitprija and Sitprija, 2012).

Cardiovascular and haemostatic disturbances

Cardiovascular effects of snake envenoming include haemorrhage, coagulopathy, decreased platelet numbers, cardiovascular shock and collapse (Frangieh et al., 2021). While numerous studies have been conducted on neurotoxicity and other systemic effects of snake envenoming, there is a lack of understanding and clarity of the mechanisms and toxins involved in the cardiovascular effects. Thus,

the focus of this thesis is an investigation of the cardiovascular effects of snake venom – in particular hypotensive components. The following sections include an overview of the hypotensive components of snake venom as well as an overview of the eight snakes that have been chosen for investigation.

1.6 Hypotensive components of snake venom

As mentioned earlier, snake venom contains an array of different proteins and peptides that work together to cause rapid immobilization of prey. Hypotensive agents are present in many snake venoms and support neurotoxins in paralysing prey by causing circulatory shock. These agents may also aid in the diffusion of other snake venom components (Joseph et al., 2004, Mackessy, 2009). There are five main classes of hypotensive components present in snake venoms: i.e. kininogens, natriuretic peptide-like components, phospholipases, serine-proteases, endogenous vascular endothelial growth factors and other hypotensive agents (Péterfi et al., 2019).

Kininogens (such as bradykinin potentiating peptides) are the largest class of hypotensive peptides. These peptides (which were first extracted from *Bothrops jararaca* venom) inhibit angiotensin-converting enzyme (ACE) and cause an increase in the hypotensive effects of bradykinin (Ferreira, 1965). ACE promotes the degradation of bradykinin which is a vasodilator (Camargo et al., 2012), so inhibiting ACE leads to increased vasodilation. Furthermore, bradykinin potentiating peptides induce gamma-aminobutyric acid (GABA) and glutamate release within the central nervous system that results in hypotension (Guerreiro et al., 2009, Munawar et al., 2018).

Natriuretic peptides (NPs) are vasoactive hormones that act via two major mechanisms: vasodilation and renal blood flow regulation. This includes the inhibition of the renin-angiotensin-aldosterone system, diuresis, natriuresis, vascular permeability regulation and increase of venous capacity (Koh and Kini, 2012, Suzuki et al., 2001, Wong et al., 2017). NPs have been isolated from numerous snake venoms including *Dendroaspis angusticeps* (Lisy et al., 1999), *Crotalus durissus cascavella* (Evangelista et al., 2008), *Pseufonaja textilis* (St Pierre et al., 2006), *Bungarus flaviceps* (Siang et al., 2010, Sridharan and Kini, 2015), *Bungarus multicinctus* (Jiang et al., 2011) and *Lachesis muta* (Soares et al., 2005). These peptides decrease heart rate, induce relaxation of blood vessels and decrease arterial pressure (Evangelista et al., 2008, Péterfi et al., 2019).

There are 4 major types of **PLA_2s** (see 1.4.1) – cytosolic PLA_2s, Ca^{2+} independent PLA_2s, secreted PLA_2s and platelet-activating factor acetylhydrolases (Burke and Dennis, 2009). Snake venom PLA_2s belong to the secreted PLA_2 family which cause neurotoxicity, cardiotoxicity, inhibition of blood coagulation and interference of platelet function (Chaisakul et al., 2014, Mackessy, 2009). These

proteins are able to decrease blood pressure via the production of arachidonic acid and interaction with platelets and leukocytes (Leiguez et al., 2018, Mackessy, 2009). PLA_2s are present in colubrid, elapid and viperide venoms, and are one of the major toxic components that are responsible for an array of pharmacological effects (Xiao et al., 2017).

Snake venom serine-proteases (SVSP) are trypsin-like enzymes that cause an imbalance in the haemostatic system by disrupting the coagulation cascade, the fibrinolytic and kallikrein-kinin systems and imitating the effects of thrombin (Serrano and Maroun, 2005). Some SVSPs activate plasminogen or coagulation factor XIII while others lower blood pressure by releasing bradykinin from kininogen (Hung and Chiou, 2001). SVSPs extracted from *Trimeresurus muscrosquamatus* venom reduce blood pressure by degrading angiotension I and angiotension II (Hung and Chiou, 2001).

Vascular endothelial growth factors (VEGFs) regulate the formation and permeability of blood vessels via their interaction with kinase-linked receptors in order to maintain homeostasis (Péterfi et al., 2019). They activate endothelium-dependent vasorelaxation via the release of nitric oxide and prostaglandins (Yang et al., 2002). VEGF components present in snake venom act similar to that of human VEGF in order to regulate vascular permeability, angiogenesis and reduce blood pressure (Kaji et al., 2006, Liu et al., 2001).

Other hypotensive components present in snake venom include heparin binding factors which promote anticoagulation, hypotension and vasorelaxation (Hirsh et al., 2001, Paredes-Gamero et al., 2010), three-finger toxins which have an array of biological functions and can cause hypotension by binding to L-type calcium channels (LTCC) and interacting with adrenergic and muscuranic receptors (Ferraz et al., 2019, Kini and Doley, 2010), and 5' nucleotidases which interact with factor IX of the blood coagulation cascade and inhibit platelet aggregation (Dhananjaya et al., 2009).

1.7 Focus of this study

While there are many hypotensive agents present in snake venoms, the mechanisms and contribution to major clinical effects such as cardiovascular collapse are still unclear. Thus, the main aim of this study was to identify and characterize the cardiovascular effects of eight different snake venoms. The following snakes were chosen as they have been reported to cause cardiovascular effects such as collapse and/or hypotension in envenomed patients – *Daboia russelii* (Sri Lankan Russell's viper), *Daboia siamensis* (Javanese Russell's viper), *Pseudonaja textilis* (Brown snake), *Bitis arientans* (Puff adder) and *Echis ocellatus* (Carpet viper). *Crotalus vegrandis* (Uracoan rattle snake) was chosen as

this species is known to cause severe haemorrhage in envenomed animals however clinical data is very limited. *Bitis gabonica* (Gaboon viper) was chosen as envenoming in humans is quite rare and thus clinical cardiovascular effects are not fully known. *Oxyuranus temporalis* (Western desert taipan) was chosen as it is a new species of taipan found in Western Australia. There have been no human envenoming and thus the cardiovascular effects of this venom are unknown. Below is an overview of these eight snakes.

1.7.1 Gaboon viper (*Bitis gabonica*)

(a)

(b)

Figure 1.4: (a) *B. gabonica* (Photograph by David Williams) and (b) geographical distribution. Red shading refers to areas of highest rate of mortality and morbidity while orange shading refers to areas where the snake is found but clinical data is lacking. Source: WHO 2010 Database (https://apps.who.int/bloodproducts/snakeantivenoms/database - viewed on 03/06/2021).

Bitis gabonica, or more commonly known as the Gaboon viper, is a member of the *Bitis* genus which consists of twelve species (Marsh and Whaler, 1984). *B. gabonica* is the largest viper species and is found predominantly across equatorial Africa (Figure 1.4b) (Marsh and Whaler, 1984, Marsh et al., 2007). It lives in rainforests and woodlands that are mainly at low altitudes but have also been seen in higher altitudes of 1,500-2,100 m (Spawls, 2002). It is also sometimes found in open country. There have been reports of Gaboon vipers being spotted commonly in coffee plantations in East Africa (Cansdale, 1948), cacao plantations in West Africa (Ionides and Pitman, 1965) and cashew plantations in Tanzania, making them a threat to agricultural farmers (Mallow et al., 2003). They are also commonly found beside running or still water (Marsh and Whaler, 1984).

B. gabonica is the heaviest (average 5.25 Kg) of all venomous snakes in Africa and can grow to 2 m in length (Marsh and Whaler, 1984). It contains the longest fangs (4 cm), produces the highest quantity of venom (> 2 g in dry weight) of all venomous snakes, and it can eject up to 10 ml of venom during one strike (Marsh et al., 2007). Its body is covered in rectangle and triangle patterns of dark brown, light brown and black (Figure 1.4a). This pattern provides excellent camouflage among the forest floors, which is quite advantageous when capturing prey such as rodents and ground dwelling birds, as this snake is usually very docile and sluggish in behaviour (Marsh and Whaler, 1984). *B. gabonica* catch prey via camouflage and ambush instead of actively hunting prey. Like most other viperad species, *B. gabonica* hold onto their prey with their large fangs until the prey dies instead of releasing it and waiting for it to die. When threatened, these snakes are known to hiss but are reluctant to attack unless hurt (Marsh and Whaler, 1984).

Bites from *B. gabonica* are quite rare in the wild, with the majority of reported bites having occurred during handling of captive species (Marsh et al., 1997). Envenoming usually leads to unstable circulation, excessive bleeding, tissue damage and necrosis (Marsh et al., 1997). SAIMR (South African Institute for Medical Research) polyvalent antivenom is available for patients.

The venom has been reported to contain arginine esterases (Viljoen et al., 1979), PLA_2 components (Botes and Viljoen, 1974), thrombin-like enzymes (Pirkle et al., 1986), anti–platelet (Huang et al., 1992) and metalloproteinase (Marsh et al., 1997) activities.

1.7.2 Puff adder (*Bitis arietans*)

(a)

(b)

Figure 1.5: (a) *B. arietans* (Photograph courtesy of Venom Supplies, Tanunda, South Australia) and (b) geographical distribution. Red shading refers to areas of highest rate of mortality and morbidity while orange shading refers to areas where the snake is found but clinical data is lacking. Source: WHO 2010 Database (https://apps.who.int/bloodproducts/snakeantivenoms/database – viewed on 03/06/2021).

Bitis arietans, or Puff adder, is a member of the viper family found throughout the Middle East of sub-Sahara, Africa and parts of the Arabian Peninsula (Figure 1.5) (Barlow et al., 2013, Fernandez et al., 2014). In Africa *B. arietans* is the most common and widespread venomous snake species

(Serrano et al., 2005) and even though the number of bites due to this species is unknown, it is estimated that a large portion of the 43,000 deaths attributed to snake bite in Africa each year are due to *B. arietans* envenoming (Kasturiratne et al., 2008). Therefore, *B. arietans* are a serious public health concern in Africa. *B. arietans* are usually found in all habitats except rainforests, true deserts and tropical alpine areas (McDiarmid et al., 1999). They are usually sluggish in behaviour and rely on camouflage for both protection and hunting.

B. arietans venom is considered one of the most toxic and highly potent of any viper venom (Segura et al., 2010). Envenoming results in both local and systemic effects in the body (Laing et al., 2003, Kasturiratne et al., 2008, Warrell et al., 1975). These include swelling, blistering, arterial thrombosis, necrosis, cardiovascular effects (hypotension and bradycardia), spontaneous bleeding and thrombocytopenia (Warrell et al., 1975). The venom contains hemorrhagins that prevent platelet function and interfere with plasmatic coagulation (Rainer et al., 2010, Dennis et al., 1990). Toxins in the venom also cause increased vascular permeability which can lead to hypovolemic shock and death (Schaeffer et al., 1985). Cardiac arrhythmias observed in patients suggests that cardiotoxins could also be present in the venom (Maita et al., 2003, Omori-Satoh et al., 1995, Schaeffer et al., 1985). Polyvalent anti-venom produced in South Africa (Segura et al., 2010) is available for bitten victims. However, lack of accessibility to hospitals and envenomed patients often choosing traditional medicines leads to poor life quality and disabilities due to necrosis (Lavonas et al., 2002).

1.7.3 Carpet viper (*Echis ocellatus*)

(a)

(b)

Figure 1.6: (a) *E. ocellatus* (Photograph by David Williams) and (b) geographical distribution. Red shading refers to areas of highest rate of mortality and morbidity while orangeshading refers to areas where the snake is found but clinical data is lacking. Source: WHO 2010Database (https://apps.who.int/bloodproducts/snakeantivenoms/database - viewed on 03/06/2021).

There are 12 different species and 20 different subspecies of *Echis* found around the world (Cherlin, 1990). *Echis* spp. are part of the Viperidae family. These species have been further divided into four main classes: *E. carinatus*, *E. coloratus*, *E. ocellatus* and *E. pyramidum* (Pook et al., 2009). *E. ocellatus*, more commonly known as carpet vipers, are found in Nigeria (Figure 1.6b) (Habib, 2015) and are one of the most medically important species of venomous snakes in the savannahs of western sub-Saharan Africa (Calvete et al., 2016). *E. ocellatus* is responsible for more deaths than any other genus of snakes due to snakebite and causes thousands of deaths and permanent disability annually (Stock et al., 2007, Chippaux, 2002, Organization, 2010). It has been reported that 497 per 100,000 population are bitten by snakes in Nigeria annually, with *E. ocellatus* envenoming causing at least 66% of mortality in certain areas (Habib, 2015, Wagstaff et al., 2009).

The average size of *E. ocellatus* is 30-50 cm and the body is covered in distinctive spots termed “eye spots” that are present from head to tail (Figure 1.6a) (St and Branch, 1995). *E. ocellatus* is quite aggressive in nature. The snake forms its body into a “S” shape and rubs itself to make sound with its scales, alerting predators and prey of its presence. *E. ocellatus* hunt during the early hours of night and usually feed on small vertebrates such as mammals, birds, lizards and amphibians (Pook et al., 2009).

E. ocellatus envenoming causes an array of clinical symptoms such as localised edema, haemorrhage and necrosis at the wound, systemic haemorrhage, intravascular coagulation and fibrinolysis (Warrell et al., 1977). The venom contains a prothrombin-activating procoagulant, hemorrhagin and cytolytic fractions that cause haemorrhage, shock, necrosis and incoaguable blood (Habib, 2015). It has been reported that cardiotoxicity and renal failure may also occur following envenoming (Habib, 2015).

1.7.4 Uracoan rattle snake (*Crotalus vegrandis*)

(a)

(b)

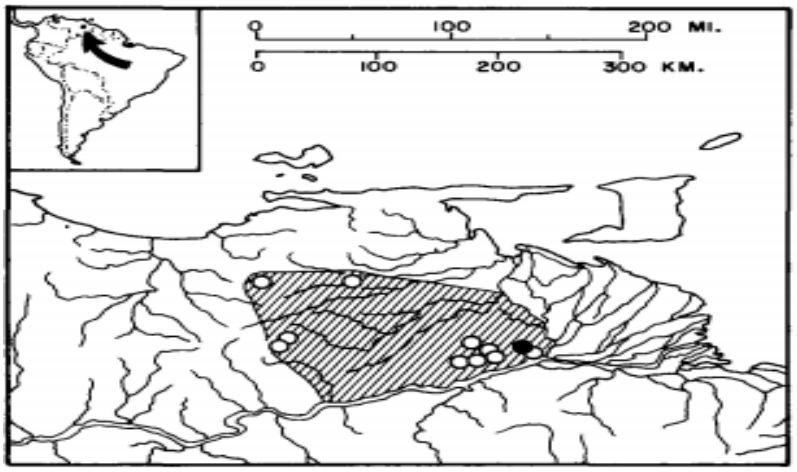

Figure 1.7: (a) *C. vegrandis* (Photograph courtesy of Venom Supplies, Tanunda, South Australia) and (b) geographical distribution. Shaded area represents estimated range and circles represent typical localities (McCranie, 1984). Source: The Reptile Database (https://reptile-database.reptarium.cz/species?genus=Crotalus&species=vegrandis- viewed on 03/06/2021)

Crotalus vegrandis, or more commonly known as the Uracoan rattlesnake, is a member of the viperid family found in Venezuela (Figure 1.7b), mainly in the states of Anzoategui and Monagas (Klauber, 1941, Pifano and Rodriguez-Acosta, 1996). While some researchers consider it to be a subspecies of *Crotalus durissus* (Shelton, 1981), others believe it is a distinct species (Harris Jr and Simmons, 1978, Hoge, 1965).

The length of an adult specimen is usually between 76-107 cm and its body colour can range from grey to reddish-brown and in some cases olive green (Figure 1.7a) (McCranie, 1984, Strimple, 1987). The dorsal scales are usually tipped white, giving an overall speckled appearance. It is also morphologically characterised by the presence of a sharp vertebral ridge, similar to that of *C. durissus* (Strimple, 1987). *C. vegrandis* feeds on rodents, small mammals and lizards (Chiszar and Radcliffe, 1976, Chiszar et al., 1977). Usually the primary defence mechanism of rattlesnakes is to hide and rattle their tail to scare away predators, however when challenged they will bite and envenom. Humans are usually envenomed if they accidently step on the snake or come too close to their habitat (Chiszar et al., 1993).

There have been a few studies on *C. vegrandis* venom; however, due to the snakes being found in a small restricted area in the savannahs of Venezuela, little is known about the clinical effects of envenoming (Viala et al., 2015). Between 1980 – 2000, there were 20 deaths recorded due to snake bite in the Monagas state (De Sousa et al., 2005). It is unknown how many are due to *C. vegrandis* bites; however, at the Manuel Nuñez Tovar Hospital in 2004 it was recorded that 68% of snake bite incidents were due to *C. vegrandis* (Caraballo et al., 2004).

C. vegrandis venom causes severe haemorrhage and local tissue damage, neurotoxicity and respiratory difficulties in envenomed animals (Rodriguez-Acosta et al., 1998, Scannone et al., 1978). The venom contains the neurotoxin crotoxin that acts pre-synaptically on muscular junctions blocking the signal transduction (Degn et al., 1991, Kaiser and Aird, 1987, Slotta and Primosigh, 1951, Viala et al., 2015). It also contains snake venom metalloproteases, such as Uracoine-1, that induce haemorrhage (Aguilar et al., 2001). Myotoxicity has also been reported, indicating the presence of crotamine-like toxins (Bober et al., 1988, Pulido-Mendez et al., 1999).

1.7.5 Sri Lankan Russell's viper (*Daboia russelii*) and Javanese Russell's viper (*Daboia siamensis*)

(a)

(b)

Figure 1.8: (a) *D. russelii* (Photograph by Mark O'Shea) and (b) geographical distribution. Red shading refers to areas of highest rate of mortality and morbidity while orange shading refers to areas where the snake is found but clinical data is lacking. Source: WHO 2010 Database (https://apps.who.int/bloodproducts/snakeantivenoms/database - viewed on 03/06/2021).

(a)

(b)

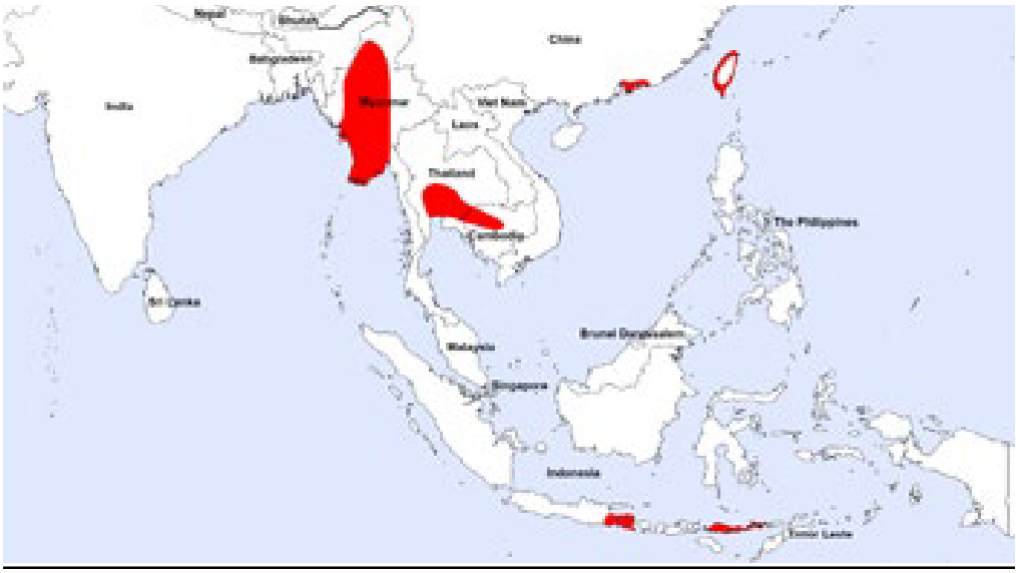

Figure 1.9: (a) *D. siamensis* (Photograph by Mark O'Shea) and (b) geographical distribution. Red shading refers to areas of highest rate of mortality and morbidity while orange shading refers to areas where the snake is found but clinical data is lacking. Source: WHO 2010 Database (https://apps.who.int/bloodproducts/snakeantivenoms/database - viewed on 03/06/2021).

Historically, viper species were divided into five subspecies based on minor differences in appearance of colour and markings. These five subspecies are distributed in ten south-Asian countries including India (*Vipera russelii russelli*), Sri Lanka (*Vipera russelii pulchella*), Indonesia (*Vipera russelii limitis*), Taiwan (*Vipera russelii formosensis*), Thailand, Burma, Bangladesh and Pakistan (*Vipera*

russelii siamensis) (Warrell, 1989). However, in 2009, a new taxonomy was proposed where the five subspecies were classified into two species. *Daboia russelii* now refers to the west of Bengal includingSri Lanka and India (Figure 1.8b), whereas *Daboia siamenses* refers to the east of Bengal including Burma, Indonesia and Thailand (Figure 1.9b) (Warrell, 2009).

Even though these two species look very similar (Figures 1.8a and 1.9a), they vary greatly in geographical distribution and venom composition. Russell's vipers are very versatile in habitation and live in all areas such as mountains, grassy terrain, rocky hills, salt scrubs, rodent burrows, wood land edges and paddy fields (Mallow et al., 2003). They are quite sluggish and reclusive by nature and are recognisable by the patterns on their skin (Warrell, 1989). When these snakes feel threatened, they coil up into a striking position and hiss loudly to warn off predators. Anatomically, Russell's vipers are very muscular which help with the rapid movement via lunging motions. Russell's vipers usually eat small vertebrates such as mice and rats, frogs, birds and lizards (Mallow et al., 2003). Adults can grow as long as 2 m long with an average fang length of 16 mm (Warrell, 1989). Dry weight from yielding venom can range between 21-268 mg in adults (Warrell, 1989). In one bite, Russell's vipers can inject about 45% of its total venom present in their glands (63±7 mg) (Pe and Cho, 1986).

Both *D. russelii* and *D. siamensis* are medically important species of snakes found in South East Asia and are responsible for the highest rate of mortality and morbidity in the region due to snake envenoming (Kasturiratne et al., 2008, WHO, 2016). Both species cause local painful swelling, venom-induced consumption coagulopathy, hypopituitarism and acute kidney injury. While *D. russelii* venom causes neuromuscular dysfunction and neurotoxicity (Silva et al., 2017). Toxins present in Russell's viper venom include PLA_2s (Venkatesh and Gowda, 2013), myotoxins *(D. russelii* only) (Silva et al., 2016a), neurotoxins (*D. russelii* only) (Silva et al., 2016b), snake venom serine protease, snake venom melloproteinase, three finger toxins (Gomes et al., 2007), C-type lectins, low molecular cytotoxins (Junqueira-de-Azevedo et al., 2006) and low molecular mass peptides (Mukherjee and Mackessy, 2013, Venkatesh and Gowda, 2013).

1.7.6 Brown snake (*Pseudonaja textilis*)

(a)

(b)

Figure 1.10: (a) *P. textilis* (Photograph by Stephen Maloney, Australian Museum) and (b) geographical distribution. Red shading refers to areas of highest rate of mortality and morbidity while orange shading refers to areas where the snake is found but clinical data is lacking. Source: WHO 2010 Database (https://apps.who.int/bloodproducts/snakeantivenoms/database - viewed on 03/06/2021).

Pseudonaja textilis, or more commonly known as the Common or Eastern brown snake, causes the highest rate of death due to snake bite in Australia (Chaisakul et al., 2013, Sutherland, 1981). It is a member of the elapid family and is found mainly in Eastern Australia (Figure 1.10b). This includes

Queensland, New South Wales, Victoria and south-eastern South Australia (Flight et al., 2006, Skejić and Hodgson, 2013). *P. textilis* occupies a range of habitats such as dry eucalyptus forests, woodlands, inner grass lands, coastal areas, and are also commonly found in open habitats, farmlands and on the outskirts of urban areas (Watharow, 2011). *P. textilis* are ground dwelling snakes that move around during the day and can be found taking shelter under logs, burrows and large rocks (Whitaker and Shine, 2003). When disturbed, they are likely to become aggressive and attack (Whitaker et al., 2000). The diet consists mainly of rodents such as mice and rats but *P. textilis* also feed on small vertebrates, frogs and other small reptiles (Shine, 1989).

An adult can grow to 2 m in length and is usually slender and of average build (Figure 1.10a) (Sutherland, 1983). The upper part of the body varies from pale to dark brown (Skinner, 2009). Compared to other Australian venomous snakes, the fangs of *P. textilis* are quite small and average 2.8 mm in length (Fairley, 1929, Skejić and Hodgson, 2013). *P. textilis* are fast moving snakes and can outpace a human adult running at full speed (Fleay, 1943).

Envenoming by *P. textilis* leads to headache, sweating, nausea and vomiting, acute kidney injury, venom-induced consumption coagulopathy, haemorrhage, and sudden cardiovascular collapse. Since 2000, there have been 23 deaths recorded following *P. textilis* envenoming (Allen et al., 2012, Welton et al., 2017). Many human bites are on the thigh area, as *P. textilis* rises up vertically off the ground before striking. This enables the snake to strike more accurately and deliver venom into its victim (Whitaker et al., 2000). *P. textilis* venom is considered the second most venomous of all terrestrial snakes based on murine LD_{50} values (Skejić and Hodgson, 2013), and is made up of a cocktail of highly toxic components – some of which have been isolated and characterised. The venom is highly potent as average venom yield is 5 mg, which is much less than other venomous snakes (Mirtschin et al., 2006).

Pseutarin C is a prothrombin activator isolated from *P. textilis* venom that consumes clotting factors such as fibrinogen, factor V and factor VIII, leading to haemorrhage and intracranial haemorrhage (Chaisakul et al., 2015, Masci et al., 1998) and may play a role in sudden collapse (Chaisakul et al., 2013). Neurotoxins have also been isolated from the venom. Interestingly, the neurotoxins from this venom do not cause paralysis or muscle weakness in humans (i.e. 'brown snake paradox'), despite being highly toxic. This may be due to low concentration of the toxins in the venom (Barber et al., 2012, Barber et al., 2013a). Other toxins isolated include textilinins which are Kunitz-type serine protease inhibitors (Masci et al., 2000), and snake venom PLA_2 (Armugam et al., 2004).

1.7.7 Western desert taipan (*Oxyuranus temporalis*)

(a)

(b)

Figure 1.11: (a) *O. temporalis* (Photograph by Bruce Budrey) and (b) geographical distribution. Orange shading refers to areas where the snake is found but clinical data is lacking. Source: WHO 2010 Database (https://apps.who.int/bloodproducts/snakeantivenoms/database - viewed on 03/06/2021).

There are three species of taipans found in Australia and Papua New Guinea, the coastal taipan (*O. scutellatus)*, the inland taipan (*O. microlepidotus)* and the western desert taipan *(O. temporalis). O. temporalis* was only discovered in 2007 in the Great Victoria Desert, 1500 km inland of Perth (Figure 1.11) (Doughty et al., 2007). Only two specimens were captured and kept in the Adelaide Zoo, thus

there is limited information about the appearance, distribution, diet and genetic variation of this species (Barber et al., 2014). Only a few studies have investigated this venom, and due to no recorded bites, there is no clinical data available.

Similar to venoms from *O. scutellatus* and *O. microlepidotus*, *O. temporalis* venom contains a substantial amount of post-synaptic neurotoxins, in particular α-neurotoxins (three-finger toxin family) (Barber et al., 2014). In 2014, a short chain post-synaptic neurotoxin (named elapitoxin-Ot1a) was isolated and pharmacologically characterised (Barber et al., 2014). Interestingly, *O. temporalis* venom appears deficient in PLA_2 neurotoxins, whereas PLA_2 toxins, i.e. taipoxin and paradoxin, are abundant in *O. scutellatus* and *O. microlepidotus* venoms (Madhushani et al., 2020, Su and Chang, 1984a, Weinstein et al., 2017).

It has been suggested that there is a relationship between toxins present in snake venom and type of prey it feeds on (Davies and Arbuckle, 2019, Lyons et al., 2020). However, despite a mammal-based diet, unlike other related taipan species that feed on mammals and contain high amounts of prothrombinase in their venom, *O. temporalis* venom also lacks both prothrombinases and procoagulant toxins ("unpublished data" (Skejic et al., 2018)). This suggests that *O. temporalis* relies mainly on post-synaptic neurotoxins to immobilise its prey ("unpublished data" (Skejic et al., 2018)). It also suggests that venom composition is not only dependent on prey type but also other factors (Daltry et al., 1996, Davies and Arbuckle, 2019, Healy et al., 2019, Zancolli et al., 2019).

1.8 Common vampire bat (*Desmodus rotundus*)

During my candidature, I had the opportunity to study venom from another animal – i.e. the common vampire bat. Below is an overview of the species.

(a)

(b)

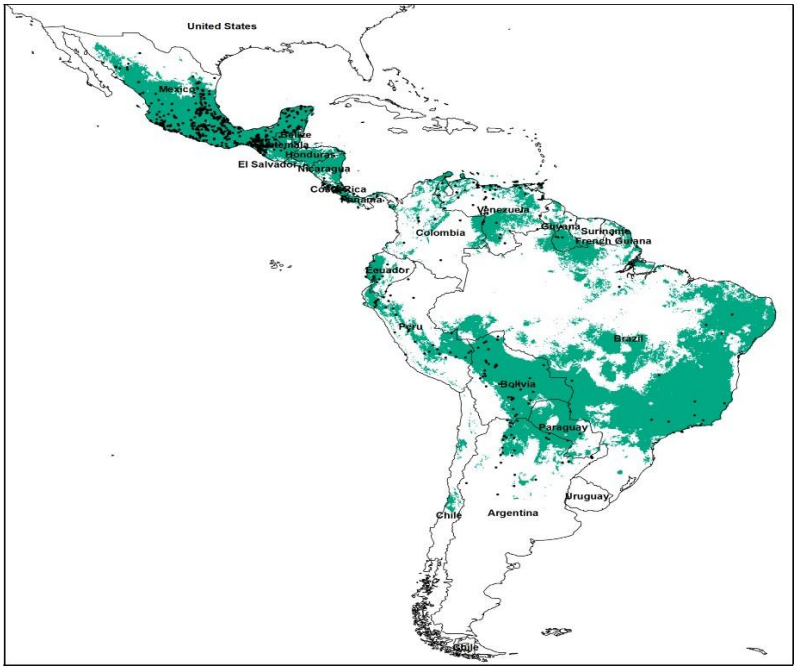

Figure 1.12: (a) *D. rotundus* (photograph by Michael Mulheisen) and (b) geographical distribution (Lee et al., 2012) (b). Source: Animal diversity web (https://animaldiversity.org/accounts/Desmodus_rotundus/ - viewed on 04/06/2021).

There are three species of vampire bats; hairly-legged vampire bat (*Diphylla ecaudata*), which feeds only on avian blood (Tuttle, 2018), the white-winged vampire bat (*Diaemus youngi*), which preys on

mammalian and avian blood (Koopman et al., 1988), and the common vampire bat (*Desmodus rotundus*) (Figure 1.12a), which feeds primarily on mammals such as livestock (Delpietro et al., 1992). Cases of humans being bitten are very rare, and death due to bat bite occurs due to rabies transmitted from the saliva and not the venom *per se* (Johnson et al., 2014, Schneider et al., 2009). These bats are found primarily in caves, tree hollows and abandoned mines in Central and South America (Figure 1.12b) (Bergner et al., 2020, Casewell et al., 2013, Lee et al., 2012). In contrast to snakes that use venom to kill their prey, the purpose of bat venom is to enable continuous feeding of blood from live animals. All three species have highly specialised saliva that contains venom which aids this hematophagous lifestyle (Low et al., 2013, Ray et al., 2018). While feeding, they lick the wound of live animals, releasing venom from the tongue which disrupts blood coagulation and allows a continuous flow of blood (Basanova et al., 2002, Greenhall, 1988, Hawkey, 2018).

D. rotundus venom contains toxins with strong anti-coagulant components which interfere with fibrin formation and proteolytic properties that break up blood clots (Hawkey, 2018, Rode-Margono and Nekaris, 2015). The venom also disrupts the coagulation cascade by inhibiting factor IXa and Xa, activation of plasminogen and inhibition of platelets (Low et al., 2013). A toxin, called draculin has been isolated and characterised. Draculin is a glycoprotein that inhibits IXa and Xa, preventing the conversion of prothrombin to thrombin which in turn inhibits the conversion of fibrinogen to fibrin (Apitz-Castro et al., 1995, Basanova et al., 2002, Fernandez et al., 1998, Fernandez et al., 1999). Plasminogen activators such as Desmokinase (Cartwright, 1974, Hawkey, 2018) and DSPA (*Desmodus rotundus* salivary plasminogen activator) (Schleuning et al., 1992, Tellgren-Roth et al., 2009) have also been isolated and studied in great detail from vampire bat venom. These activators dissolve fibrin clots, allowing a continuous blood flow from the wound site (Hawkey, 1966, Hawkey, 2018).

Recently, a new peptide was identified and synthetised from the common vampire bat that is similar in size and amino acid sequence to human calcitonin gene-related peptide (CGRP) (Low et al., 2013). In this thesis, the effect of this new peptide (named vCGRP) on blood vessels was investigated and characterized.

1.9 AIMS

The present study is aimed at exploring the cardiovascular effects of animal venoms – in particular eight different snakes and vampire bat. The toxins and mechanisms involved in cardiovascular dysfunction following envenoming are poorly understood, despite an array of clinical manifestations present. In this study, the pharmacological mechanisms and pathways involved were investigated. This involved both *in vivo* and *in vitro* experimentations.

Specific Aims

- Identifying medically relevant snake species that cause cardiovascular collapse/hypotension and investigating the mechanisms involved

- Investigating and characterizing the hypotensive effects of *D. russelii* venom

- Characterizing the vasodilatory effects of *D. rotundus* vCGRP

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Addendum to page 19

1.2.4 Atractaspididae

The Atractaspididae genus consists of fifteen to eighteen species (Golay et al., 1993, Spawls and Branch, 1995), mainly found is Sub-Saharan Africa as well as Israel, Palestine and Arabian Peninsula (Spawls and Branch, 1995). They are more generally known as burrowing asp, burrowing vipers, stiletto snakes and mole vipers (Tilbury and Verster, 2016). They are small, black and thin in appearance and quite secretive in nature, though bites to humans have been recorded (Tilbury and Verster, 2016). Unlike other venomous snakes that envenom with two fangs at a time, burrowing asp are known to envenom their prey with a backward stab using a single fang (Corkill et al., 1959, Golani and Kochva, 1988, Visser, 1975). This unusual behavior is correlated with unusual cephalic anatomical features and despite having a diverse and unique morphology, there is little research on this species (Deufel and Cundall, 2003, Shine et al., 2006).

The venom of burrowing asp contains both low molecular weight toxins and high molecular weight toxins such as hemorrhagins that cause severe hemorrhaging in envenomed patients (Ovadia, 1987). Envenomation also leads to local pain, local blistering and necrosis, muscle pain, nausea, abdominal pain, vomiting and diarrhea. Respiratory distress, hypertension, and neurological symptoms have also been reported (Gunders et al., 1960, Tilbury and Verster, 2016). Death due to envenoming is usually rapid and due to neurotoxic effects (Weiser et al., 1984). Interestingly the venom of burrowing asp is very viscous and contain 50-100 different molecular compounds (Tilbury and Verster, 2016). This includes three-finger toxins, c-type lectins, serine proteases, PLA_2s, metalloproteases, and other toxins (Terrat et al., 2013). The venom also contains a class of toxins called sarafotoxins that is unique to the genus and causes cardiovascular disruptions (Ducancel, 2002, Marshall and Johns, 1999).

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CHAPTER 2- An *in vivo* examination of the differences between rapid cardiovascular collapse and prolonged hypotension induced by snake venom

The focus of this Chapter was to investigate the cardiovascular effects of the eight snakes listed in section 1.5 of the Introduction. Following on from previous studies in our laboratory, *in vivo* studies were conducted using anaesthetised rats. Blood pressure was monitored to determine whether the venoms had an effect on blood pressure and, if so, to identify common features/characteristics of these responses. It was observed that there were two distinct cardiovascular effects produced by subgroups of venoms – i.e. "rapid cardiovascular collapse" and "prolonged hypotension". *In vitro* experiments using isolated mesenteric arteries mounted in a myograph and biochemical assays were also conducted to investigate whether common mechanisms of action / venom components were present between different species of snakes.

The study was published as a research article in the journal, Scientific Reports

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An *in vivo* examination of the differences between rapid cardiovascular collapse and prolonged hypotension induced by snake venom

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We investigated the cardiovascular effects of venoms from seven medically important species of snakes: Australian Eastern Brown snake (*Pseudonaja textilis*), Sri Lankan Russell's viper (*Daboia russelii*), Javanese Russell's viper (*D. siamensis*), Gaboon viper (*Bitis gabonica*), Uracoan rattlesnake (*Crotalus vegrandis*), Carpet viper (*Echis ocellatus*) and Puff adder (*Bitis arietans*), and identified two distinct patterns of effects: i.e. rapid cardiovascular collapse and prolonged hypotension. *P. textilis* (5 µg/kg, i.v.) and *E. ocellatus* (50 µg/kg, i.v.) venoms induced rapid (i.e. within 2 min) cardiovascular collapse in anaesthetised rats. *P. textilis* (20 mg/kg, i.m.) caused collapse within 10 min. *D. russelii* (100 µg/kg, i.v.) and *D. siamensis* (100 µg/kg, i.v.) venoms caused 'prolonged hypotension', characterised by a persistent decrease in blood pressure with recovery. *D. russelii* venom (50 mg/kg and 100 mg/kg, i.m.) also caused prolonged hypotension. A priming dose of *P. textilis* venom (2 µg/kg, i.v.) prevented collapse by *E. ocellatus* venom (50 µg/kg, i.v.), but had no significant effect on subsequent addition *of D. russelii* venom (1 mg/kg, i.v). Two priming doses (1 µg/kg, i.v.) of *E. ocellatus* venom prevented collapse by *E. ocellatus* venom (50 µg/kg, i.v.). *B. gabonica*, *C. vegrandis* and *B. arietans* (all at 200 µg/kg, i.v.) induced mild transient hypotension. Artificial respiration prevented *D. russelii* venom induced prolonged hypotension but not rapid cardiovascular collapse from *E. ocellatus* venom. *D. russelii* venom (0.001–1 µg/ml) caused concentration-dependent relaxation (EC_{50} = 82.2 ± 15.3 ng/ml, R_{max} = 91 ± 1%) in pre-contracted mesenteric arteries. In contrast, *E. ocellatus* venom (1 µg/ml) only produced a maximum relaxant effect of 27 ± 14%, suggesting that rapid cardiovascular collapse is unlikely to be due to peripheral vasodilation. The prevention of rapid cardiovascular collapse, by 'priming' doses of venom, supports a role for depletable endogenous mediators in this phenomenon.

Snake venoms act as a defence against predators, aid in the capture and paralysis of prey, and assist in the digestion of prey[1]. They contain a multitude of toxins with a wide range of activities that target vital physiological processes. Many of the toxins responsible for the clinical manifestations of envenoming in humans have been extensively studied and pharmacologically/biochemically characterised. These venom components include neurotoxins[2–4], myotoxins[5–7], and components with pro-coagulant, anticoagulant, haemolytic and local tissue necrotic activity[8–10]. However, the nature and activity of the toxins affecting the cardiovascular system are less well understood.

There are a number of cardiovascular effects associated with snake envenoming, including hypotension, myocardial infarction, cardiac arrest, hypertension, brady- or tachy-cardia and atrial fibrillation[10–13]. Identifying the mechanism(s) responsible for venom-induced cardiovascular collapse has garnered more interest in recent

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Species (scientific name)	Species (common name)	Dose (μg/kg, i.v.)	Maximum decrease in MAP* (%)	Classified as 'rapid cardiovascular collapse'	PLA_2 activity (nmol/min/ml)	Procoagulant activity (Log EC_{50}) (ng/ml)
D. russelii	Sri Lankan Russell's viper	100	45 ± 8	No	1,334 ± 105	3.64 ± 0.12
D. siamensis	Javanese Russell's viper	100	35 ± 7	No	10,237 ± 1084	3.09 ± 0.05
B. arietans	Puff adder	200	17 ± 2	No	378 ± 46	N/A
C. vegrandis	Uracoan rattlesnake	200	11 ± 1	No	1,077 ± 38	4.75 ± 0.04
B. gabonica	Gaboon viper	200	23 ± 3	No	3,498 ± 354	N/A
E. ocellatus	Carpet viper	50	100	Yes	111 ± 8	3.26 ± 0.06
P. textilis	Brown snake	5–10	100	Yes	473 ± 3	1.29 ± 0.05

Table 1. Summary of the effects and activity of snake venoms (n = 3–6). *Within 10 min of injection. MAP, mean arterial pressure; PLA_2, phospholipase A_2.

years. We have previously defined 'cardiovascular collapse' as a sudden drop in recordable blood pressure[14] following the administration of venom, to a laboratory animal or after human envenoming. The most common snakes responsible for this phenomenon are the brown snakes (*Pseudonaja* spp.)[15] and, less commonly, taipans (*Oxyuranus* spp.)[14] and tiger snakes (*Notechis* spp.)[16]. In some cases, patients spontaneously recover after collapse or respond well to basic and advanced life support[17,18]. In some cases of envenoming, particularly by brown snakes *(Pseudonaja spp.)*, the collapse can be fatal[16,17]. Indeed, in Australia, cardiovascular collapse is the leading cause of death due to snake envenoming[19].

A number of hypotheses have been proposed to explain the cause of the cardiovascular collapse associated with snake envenoming. Previous studies have postulated that cardiovascular collapse may be due to prothrombin activators or pro-coagulant toxins present in snake venoms[20,21]. We have recently demonstrated that *in vivo* cardiovascular collapse can be caused by death adder (*Acanthophis rugosus*) venom, despite a lack of pro-coagulants in this venom. This suggests that pro-coagulant toxins are not required to induce collapse[15]. Furthermore, administering small 'priming' doses of *A. rugosus* venom, prior to *P. textilis* venom, prevented subsequent cardiovascular collapse. This indicated that the release of depletable endogenous mediators most likely contribute to cardiovascular collapse. We also showed that the protective effect of priming doses of venom is transient (i.e. lasting up to approximately 1 hour), indicating replenishment of mediators[15]. This suggests that clotting factors are not directly involved in cardiovascular collapse, given the longer time period required for their resynthesis. Commercial polyvalent antivenom demonstrated a protective effect on cardiovascular collapse *in vivo*, supporting a role for antigenic venom components in cardiovascular collapse[15].

To further investigate this phenomenon, in the present study we examined the cardiovascular activity of seven medically important snake venoms: Australian Eastern Brown snake (*Pseudonaja textilis*), Sri Lankan Russell's viper (*Daboia russelii*), Javanese Russell's viper (*D. siamensis*), Gaboon viper (*Bitis gabonica*), Uracoan rattlesnake (*Crotalus vegrandis*), Carpet viper (*Echis ocellatus*) and Puff adder (*Bitis arietans*). We identified the species which caused cardiovascular collapse *in vivo* to further investigate the possible mechanisms for this phenomenon.

Results

***In vivo* experiments.** For these experiments 200 μg/kg (i.v.) was chosen as a standard dose for all venoms, unless a lower dose caused a similar response (i.e. *D. siamensis* 100 μg/kg, i.v.; *E. ocellatus* 50 μg/kg, i.v.; *P. textilis* 5 μg/kg, i.v.).

The mean blood pressure and heart rate of rats prior to administration of venoms were 97 ± 16 mmHg and 255 ± 63 b.p.m., respectively.

B. gabonica (200 μg/kg, i.v.), *B. arietans* (200 μg/kg, i.v.), *C. vegrandis* (200 μg/kg, i.v.) and *D. siamensis* (100 μg/kg, i.v.) venoms caused relatively minor hypotensive responses (i.e. between 11 to 35% decrease) in anaesthetised rats (Table 1). *D. russelii* (100 μg/kg, i.v) caused prolonged hypotension (45 ± 8% decrease) (Table 1). *P. textilis* (5 μg/kg, i.v.) and *E. ocellatus* (50 μg/kg, i.v.) venoms induced rapid cardiovascular collapse within 2 min of venom administration (Fig. 1a; Table 1).

To investigate the effects of artificial respiratory support, a higher dose of *D. russelii* venom (1 mg/kg, i.v.) was used, which caused a 100% decrease in blood pressure. This hypotensive effect (i.e. 100%) of *D. russelii* venom (1 mg/kg, i.v.) was significantly attenuated by artificial respiratory support, reducing the hypotensive effect to 42% (Fig. 2a). In contrast, the rapid cardiovascular collapse induced by *E. ocellatus* venom (50 μg/kg, i.v.) was not attenuated by artificial respiratory support (Figs. 1b and 2b).

To explore the effect of priming doses on both types of hypotensive responses, low dose *P. textilis* venom (2 μg/kg, i.v.) was administered 10 min prior to venom administration. A priming dose of *P. textilis* venom (i.e. 2 μg/kg, i.v.) had no significant effect on the subsequent addition of *D. russelii* venom (1 mg/kg, i.v.; Fig. 2a). In contrast, a priming dose of *P. textilis* venom (2 μg/kg, i.v.) prevented rapid cardiovascular collapse induced by *E. ocellatus* venom (50 μg/kg, i.v.; Fig. 2b), as did two sequential priming doses, but not one, of *E. ocellatus* venom (1 μg/kg, i.v.; Figs. 1c and 2b).

To further investigate the above effects of the venoms, a representative venom that caused collapse (i.e. *P. textilis*) and a representative venom that caused hypotension (i.e. *D. russelii*) were injected intramuscularly. Venom doses were increased to better mimic a bite scenario. *P. textilis* venom (20 mg/kg, i.m.; Table 2) caused collapse within 10 min of administration to the left bicep femoris muscle. *D. russelii* venom (50 mg/kg or 100 mg/kg, i.m.; Table 2) caused hypotension, but not collapse, within 30 min of administration.

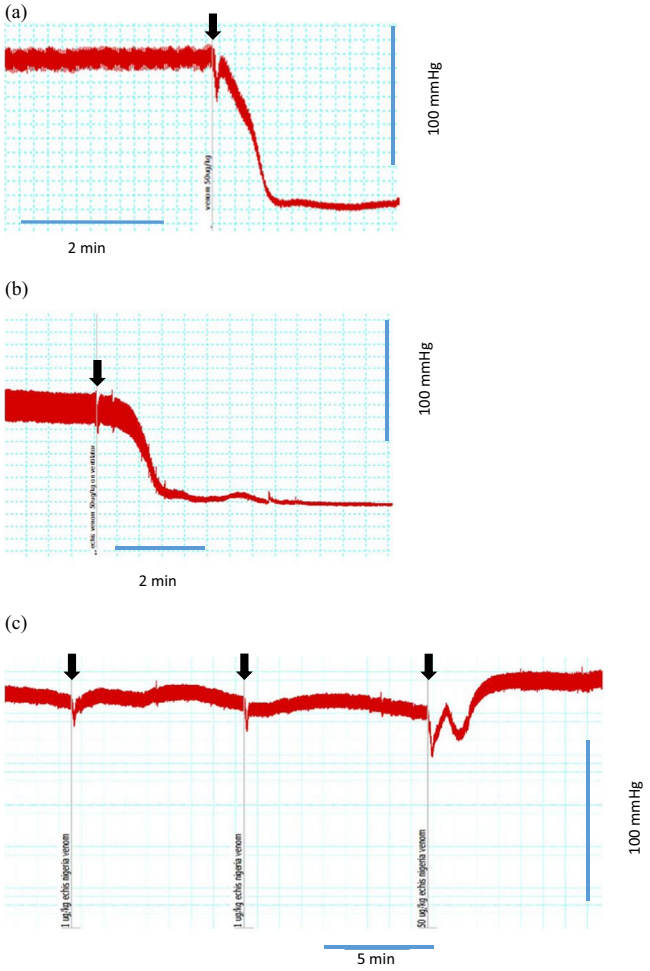

Figure 1. Traces showing rapid cardiovascular collapse induced by *E. ocellatus* venom (50 µg/kg, i.v.) in anaesthetised rats in the (**a**) absence and (**b**) presence of artificial respiration. **(c)** Trace showing the response to *E. ocellatus* venom (50 µg/kg, i.v.) after two sequential priming doses of *E. ocellatus* venom (1 µg/kg, i.v). Venom additions indicated by arrows.

PLA_2 assay. All venoms had PLA_2 activity. *D. siamensis* venom had the highest PLA_2 activity, followed by *B. gabonica, D. russelii* and *C. vegrandis* venoms. *P. textilis, B. arietans* and *E. ocellatus* venoms had low PLA_2 activity (Table 1).

Pro-coagulant assay. *P. textilis* venom had the most potent pro-coagulant activity (i.e. $\log EC_{50} = 1.29 \pm 0.05$ ng/ml; Fig. 3; Table 1), followed by *D. russelii, D. siamensis* and *E. ocellatus* venoms. *C. vegrandis* venom ($\log EC_{50} = 4.75 \pm 0.04$ ng/ml) had less pro-coagulant activity, and *B. arietans* and *B. gabonica* venoms had no detectable pro-coagulant activity (Fig. 3; Table 1).

***In vitro* myography experiments.** *D. russelii* venom (1–1000 ng/ml) was a potent vasorelaxant ($EC_{50} = 82.2 \pm 15.3$ ng/ml, $R_{max} = 91 \pm 1\%$; Fig. 4a) in small mesenteric arteries. *D. siamensis* venom was a less potent vasodilator than *D. russelii* venom with an EC_{50} value of ~700 ng/ml and a relaxation response at 1000 ng/ml of $66 \pm 15\%$. *P. textilis* venom caused < 50% relaxation ($38.6 \pm 9\%$) whilst, *E. ocellatus, B. arientans, B. gabonica and C. vegrandis* venoms induced < 30% relaxation (Fig. 4).

Discussion

We have demonstrated two distinct patterns of cardiovascular effects caused by the intravenous administration of different snake venoms. The first group of venoms cause a rapid decrease in blood pressure, often without recovery. We refer to this as 'rapid cardiovascular collapse' and it is the same phenomenon that we have previously described with Australian elapid venom[15]. A defining feature of this hypotensive response is that it is attenuated by sub-toxic 'priming' doses of venom of the same, or different snake species[15]. Snake venoms reported to induce this effect include *P. textilis* and *E. ocellatus* in this study, and previously, *O. scutellatus* (Coastal taipan)[14]. The second group of venoms, which include *D. russelii* and *D. siamensis*, caused a slower and prolonged decrease in blood pressure, with recovery occurring in most cases. In contrast to the first group, the drop in blood pressure is not prevented by prior administration of priming doses. We refer to this effect as 'prolonged hypotension'.

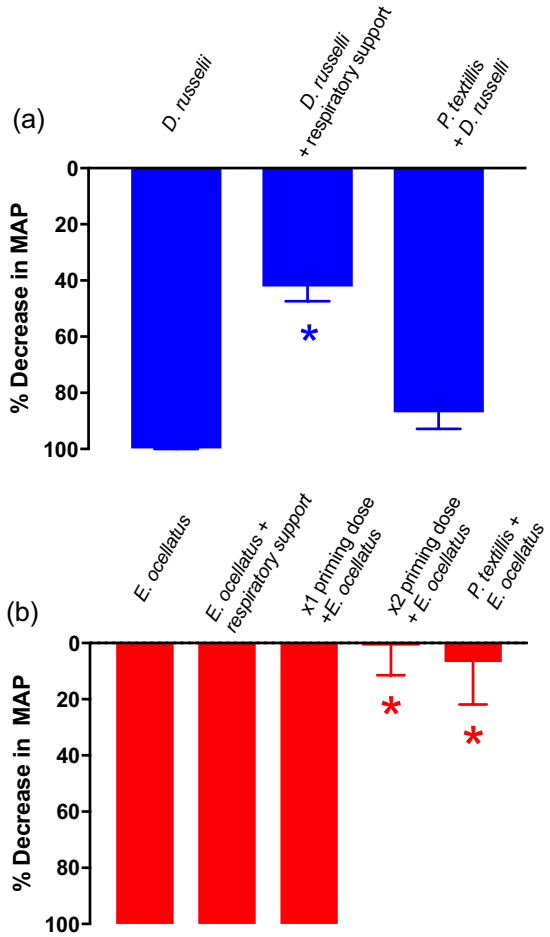

Figure 2. (**a**) The effects of *D. russelii* (1 mg/kg, i.v.) venom on the mean arterial blood pressure (MAP) of anesthetised rats in the presence (n = 5) or absence (n = 4) of artificial respiration, and in the presence of prior 'priming' with *P. textilis* venom (2 µg/kg, i.v., n = 6). (**b**) The effects of *E. ocellatus* (50 µg/kg, i.v.) venom on MAP of anesthetised rats in the presence (n = 5) or absence (n = 4) of artificial respiration, and in the presence of prior 'priming' with either *P. textilis* venom (2 µg/kg, i.v., n = 5), or one or two sequential doses of *E. ocellatus* venom (1 µg/kg, i.v., n = 3–4) venom. *P < 0.05 significantly different from response to same venom alone.

Species (scientific name)	Species (common name)	Dose (mg/kg, i.m.)	Maximum decrease in MAP (%)	Classified as 'rapid cardiovascular collapse'
D. russelii	Sri Lankan Russell's viper	50 100	27 ± 13 52 ± 9	No
P. textilis	Brown snake	20	100	Yes

Table 2. Summary of the effects of venom (n = 4) on mean arterial blood pressure following i.m. administration.

We have previously postulated that the attenuation of the hypotensive effect with prior administration of smaller sub-toxic doses of venom is due to the pre-release, and depletion, of mediators which induce collapse[15]. This phenomenon was observed in the current study in which smaller priming doses of *E. ocellatus* venom or *P. textilis* venom prevented cardiovascular collapse caused by a larger dose of *E. ocellatus* venom. This suggests that these venoms are inducing their cardiovascular effects via a common mechanism.

For a high dose of *D. russelii* venom (i.e. 1 mg/kg), a response similar to rapid cardiovascular collapse occurred. However, when the rat was placed on a ventilator prior to administration of venom, this so called 'collapse' was prevented. In contrast, when rats administered *E. ocellatus* venom were placed on the ventilator, rapid cardiovascular collapse still occurred. The reasons for the protective effects of supportive respiration are unclear. We have previously shown that the neurotoxins present in *D. russelii* venom are relatively weak[22]. However, given that a rat has approximately 64 ml of circulating blood per kg body weight, an intravenous dose of 1 mg/kg of venom leads to a blood concentration of approximately 16 µg/ml. This very high venom concentration may be sufficient to cause paralysis of the diaphragm given that a 30 ng/ml concentration of the same venom caused complete

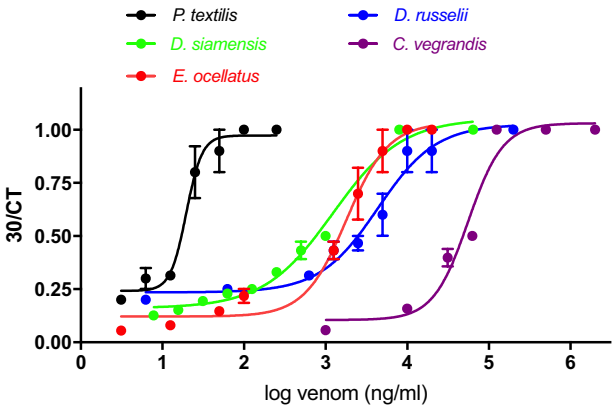

Figure 3. The pro-coagulant effects of venoms on the clotting time of fresh frozen plasma (n = 5–6).

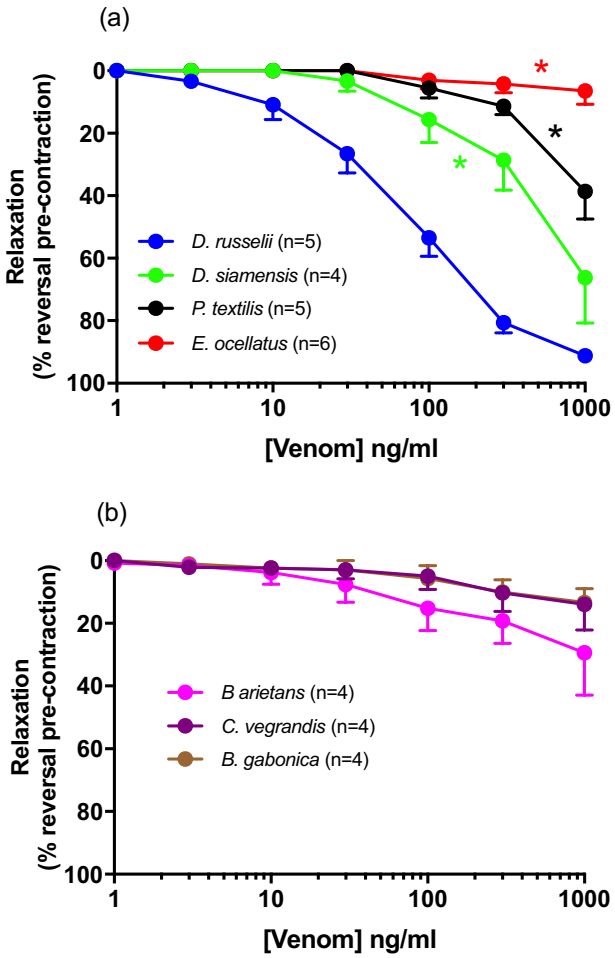

Figure 4. Cumulative concentration-response curves to venom (1 ng/ml − 1 µg/ml, n = 4–6) in rat small mesenteric arteries. Values are expressed as % reversal of pre-contraction and given as mean ± SEM, where *n* = number of animals. *P < 0.05, concentration-response curve significantly different as compared to *D. russelii*.

neuromuscular blockade in the chick biventer nerve-muscle preparation[22]. Therefore, it could be argued that artificial respiration is preventing or overcoming the paralytic effects of the neurotoxins on the rat diaphragm. The different effects of supportive respiration on the cardiovascular effects of the venoms also supports the fact that collapse due to *E. ocellatus* venom occurs via a different mechanism. These studies were conducted *in vivo* using ketamine/xylazine as anesthesia, which may have affected the blood pressure, although ketamine is more likely to cause a slight increase in blood pressure.

To ensure that these cardiovascular effects seen in the *in vivo* model occurs in an actual snake bite, the effects of *P. textilis* venom and *D. russelii* venom were also tested via intramuscular administration. At 20 mg/kg (i.m.), *P. textilis* venom caused collapse within 10 min of administration. This delay in response is likely to be due to the time it takes for the venom to be absorbed. In contrast, when *D. russelii* venom was administered via intramuscular injection prolonged hypotension occurred, similar to that observed when venom was administrated intravenously. Even at 100 mg/kg concentration, collapse did not occur, further highlighting that both collapse and hypotension are not dose-dependent responses but represent two distinct cardiovascular effects.

There are many factors that could lead to venom-induced hypotension[11], as distinct from cardiovascular collapse. Some snake venoms have highly evolved toxins such as calciseptine, FS2 toxins, C10S2C2 and S4C8 which block L-type Ca^{2+} currents[23,24]. Increasing capillary permeability protein (ICPP), isolated from Blunt-nosed viper *(V. lebtina)* venom is similar in potency and structure to vascular endothelial growth factor (VEGF) and is responsible for increasing vascular permeability[25]. Natriuretic peptides found in Green Mamba *(D. angusticeps)* venom[26] and bradykinin potentiating peptides found in *Bothrops spp*. are also potent vaso-relaxants[10,27,28]. In the current study, *D. russelii* venom caused concentration-dependent relaxation of rat small mesenteric arteries suggesting peripheral vasodilation contributes to the prolonged hypotension observed *in vivo*. *D. siamensis* venom was also an efficacious dilator of rat small mesnteric arteries, though less potent than *D. russelii* venom. In contrast, the venoms which had a modest hypotensive effect *in vivo* (*B. arientans, C. vegrandis and B. gabonica*) were poor vasorelaxants of isolated mesenteric arteries. Although vasorelaxant responses can exhibit heterogeneity throughout the vasculature, the mesenteric vascular bed was chosen for this study given it makes a significant contribution to overall total peripheral resistance, receiving 25% of total cardiac output. As such, characterising vasorelaxation responses in these small mesenteric arteries (approx. 300microns in diameter), is of physiological relevance to blood pressure control. Gaboon viper *(B. gabonica)* venom has been shown to induce vasodilation resulting in a drop in peripheral resistance, leading to reduction in stroke volume due to cardiotoxins[29]. In another study using isolated heart preparations, Rhinoceros viper *(B. nasicornis)* venom produced an increase in left ventricular pressure, pacemaker activity and heart rate, indicating that the venom contains toxins that disrupt $[Ca^{2+}]$ and ion conductance[30].

PLA_2 toxins are ubiquitous components of snake venoms and display an array of activities including neurotoxicity, myotoxicity, cardiotoxicity, anti-coagulation, haemolytic, hypotensive and local tissue necrotic activity[31]. Interestingly, *P. textilis* and *E. ocellatus* venom, which induced rapid cardiovascular collapse, had the lowest PLA_2 activity, whereas *Daboia spp*. had the highest amount of PLA_2 activity. Therefore, there does not seem to be a link between PLA_2 activity and cardiovascular collapse, although these toxins may contribute, directly or indirectly, to prolonged hypotension via vasodilation.

Daboia spp. and *Pseudonaja spp*. contain pro-coagulants factors in their venom[32]. While both *P. textilis* and *E. ocellatus* venoms caused rapid cardiovascular collapse *in vivo*, *P. textilis* venom was most potent in causing coagulation while *E. ocellatus* venom possessed comparatively less pro-coagulant activity. *D. russelii* and *D. siamensis* venom also caused coagulation. *D. russelii* and *D. siamensis* venoms are known to contain Factor X[33,34] while both *E. ocellatus*[35] and *P. textilis* venom contain prothrombin activators[21,36,37]. Therefore, pro-coagulant activity is unlikely to be directly related to the cardiovascular collapse induced by the venoms.

In conclusion, we have shown that the *in vivo* cardiovascular effects of venom include, at least, two distinct phenomena i.e. rapid cardiovascular collapse and prolonged hypotension and that both effects involve different mechanisms. Rapid cardiovascular collapse has a sudden onset and appears to be mediated by depletable endogenous mediators. In contrast, prolonged hypotension has a slower onset and appears to be due mainly to vasodilation.

Methods

Materials. Drugs and materials used were ketamine (Ceva Animal Health, Australia), xylaxine (Troy Laboratories Pty, Ltd, Australia), heparin (Hospira, Germany), bovine serum albumin (Sigma, USA), and fresh frozen plasma (Australian Red Cross). *D. siamensis, P. textilis, B. arietans, B. gabonica* and *C. vegrandis* venoms were obtained from Venom Supplies (Australia). *D. russelii* venom was a gift from Professor A. Gnanadasan (University of Colombo). *E. ocellatus* venom was a gift from the Liverpool School of Tropical Medicine. For procoagulant assays, venom (1 mg/mL) was prepared in 0.5% bovine serum albumin/tris-buffered saline and stored at −20 °C. Dilutions were prepared in 0.5% BSA/TBS immediately before use.

Animal experiments were approved by the Monash University Ethics Committee (MARP/2014/097 and MARP/2017/147). All experiments were performed in accordance with relevant guidelines and regulations.

Anaesthetised rats. Male Sprague-Dawley rats (280–350 g) were anaesthetised with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Ketamine/xylazine cocktail was used as it provides sedation and muscle relaxation as well as deep analgesia and anesthesia without compromising blood pressure. A midline incision was made and a cannula inserted into the trachea for mechanical ventilation (~1 ml/100 g of body weight at 55 strokes/min) if required. Cannulae were inserted into the left jugular vein for administration of venom and the right carotid artery to record arterial blood pressure. The arterial cannula was connected to a pressure transducer. Blood pressure was then allowed to stabilise for approximately 10–15 min. Body temperature was maintained at 37 °C using an overhead lamp and heated dissection table. Venom was administered via the jugular vein followed by flushing with saline or via a bolus administration into the left bicep femoralis muscle. Responses to venom were measured as percentage change in mean arterial pressure (MAP).

Myograph experiments. Male Sprague-Dawley rats (200–250 g) were euthanized by CO_2 inhalation (95% CO_2, 5% O_2) followed by cervical dislocation. Small mesenteric arteries (third-order branch of the superior

mesenteric artery) were isolated, cut into 2 mm lengths, and mounted in isometric myograph baths. Vessels were maintained in physiological salt solution, composed of (in mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.8 KH_2PO_4, 2.5 $CaCl_2$, 11 glucose, and 0.026 EDTA, at 37 °C and supplied with carbogen (95% O_2; 5% CO_2). The mesenteric arteries were allowed to equilibrate for 30 min under zero force and then a 5 mN resting tension was applied. Changes in isometric tension were recorded using Myography Interface Model 610 M version 2.2 (ADInstruments, Pty Ltd, USA) and a chart recorder (Yokogawa, Japan). Following a 15 min equilibration period at 5 mN, the mesenteric arteries were contracted maximally (F_{max}) using a K^+ depolarizing solution [K^+-containing physiological salt solution (KPSS); composed of (in mM) 123 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose]. The integrity of the endothelium was confirmed by relaxation to acetylcholine (ACh, 10 µM) in tissues pre-contracted with the thromboxane A_2 mimetic, U46619 (1 µM), then washed with physiological salt solution and the tension allowed to return to baseline. Relaxation of >80% to ACh was used to indicate vessels with an intact endothelium. There were no significant differences in response to ACh between the groups studied. If endothelial damage was evident (ACh relaxation <80%) then the vessel was not used for experimentation. Cumulative concentration-response curves to venom (1 ng/ml-1µg/ml) were constructed in vessels pre-contracted with titrated concentrations of U46619 (~50% F_{max}). Sodium nitroprusside (SNP, 10 µM) was added at the end of each concentration-response curve to ensure maximum relaxation. Only one concentration-response curve to venom was obtained in each vessel segment[38,39].

Pro-coagulation assay. Aliquots (10 ml) of fresh frozen plasma were thawed at 37 °C, then spun at 2500 rpm for 10 min. Venom solutions (100 µL) were placed in the wells of a 96 well microtitre plate at room temperature or at 37 °C in a BioTek ELx808 plate reader. Plasma (100 µL) and calcium (0.2 M/ml) were then added simultaneously to each well using a multichannel pipette. After a 5 s shake step for mixing, the optical density at 340 nm was monitored every 30 s over 20 min[40].

PLA_2 assay. PLA_2 activity of the venoms was determined using a secretory PLA_2 colourmetric assay kit (Cayman Chemical; MI, USA) according to manufacturer's instructions. This assay used 1, 2-dithio-analogue of diheptanoyl phosphatidylcholine, which serves as a substrate for PLA_2 enzymes. Free thiols generated following the hydrolysis of the thioester bond at the *sn*-2 position by PLA_2 are detected using DTNB (5, 5′-dithio-bis-[2-nitrobenzoic acid]). Colour changes were monitored at 405 nm in a fusion α microplate reader (PerkinElmer; MA, USA), sampling every minute for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolysed per minute per milligram of enzyme[2].

Statistical analysis. For the anaesthetized rat experiments, pulse pressure was defined as the difference between systolic and diastolic blood pressures. Mean arterial pressure (MAP) was calculated as diastolic blood pressure plus one-third of pulse pressure. These data were tested using a D'Agostino-Pearson normality test and found to be normally distributed. Therefore, differences in MAP between treatment groups were analysed using a one-way ANOVA with Dunnett's multiple comparison test. Sample sizes are based on the number of animals required to provide >85% power to detect an effect size of 35% with a confidence level (α) of 5% for the *in vivo* endpoint measure of blood pressure (standard deviation (SD) <15%). This ensured that experimental design was sufficiently powered.

For the myography experiments, blood vessel relaxation was expressed as a percentage reversal of the U46619 pre-contraction. Individual relaxation curves to *D. russelii* venom were fitted to a sigmoidal logistic equation and EC_{50} values (concentration of agonist resulting in a 50% relaxation) calculated[41]. Where EC_{50} values could not be obtained, concentration-response curves to venoms were compared by means of a two-way repeated measures ANOVA (n = number of artery segments from separate animals). Data represent the mean ± SEM (error bars on graph). Statistical significance was defined as $P < 0.05$. All data analysis was performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA, USA).

For the coagulation assay, responses were plotted as 30 s/[clotting time(s)] against the logarithm of the venom concentration. This provided a normalised measure of the clotting effect and produced normalised concentration-clotting curves, which were fitted with a standard sigmoidal curve (Hill slope = 1) to calculate the effective concentration 50 (EC_{50}). The EC_{50} is the concentration of venom that resulted in a pro-coagulant effect halfway between no clotting effect and maximal clotting effect[42].

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Author contributions

R.K., W.C.H., B.K.-H. and G.K.I. designed the study. R.K., W.C.H. and B.K.-H. conducted experiments. R.K. analyzed the data. W.C.H. and R.K. prepared the figures. R.K. wrote the initial manuscript text. B.K.-H., A.S., S.K., G.K.I. and W.C.H. reviewed the manuscript and contributed to experimental design.

Competing interests
The authors declare no competing interests.

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Appendix: Activity of Western desert taipan (*O. temporalis*) venom

Introduction

In addition to the snake venoms included in the preceding manuscript, I was fortunate enough to gain access to a limited quantity of Western desert taipan (*Oxyuranus temporalis*) venom. This species was only discovered in 2007 in Western Australia, and only a handful of specimens have been captured (Barber et al., 2016, Brennan et al., 2012, Doughty et al., 2007, Shea, 2007). A detailed introduction to this species, and previous work on the venom, has been included in Chapter 1. There have been no reported bites in humans from *O. temporalis*, and thus no clinical data is available (Barber et al., 2016). I was interested in investigating whether the venom would cause any cardiovascular effects in the anaesthetised rat model. Thus, the same experiments that were conducted on the other snake venoms were also conducted using *O. temporalis* venom. However, given this species is not clinically important due to the apparent small number of specimens in the wild, the data was not included in the published manuscript.

Methods

Venom

O. temporalis venom was obtained from Adelaide Zoo, South Australia.

Anaesthetised rats

As per described in the preceding manuscript (Kakumanu et al., 2019).

PLA_2 assay

As per described in the preceding manuscript (Kakumanu et al., 2019).

Pro-coagulant activity

As per described in the preceding manuscript (Kakumanu et al., 2019).

Myograph experiments

As per described in the preceding manuscript (Kakumanu et al, 2019).

Statistical analysis

As per described in the preceding manuscript (Kakumanu et al., 2019).

Results

In vivo experiments

O. temporalis venom (100 µg/kg, i.v.) caused a relatively small decrease in blood pressure (i.e. 24%) in anaesthetised rats (Table 1) within 10 min of administration (n=4). A PowerLab trace (Figure 2.1) has been provided to illustrate the effects of the venom on blood pressure.

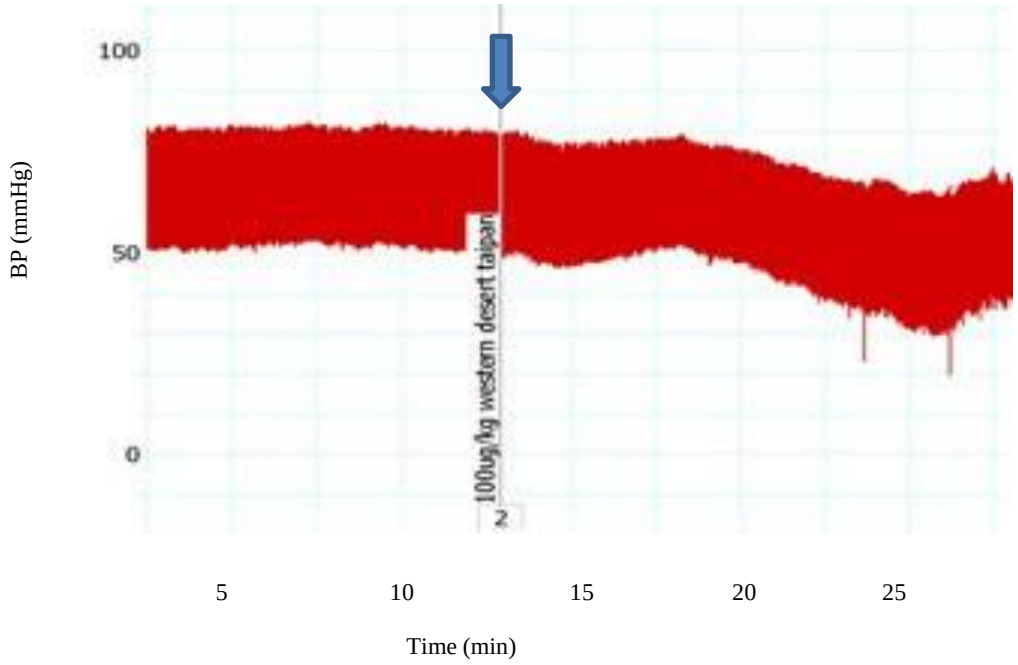

Figure 2.1: Trace showing the effect of *O. temporalis* venom (100 µg/kg, i.v.) in an anaesthetised rat. Venom addition indicated by arrow.

PLA_2 activity

O. temporalis venom displayed low PLA_2 activity (273 ± 22 nmol/min/ml) compared to the other snake venoms (Table 1 from (Kakumanu et al., 2019).

Pro-coagulant activity

O. temporalis venom displayed low pro-coagulant activity ($LogEC_{50}$ = 4.65 ± 0.06; Figure 2.1) compared to the other snake venoms (Table 1, Figure 3 from (Kakumanu et al., 2019).

Table 2.1: Summary of the activity of *O. temporalis* venom (n=3-5).

Species (scientific name)	Species (common name)	Dose (µg/kg, i.v.)	Maximum decrease in MAP* (%)	Classified as 'rapid cardiovascular collapse'
O. temporalis	Western desert taipan	100	24 ± 3.4	No

*** Within 10 min of injection. MAP, mean arterial pressure; PLA_2, phospholipase A_2.**

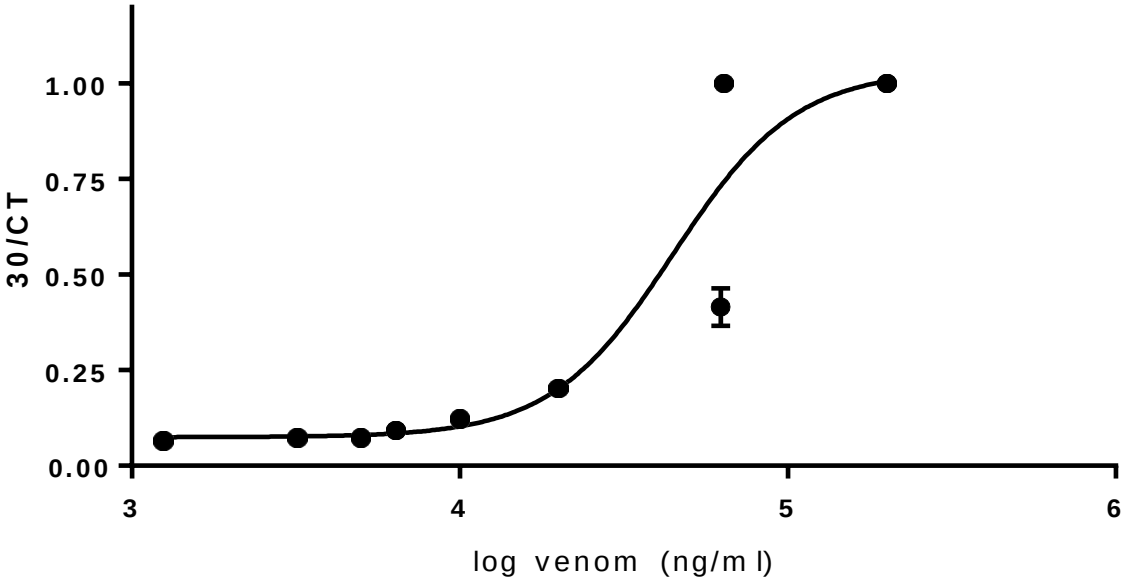

Figure 2.2: **The pro-coagulant effects of *O. temporalis* venom on the clotting time of fresh frozen plasma (n = 5).**

***In vitro* myograph experiments**

O. temporalis induced less than 20% relaxation in small mesenteric arteries (Figure 2.3).

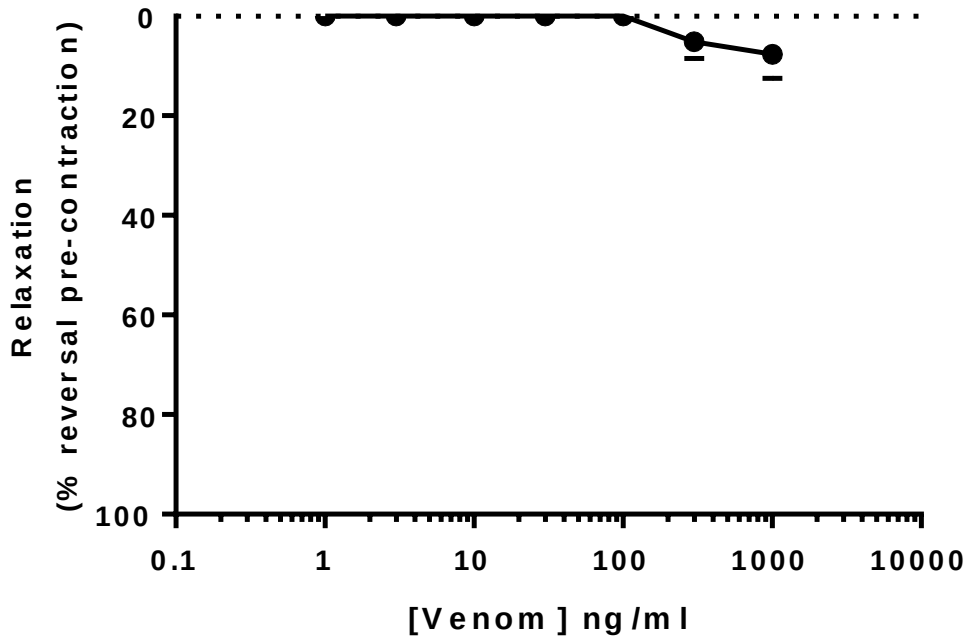

Figure 2.3: Cumulative concentration-response curve to venom (1 ng/ml – 1 µg/ml, n = 5) in rat small mesenteric arteries. Values are expressed as % reversal of pre-contraction and given as mean ± SEM, where n = number of animals.

Discussion

There are three species of taipans found in Australia and Papua New Guinea, i.e. the coastal taipan (*O. scutellatus),* the inland taipan (*O. microlepidotus)* and, the recently discovered, Western desert taipan *(O. temporalis). O. scutellatus* and *O. microlepidotus* venoms are highly potent and, based on murine LD_{50} values, are regarded as some of the most toxic venoms in the world. Envenoming in humans results in neurotoxicity, venom-induced consumption coagulopathy, renal failure, thrombotic microangiopathy, haemolytic anaemia and mild rhabdomyolysis (Chaisakul et al., 2014, Johnston et al., 2017). Similar to *O. scutellatus* and *O. microlepidotus* venoms, *O. temporalis* venom contains a substantial quantity of post-synaptic neurotoxins, with α-elapitoxin-Ot1a appearing to be the primary short-chain neurotoxin (Barber et al., 2014). The cardiovascular activity of *O. temporalis* venom is unknown.

In contrast to the venoms that caused cardiovascular collapse in the anesthetised rat model – i.e. *E. ocellatus* and *P. textilis*, *O. temporalis* venom caused a slower hypotensive response. Interestingly,

our laboratory has previously shown that both *O. scutellatus* (Chaisakul et al., 2012) and *O. microlepidotus* (Bell et al., 1998) venoms cause rapid cardiovascular collapse in anesthetised rats. "Priming" with sub-lethal doses of venom prevented collapse from *O. scutellatus* venom (Chaisakul et al., 2012). A PLA_2 component, subsequently isolated from *O. scutellatus* venom, was shown to contribute to the cardiovascular activity observed *in vivo* (Chaisakul et al., 2014). This fraction (i.e. OSC3) was isolated using size-exclusion HPLC and further separated into two toxins (OSC3a and OSC3b). Individually, these toxins caused a moderate hypotensive response *in vivo*, but not collapse. OSC3 was also shown to relax mesenteric arteries. OSC3 makes up 14.2% of the whole venom and thus it was suggested that cardiovascular collapse could be due to this toxin acting in a synergistic manner with the other venom components. Interestingly, when OSC3a and OSC3b were administered a second time in the same animal, there was no hypotension, indicating that the effect of the toxins could be mediated by depletable endogenous mediators or susceptible to tachyphylaxis (Chaisakul et al., 2014).

Previously, PLA_2 components isolated from snake venom have been shown to release histamine from human colon, tonsils, mast cells and lung cells (Wei et al., 2006, Wei et al., 2010). Similarly, pre-administration of mepyramine (H1 receptor antagonist) and heparin (which can prevent histamine, bradykinin and prostaglandin release; (Carr, 1979, Inase et al., 1993) markedly attenuated the hypotensive effects of OSC3a and OSC3b indicating that these toxins may be releasing endogenous mediators such as histamine or bradykinin (Chaisakul et al., 2014). OSC3a and OSC3b were also shown to contain high PLA_2 activity (Chaisakul et al., 2014). In contrast, in the current study, *O. temporalis* venom displayed very little PLA_2 activity (Table 2.1), indicating that the weaker hypotension observed *in vivo* may be a result of lower levels of PLA_2 components in the venom compared to *O. scutellatus* venom.

Studies using *O. microlepidotus* have suggested that the venom contains a component that promotes synthesis of arachidonic acid metabolites, and thus causes collapse via the eicosanoid pathway, and a component that causes vasorelaxation (Bell et al., 1998). In contrast, in the current study, *O. temporalis* venom produced virtually no vasorelaxation (Figure 2.3) at the concentrations tested indicating that the venom may not contain toxins that affect vascular smooth muscle or induce release of vasodilator mediators from endothelial cells. Interesting, *O. temporalis* venom contained the lowest pro-coagulant activity in comparison to the other venoms (i.e. compared to the venoms described in the preceding manuscript; Kakumanu et al., 2019), which is similar to previous studies (Barber et al., 2014, Zdenek et al., 2019). While *O. temporalis* venom has been shown to contain potent post-synaptic neurotoxins (Barber et al., 2016), no cardiovascular toxins have been previously

investigated. Since the venom did not cause cardiovascular collapse, further research was not conducted. Isolation and characterisation of the pro-coagulant toxin/s will be conducted in the future.

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CHAPTER 3- *D. russelii* venom mediates vasodilatation of resistance like arteries via activation of K_v and K_{Ca} channels

In Chapter 2, the *in vivo* and *in vitro* cardiovascular effects of various snake venoms were investigated. In particular, we observed that *P. textilis* and *E. ocellatus* venoms caused rapid cardiovascular collapse in an anesthetised rat model while, in contrast, *D. russelii* venom caused a prolonged hypotensive response. Further *in vitro* studies showed that *D. russelii* venom was a potent vasodilator of small blood vessels, indicating that the prolonged hypotension observed *in vivo* could be due to vasorelaxation of blood vessels. Therefore, this Chapter focuses on further exploration of the vasodilatory effects of *D. russelii* venom. Myograph experiments were conducted using rat small mesenteric arteries. By testing different pathways involved in smooth muscle vasorelaxation, it was determined that the venom acts via potassium channels, in particular K_v and K_{Ca} channels.

The study was published as a research article in the journal, Toxins

KAKUMANU, R., KURUPPU, S., RASH, L. D., ISBISTER, G. K., HODGSON, W. C. & KEMP-HARPER, B. K. 2019b. *D. russelii* venom mediates vasodilatation of resistance like arteries via activation of Kv and K_{Ca} channels. *Toxins*, 11**,** 197-207.

toxins

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Article

D. russelii Venom Mediates Vasodilatation of Resistance Like Arteries via Activation of K_v and K_{Ca} Channels

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Abstract: Russell's viper (*Daboia russelii*) venom causes a range of clinical effects in humans. Hypotension is an uncommon but severe complication of Russell's viper envenoming. The mechanism(s) responsible for this effect are unclear. In this study, we examined the cardiovascular effects of Sri Lankan *D. russelii* venom in anaesthetised rats and in isolated mesenteric arteries. *D. russelii* venom (100 μg/kg, i.v.) caused a 45 ± 8% decrease in blood pressure within 10 min of administration in anaesthetised (100 μg/kg ketamine/xylazine 10:1 ratio, i.p.) rats. Venom (1 ng/mL–1 μg/mL) caused concentration-dependent relaxation (EC_{50} = 145.4 ± 63.6 ng/mL, R_{max} = 92 ± 2%) in U46619 pre-contracted rat small mesenteric arteries mounted in a myograph. Vasorelaxant potency of venom was unchanged in the presence of the nitric oxide synthase inhibitor, L-NAME (100 μM), or removal of the endothelium. In the presence of high K^+ (30 mM), the vasorelaxant response to venom was abolished. Similarly, blocking voltage-dependent (K_v: 4-aminopryidine; 1000 μM) and Ca^{2+}-activated (K_{Ca}: tetraethylammonium (TEA; 1000 μM); SK_{Ca}: apamin (0.1 μM); IK_{Ca}: TRAM-34 (1 μM); BK_{Ca}; iberiotoxin (0.1 μM)) K^+ channels markedly attenuated venom-induced relaxation. Responses were unchanged in the presence of the ATP-sensitive K^+ channel blocker glibenclamide (10 μM), or H1 receptor antagonist, mepyramine (0.1 μM). Venom-induced vasorelaxtion was also markedly decreased in the presence of the transient receptor potential cation channel subfamily V member 4 (TRPV4) antagonist, RN-1734 (10 μM). In conclusion, *D. russelii*-venom-induced hypotension in rodents may be due to activation of K_v and K_{Ca} channels, leading to vasorelaxation predominantly via an endothelium-independent mechanism. Further investigation is required to identify the toxin(s) responsible for this effect.

Keywords: *D. russelii* venom; hypotension; potassium channels; vasodilatation

1. Introduction

Snake bite is a globally important health issue [1,2]. Snake venom has three purposes: a defensive mechanism against predators, an aid to capture prey, and/or a tool to deter/challenge competitors [3].

Venom is a complex cocktail of toxins and enzymes that have a wide range of biological activities targeting major physiological pathways and organs [4]. Ninety to ninety-five percent of snake venom consists of proteins and peptides, many of which are toxic to humans [5]. These components often possess enzymatic activity and ligand binding abilities that, in combination and/or separately, result in the clinical envenoming symptoms in humans and other organisms [3,6]. While most components of snake venom, such as neurotoxins [7–9], myotoxins [10–12], pro-coagulants, and anticoagulant, haemolytic and local tissue necrotic factors [13–15] have been studied in detail, toxins targeting the cardiovascular system are less well understood.

We have previously demonstrated two distinct cardiovascular activities due to snake envenoming: "cardiovascular collapse" (defined as an irreversible rapid drop in blood pressure) [16,17] versus "prolonged hypotension" (defined as a gradual decline in blood pressure that is reversible) [18]. A number of hypotheses have been postulated in regards to the mechanism(s) of "cardiovascular collapse." This includes the potential involvement of prothrombin activators or pro-coagulant toxins present in snake venoms [19,20]. However, we have previously demonstrated, using an in vivo animal model, that death adder (*A. rugosus*) venom causes collapse even though it contains no pro-coagulant toxins [16]. The release of depletable endogenous mediators has also been postulated to induce "cardiovascular collapse" [16].

Hypotension is defined as a blood pressure that is below the expected normal range for an individual in a given environment. Factors that can affect blood pressure include age, weight, medications, dehydration, or underlying medical conditions. Physiologically, hypotension can occur due to reduced systemic vascular resistance, reduced cardiac output, hypovolemia, vascular obstruction, or blood volume redistribution [21]. Known snake toxins that can affect blood pressure include natriuretic peptides, bradykinin-potentiating peptides, incretin mimetics, and sarafotoxins [22].

In this study, we examined in more detail the hypotensive effects of snake venom and pharmacologically characterized the vasodilatory effects of Sri Lankan Russell's viper (*D. russelii*) venom. *D. russelii* is regarded as one of the most medically important venomous snakes, as it causes the highest rate of mortality and morbidity due to snake bite in Asia [23,24]. It is mainly found in South Asia and is responsible for 73% of snake envenoming cases in the Anuradhapura District, Sri Lanka [25,26]. Clinical manifestations of envenoming include neuromuscular paralysis, coagulopathy, acute kidney failure, and hypotension [27]. Since the venom consists mainly of neurotoxins and myotoxins (~80%), these toxins have been well characterized pharmacologically [11,24,28], but the cardiovascular effects of this venom are less clear.

2. Results and Discussion

D. russelii is one of the most medically important species of snakes found in South East Asia, and is responsible for the highest rate of mortality and morbidity in this region due to snake envenoming [28–31]. The clinical syndromes include coagulopathy, mild neurotoxicity, acute kidney injury, bleeding, and hypotension [11,29,32]. While the neurotoxicity [24,28], acute kidney injury, and coagulopathy [33–35] have been studied in great depth, little is known about the cardiovascular effects. In the current study, we investigated the prolonged hypotensive effect of *D. russelii* venom both in vivo and in vitro in rodents.

2.1. In Vivo Studies

D. russelii venom (100 μg/kg, i.v., $n = 4$) caused prolonged (30 min) hypotension (45 ± 8% decrease in mean arterial pressure) within 10 min of administration (Figure 1). In addition, the heart rate of rats decreased ~20% i.e. from 289 ± 85 b.p.m. ($n = 4$), just prior to venom administration, to 242 ± 71 b.p.m. ($n = 4$) at the time of maximum decrease in blood pressure.

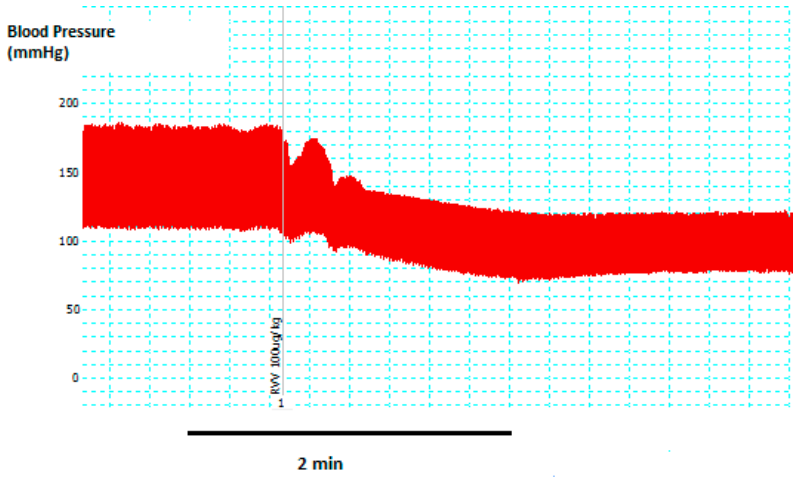

Figure 1. Original recording showing the hypotensive response to *D. russelii* venom (100 g/kg, i.v.) in an anaesthetized rat. Venom (RVV; Russell's viper venom) was added as indicated by the line on the trace.

2.2. Vasorelaxant Responses to D. russelii Venom

We next identified *D. russelii* venom as a potent dilator (EC_{50} = 145.4 ± 63.6 ng/mL, R_{max} = 92 ± 2%; Figure 2) of isolated rat small mesenteric arteries. Interestingly, vasorelaxation was unchanged following endothelial denudation (EC_{50} = 137.6 ± 51.6 ng/mL, R_{max} = 87 ± 7%) or inhibition of nitric oxide synthase by L-NAME (100 µM, EC_{50} = 130.7 ± 72.5 ng/mL, R_{max} = 91 ± 5%), indicating that the venom is likely directly targeting the vascular smooth muscle to cause relaxation. Thus, we next sought to characterize the mechanism(s) via which the venom mediates endothelium-independent vasorelaxation.

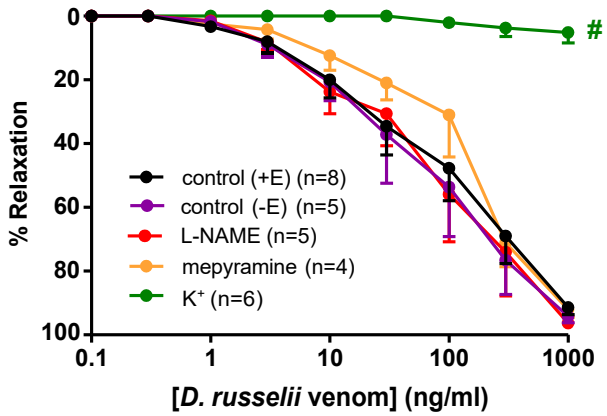

Figure 2. Cumulative concentration-response curves to *D. russelii* venom in rat small mesenteric arteries in the absence or presence of L-NAME (100 µM), 30 mM K^+, mepyramine (0.1 µM), or following endothelial denudation (−E). Values are expressed as % reversal of U46619 pre-contraction and given as mean ± SEM, where *n* = number of animals. # $p < 0.05$, response at 1000 ng/mL versus control (+E) (one-way ANOVA, Bonferonni's post-hoc test).

In the presence of the histamine H1 receptor antagonist, mepyramine (0.1 µM), there was no change in vasorelaxation (EC_{50} = 159.4 ± 82.3, R_{max} = 76 ± 10%) compared to venom alone, indicating that histamine does not appear to play a role in *D. russelii*-venom-induced vasorelaxation. In contrast, raising the extracellular concentration of K^+ to 30 mM abolished the vasorelaxation effects of venom (R_{max} = 5 ± 3%, $p < 0.05$, Table 1), suggesting that the venom also modulates relaxation of small resistance-like arteries in part via activation of K^+ channels.

Table 1. Effect of treatments on *D. russelii*- or GSK1016790A-induced vasorelaxation in rat small mesenteric arteries.

Treatment	*D. russelii* Venom		
	EC_{50} (ng/mL)	R_{max} (%)	*n*
Control (+E)	145.4 ± 63.6	92 ± 2	8
Control (−E)	137.6 ± 51.6	87 ± 7	5
L-NAME	130.7 ± 72.5	91 ± 5	5
Mepyramine	159.4 ± 82.3	76 ± 10	4
K^+	ND	5 ± 3 #	5
Control	276.5 ± 69.3	88 ± 2	15
TEA	ND	50 ± 14 #	7
Apamin	ND	11 ± 5 #	8
Iberiotoxin	ND	29 ± 6 #	8
TRAM-34	ND	29 ± 15	6
Control	328.7 ± 110.5	89 ± 2	9
Glibenclamide	237.7 ± 62.1	84 ± 4	4
4-Aminopyridine	ND	7 ± 6 #	5
Control	273.6 ± 57.6	86 ± 4	6
RN-1734	ND	50 ± 7 *	6
	GSK1016790A		
Control (+E)	2.8 ± 0.7 μM	84 ± 5	6
Control (−E)	1.3 ± 0.6 μM	77 ± 10	4
RN-1734	3.5 ± 0.9 μM	79 ± 3	5

Values as % reversal of the level of pre-contraction; +E = endothelium intact; –E = endothelium denuded; ND = Not determined; # $p < 0.05$, 1-way ANOVA as compared to control * $p < 0.05$, student's unpaired t-test.

2.3. Contribution of Potassium Channels to D. russelii-Mediated Vasorelaxation

It is well known that potassium channels play an integral role in maintaining the membrane potential and therefore, contractile tone in smooth muscle cells [36]. The distribution and nature of potassium channels vary depending upon the size of the vessel, as well as the type of vascular bed [37,38]. There are at least four different subtypes of potassium channels present in mesenteric arterial smooth muscle cells. These include inward rectifier (K_{IR}), voltage-gated (K_V), ATP sensitive (K_{ATP}), and Ca^{2+}-activated (K_{Ca}) potassium channels [36,39]. When K^+ channels are activated, it leads to vascular smooth muscle cell hyperpolarization and relaxation, thereby leading to a decrease in blood pressure and increased blood flow [36].

Whilst there are many different venoms that are known to contain potassium channel inhibiting peptides, the current study has identified an apparent ability of *D. russelii* venom to activate potassium channels.

Vasorelaxation to *D. russelii* venom in rat small mesenteric arteries was unchanged in the presence of the ATP-sensitive K^+ channel inhibitor, glibenclamide (10 μM; EC_{50} = 237.7 ± 62.1, R_{max} = 84 ± 4%, Figure 3a). However, the voltage-gated K^+ channel inhibitor 4-aminopyridine, (1000 μM) abolished venom-induced relaxation, reducing the response at 1000 ng/mL to 7 ± 6% ($p < 0.01$; Table 1). The non-selective K_{Ca} channel blocker TEA, (1000 μM, R_{max} = 50 ± 14%), markedly attenuated venom-induced vasorelaxation. Similarly, blocking large (BK_{Ca}; iberiotoxin, 0.1 μM, R_{max} = 29 ± 6%), intermediate (IK_{Ca}; TRAM-34 1 μM, R_{max} = 29 ± 15%), and small (SK_{Ca};

apamin, 0.1 μM, R_{max} = 11 ± 5%, Figure 3b, Table 1) Ca^{2+}-activated K^+ channels significantly inhibited venom-induced vasorelaxation.

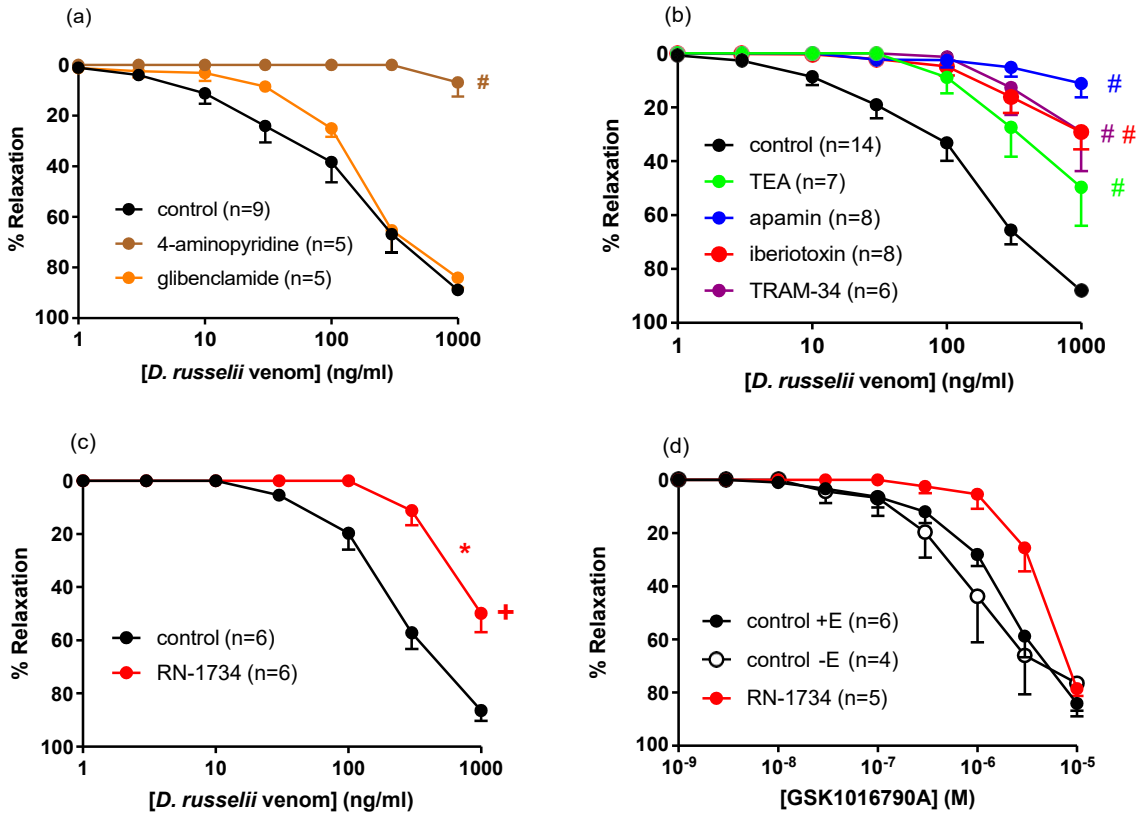

Figure 3. Cumulative concentration–response curves to *D. russelii* venom in rat small mesenteric arteries in the absence or presence of (**a**) 4-aminopyridine (1000 μM) or glibenclamide (10 μM), (**b**) TEA (1000 μM), apamin (0.1 μM), iberiotoxin (0.1 μM) or TRAM-34 (1 μM), or (**c**) RN-1734 (10 μM). (**d**) Cumulative concentration–response curves to GSK1016790A in the absence (control +E) or presence of RN-1734 (10 μM) or in endothelium-denuded vessels (control −E). Values are expressed as % reversal of pre-contraction and given as mean ± SEM, where *n* = number of animals. # $p < 0.05$, response at 1000 ng/mL versus control (one-way ANOVA, Bonferonni's post-hoc test), + $p < 0.05$, response at 1000 ng/mL versus control (student's unpaired t-test), * $p < 0.05$ vs control concentration-response curve (two-way repeated measures ANOVA).

These findings suggest the involvement of both K_{Ca} and K_v channels in *D. russelii*-venom-induced vasorelaxation. Given both K_{Ca} and K_v channels are sensitive to Ca^{2+}, our observations raise the interesting possibility that the venom may modulate intracellular Ca^{2+} levels, thereby indirectly modulating K^+ channel function. Indeed, our observation that the vasorelaxant effect of the venom was attenuated in the presence of the TRPV4 antagonist, RN-1734 (10 μM), further supports this concept. Specifically, in the presence of RN-1734, the potency of the venom was decreased approximately five-fold, and the response to 1000 ng/mL significantly reduced from 86 ± 4 to 50 ± 7% (Figure 3c). The TRPV4 agonist, GSK1016790A, also caused concentration-dependent relaxation (EC_{50} = 2.8 ± 0.7 μM, R_{max} = 84 ± 5%) in rat small mesenteric arteries, a response which was unchanged following endothelial denudation (EC_{50} = 1.3 ± 0.6 μM, R_{max} = 77 ± 10%). In the presence of RN-1734 (10 μM), there was an apparent two-fold decrease in the potency of GSK1016790A, yet this change failed to reach statistical significance (EC_{50} = 3.5 ± 0.9 μM, Figure 3d, Table 1). The greater inhibitory effect of RN-1734 against *D. russelii*-venom-induced vasorelaxation, as compared to GSK1016790A, may reflect a lower efficacy of the venom as a TRPV4 activator or an ability of RN-1734

to target TRPV4-independent signaling pathways potentially activated by the venom, identification of which are beyond the scope of the current study.

Whilst TRPV4 receptors are abundantly expressed on the endothelium [40], they are also present on the vascular smooth muscle [40,41]. Here, activation of TRPV4 leads to Ca^{2+} influx, the generation of Ca^{2+} sparks from the sarcoplasmic reticulum, and the subsequent activation of BK_{Ca} and vasorelaxation [42,43]. As such, *D. russelii* venom may cause vasorelaxation of resistance arteries in part via activation of vascular smooth muscle TRPV4 and opening of K_{Ca}. Future patch-clamp studies are required to support this hypothesis, and the potential for the venom to directly activate K_{Ca} and K_v channels remains.

3. Conclusions

D. russelii venom is known to cause an array of clinical manifestations in envenomed patients. These include coagulopathy, mild neurotoxicity, acute kidney injury, and sometimes severe hypotension. In this study, we have demonstrated that the hypotensive response may be indicative of a vasodilatory action of the venom at the level of the resistance vasculature. We have shown that *D. russelii* venom is a potent dilator of resistance-like arteries, mediating its response via activation of K_v and K_{Ca} channels, which may be modulated, in part, via signaling downstream of TRPV4. Future studies, including electrophysiological experiments and separation of venom components using HPLC, as well as investigating the potential contribution of other potassium channels such as K_{IR} channels and Na^+/K^+-ATPase, will aid in identifying the toxin(s) responsible for relaxation and provide further insight into the underlying mechanisms.

4. Materials and Methods

4.1. In Vivo Blood Pressure Experiments

Animal experiments were approved by the Monash University Ethics Committee (MARP/2014/097). Sprague-Dawley male rats (weight 280–350g) were anaesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). A midline incision was made and cannulae inserted into the trachea for mechanical ventilation, if required. Cannulae were also inserted into the left jugular vein for administration of venom and the right carotid artery to record arterial blood pressure. The arterial cannula was connected to a pressure transducer (PowerLab/400 system, ADInstruments Inc, Sydney, NSW, Australia). Blood pressure was then allowed to stabilize for approximately 10–15 min. Body temperature was maintained at 37 °C using an overhead lamp and heated rat table. Venom (100 µg/kg) was administrated via the jugular vein followed by flushing with saline. Responses to the venom were measured as percentage change in mean arterial pressure (MAP).

4.2. Isolation of Rat Small Mesenteric Arteries

Male Sprague-Dawley rats (200–250g) were euthanized via CO_2 inhalation (95% CO_2, 5% O_2), followed by exsanguination. Small mesenteric arteries (second-order branch of the superior mesenteric artery) were isolated, cut into 2 mm lengths, and mounted on 40 µm wires in small vessel myographs [44]. Vessels were maintained in physiological salt solution (composed of (in mM) 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.8 KH_2PO_4, 2.5 $CaCl_2$, 11 glucose and 0.026 EDTA) at 37 °C, and were bubbled with carbogen (95% O_2, 5% CO_2). In a subset of arteries, the endothelium was gently denuded via insertion of a 40 µm wire inside the lumen and rubbing the vessel walls. The mesenteric arteries were allowed to equilibrate for 30 min under zero force and then a 5 mN resting tension was applied. Changes in isometric tension were recorded using Myograph Interface Model 610 M v2.2 (DMT, Aarhus, Denmark) and PowerLab/835 (ADInstruments Inc). Data were recorded with the data acquisition program Chart (v5, ADInstruments). Following a 30 min equilibration period at 5 mN, the mesenteric arteries were contracted maximally (F_{max}) using a K^+ depolarizing solution (K^+-containing physiological salt solution (KPSS); composed of (in mM) 123 KCl, 1.17 $MgSO_4$,

1.18 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 11 glucose). The integrity of the endothelium was confirmed by relaxation to acetylcholine (ACh, 10 μM) in tissues pre-contracted with the thromboxane A_2 mimetic, U46619 (1 μM). Vessels with a relaxation response to ACh < 20% were considered endothelium-denuded, while vessels with a relaxation response to ACh > 80% were considered endothelium-intact. Arteries were washed with physiological salt solution and the tension was allowed to return to baseline.

4.3. Vasorelaxation Experiments

Cumulative concentration–response curves to venom (1 ng/mL–1 μg/mL) were constructed in vessels pre-contracted submaximally to ~50% F_{max} with titrated concentrations of U46619 (0.01–0.2 μM). Responses to venom were obtained in endothelium-intact mesenteric arteries pre-incubated for 30 min with either 30 mM K^+ [45], mepyramine (0.1 μM), L-NAME (100 μM) [44], TEA (1000 μM) [46], iberiotoxin (0.1 μM), apamin (0.1 μM), TRAM-34 (1 μM), 4-aminopyridine (1000 μM) [44], glibenclamide (10 μM) [47,48] or RN-1734 (10 μM). Cumulative concentration-response curves were also constructed to venom alone (1 ng/mL–1 μg/mL) or GSK1016790A in the presence and absence of RN-1734 (10 μM). In a subset of endothelium-denuded arteries, the vasorelaxation of venom and GSK1016790A were also examined. Sodium nitroprusside (SNP; 10 μM) [49] was added at the end of each concentration response curve to ensure maximum relaxation. Only one concentration–response curve to venom was obtained in each vessel segment due to tachyphylaxis [49,50].

4.4. Data Analysis and Statistical Procedures

4.4.1. In Vivo

For the anaesthetized rat experiments, pulse pressure was defined as the difference between systolic and diastolic blood pressures. Mean arterial pressure (MAP) was calculated as diastolic blood pressure plus one-third of pulse pressure. Heart rate (HR) was determined from the blood pressure trace.

4.4.2. In Vitro

Blood vessel relaxation was expressed as a percentage reversal of the U46619 pre-contraction (i.e., cumulative relaxation responses to venom or GSK1016790A were measured as a change in tension from the stable U46619 contraction, and expressed as a percentage of this contractile response). Individual relaxation curves were fitted to a sigmoidal logistic equation and EC_{50} values (concentration of agonist resulting in a 50% relaxation) were calculated. Statistical comparisons between the experimental groups' mean EC_{50} and maximum relaxation (R_{max}) values were made using either a student's unpaired t-test or a one-way ANOVA with Bonferroni's post hoc comparison. Where EC_{50} values could not be obtained, concentration–response curves were compared by means of a two-way ANOVA (repeated measures). n = Number of artery segments from separate animals. Data represent the mean ± SEM (error bars on graph). Statistical significance was defined as $p < 0.05$. All data analysis was performed using GraphPad Prism v5.02 (GraphPad Software, San Diego, CA, USA) [44].

4.5. Reagents

Reagents and their sources were: U46619 (Cayman Chemical company, Ann Arbor, MI, USA), glibenclamide, TEA, 4-aminopyridine, TRAM-34, mepyramine, L-NAME, SNP, isoprenaline, ACh, RN-1734 and GSK1016790A (Sigma-Aldrich, St Louis, MO, USA), and iberiotoxin (In vitro Technologies, Melbourne, VIC, Australia). Stock solutions of U46619 (1 mM) were made up in absolute ethanol. All subsequent dilutions of stock solutions were in distilled water. All other drugs were dissolved in distilled water, and all dilutions were prepared fresh daily.

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Appendix: Activity of Sri Lankan Russell's viper (*D. russelii*) venom

Introduction

In the previous Chapter, the cardiovascular effects of eight different snake venoms were investigated. In particular, Sri Lankan Russell's viper (*D. russelii*) venom was found to be a potent vasodilator in rat small mesenteric arteries and to cause prolonged hypotension in an anaesthetised rat model. *D. russelii* is one of the most venomous snakes in Asia and causes the highest rate of mortality and morbidity due to snake bite in South Asia (De Silva and Ranasinghe, 1983, Silva et al., 2017). In the Anuradhapura district, Sri Lanka, it is responsible for more than 70% of snake envenomings (Alirol et al., 2010, Phillips et al., 1988). *D. russelii* venom causes a wide range of clinical effects including cardiovascular disruptions (Warrell, 1989). A detailed introduction to this species, and previous studies on the venom, have been included in Chapter 1 and the preceding two published manuscripts. This additional Chapter focussed on characterising the mechanism by which *D. russelii* venom caused vasodilation *in vitro*. Further studies were conducted to isolate and characterise the toxin(s) responsible for both the vasodilation and hypotension observed *in vivo*. The data was not included in the published manuscript as the specific toxin(s) responsible were not identified.

Methods

Venom

D. russelii venom was a gift from Professor A. Gnanadasan (University of Colombo).

Size-exclusion HPLC

Venom (3 mg) was applied to a Superdex G-75 column (13 µm; 10 x 300mm^2) equilibrated with ammonium acetate buffer (0.1M; pH 6.8). The sample was eluted at a flow rate of 0.5 ml/min and the output was monitored at 280nm. The fractions were collected and pooled, frozen at -80°C and then freeze-dried to remove the solvent (Chaisakul et al., 2012). When required for *in vivo*/*in vitro* screening, freeze-dried components were reconstituted with Milli-Q water and protein content was determined using a BCA protein assay kit.

BCA Assay

Venom (25 µl) diluted 5-fold in Milli-Q water was aliquoted in triplicate onto a 96-well micro-titre plate. BSA solutions diluted from 1 to 0.025 mg/ml were used as reference standards and Milli-Q water used as the blank. Absorbance was measured at 562 nm utilizing the fusion α-plate reader (PerkinElmer., Massachusetts, USA).

In vivo blood pressure experiments

As per described in the preceding manuscript (Kakumanu et al., 2019).

Isolation of rat small mesenteric arteries

As per described in the preceding manuscript (Kakumanu et al., 2019).

Vasorelaxation experiments

As per described in the preceding manuscript (Kakumanu et al., 2019).

Results

***In vivo* experiments**

D. russelii venom was separated into eight fractions (labelled A-H) using size-exclusion HPLC (Figure3.1).

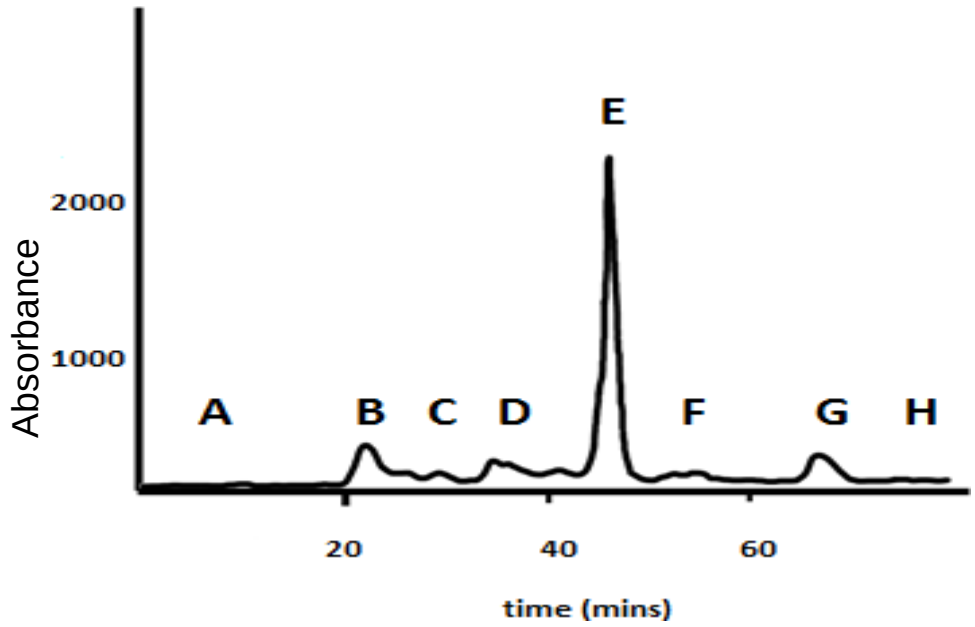


Figure 3.1: HPLC size-exclusion profile of *D. russelii* venom. Alphabetical letters indicate the different fractions separated according to size.

The protein content of each fraction was determined using a BCA Assay (Table 3.1).

Table 3.1: Quantity of protein in each fraction of *D. russelii* venom obtained via BCA assay.

Fraction	Protein Quantity (µg/mL)
A	0
B	1396
C	550
D	4709
E	6861
F	0
G	0
H	0

Each fraction was administrated intravenously into anaesthetised rats and blood pressure was monitored. In preliminary experiments, none of the individual fractions (50-100 µg/kg, i.v.) when administered alone caused hypotension (Table 3.2).

Table 3.2: Summary of the effects of each fraction of *D. russelii* venom on blood pressure of anaesthetised rats. N = 2-3.

Fraction	Dose (µg/kg, i.v.)	Effect on blood pressure
A	50	No change
B	100	No change
C	100	No change
D	100	No change
E	100	No change
F	50	No change
G	100	No change
H	50	No change

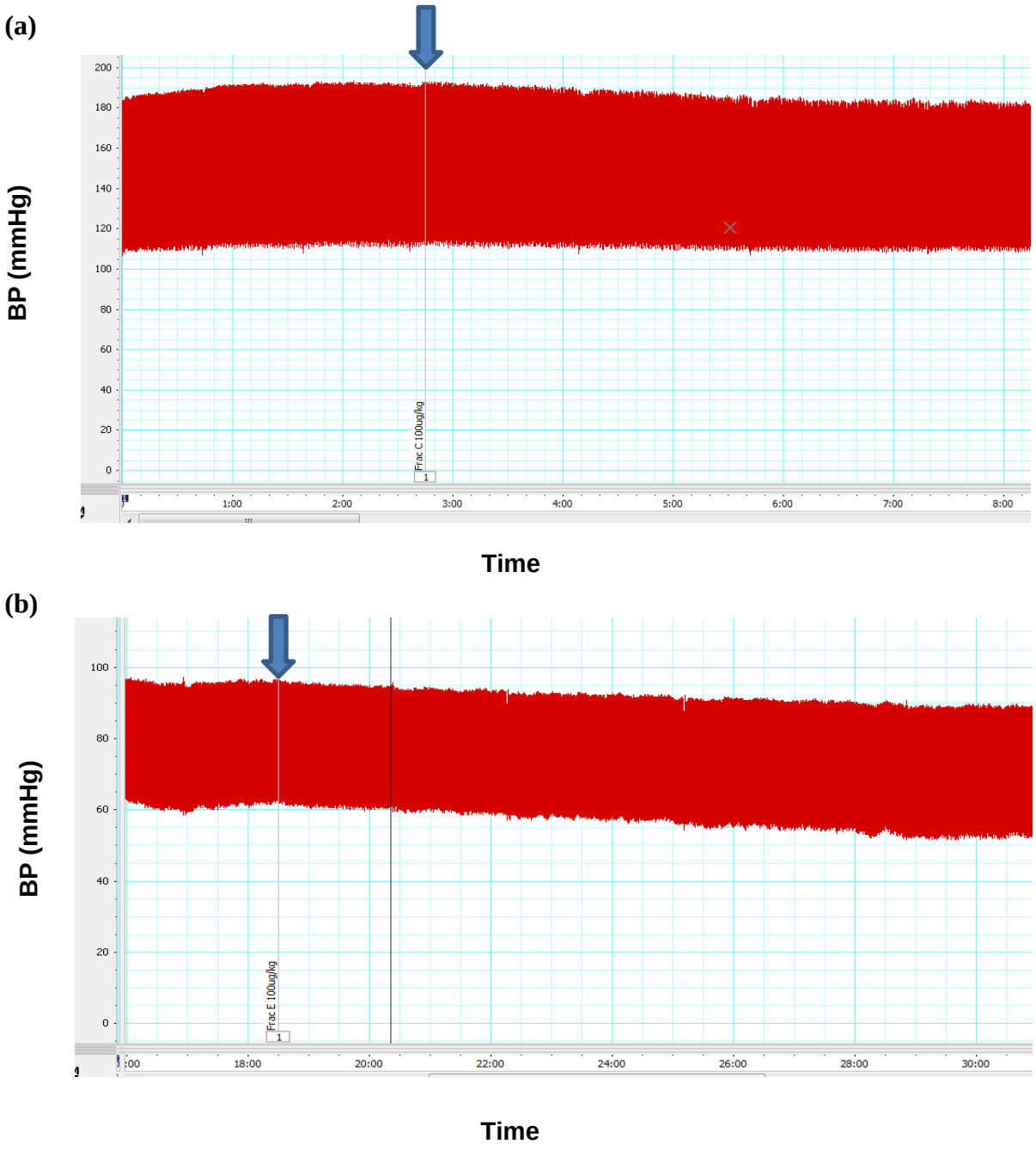
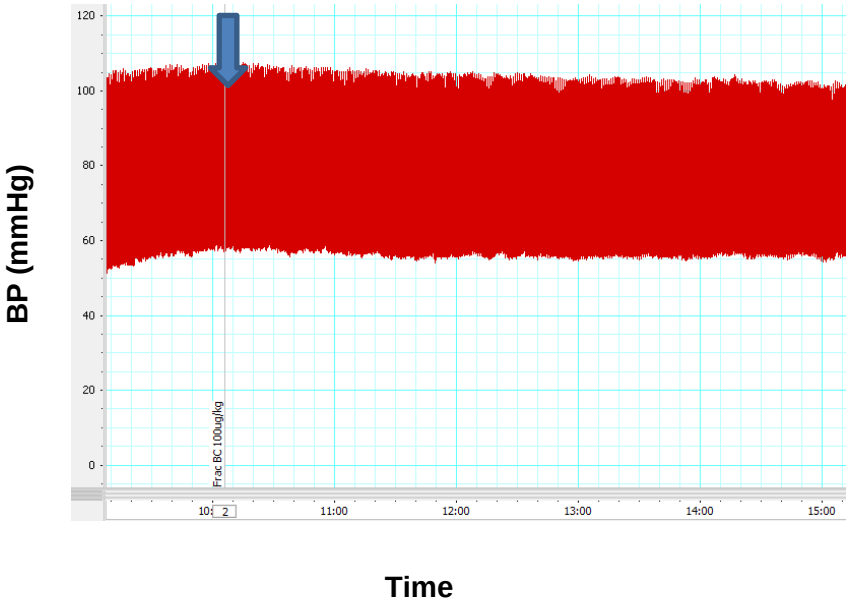


Figure 3.2: Trace showing the effects of *D. russelii* (a) fraction C (100 µg/kg, i.v.) and (b) fraction E (100 µg/kg, i.v.) on anaesthetised rats. Addition of fraction indicated by arrow.

Different combinations of the fractions (100 µg/kg, i.v.) also did not markedly affect blood pressure with only small decreases in blood pressure observed (Table 3.3).

(a)



(b)

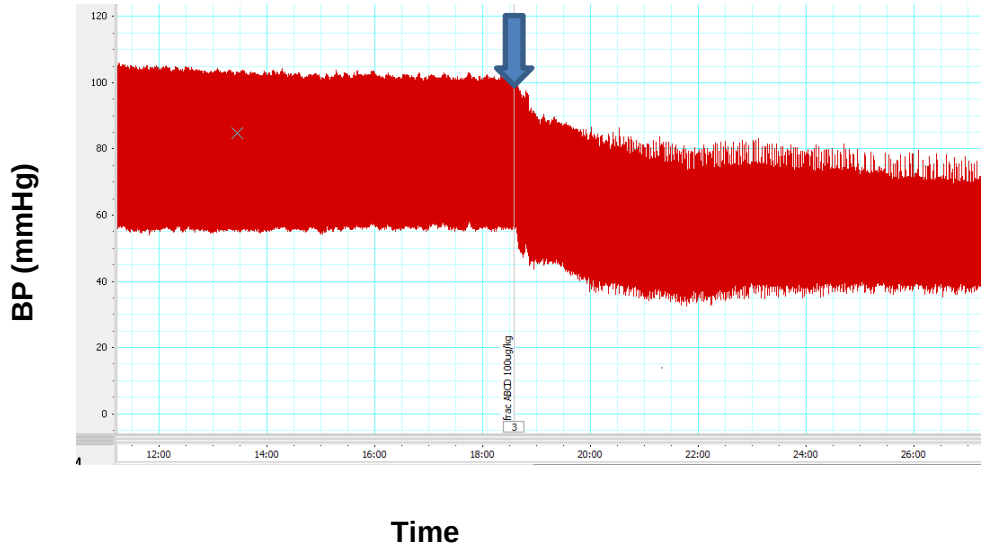


Figure 3.3: Trace showing the effects of *D. russelii* combined fractions (a) B and C (100 µg/kg, i.v.) and (b) A, B, C and D (100 µg/kg, i.v.) on anaesthetised rats. Venom addition indicated by arrow.

Table 3.3: Summary of the effects of different combinations of *D. russelii* venom fractions on blood pressure of anaesthetised rats. N = 2-3

Fraction combination	Dose (µg/kg, i.v.)	Effect on blood pressure
BC	100	No change
ABCD	100	Small decrease
EFGH	100	Small decrease
ABCDFGH	100	Small decrease
ADEFGH	100	Small decrease

As a positive control, whole venom (100 µg/kg, i.v.) was shown to cause prolonged hypotension. When the eight fractions were recombined following separation, the recombined fractions caused prolonged hypotension, similar to the crude venom. The HPLC buffer and saline did not affect blood pressure.

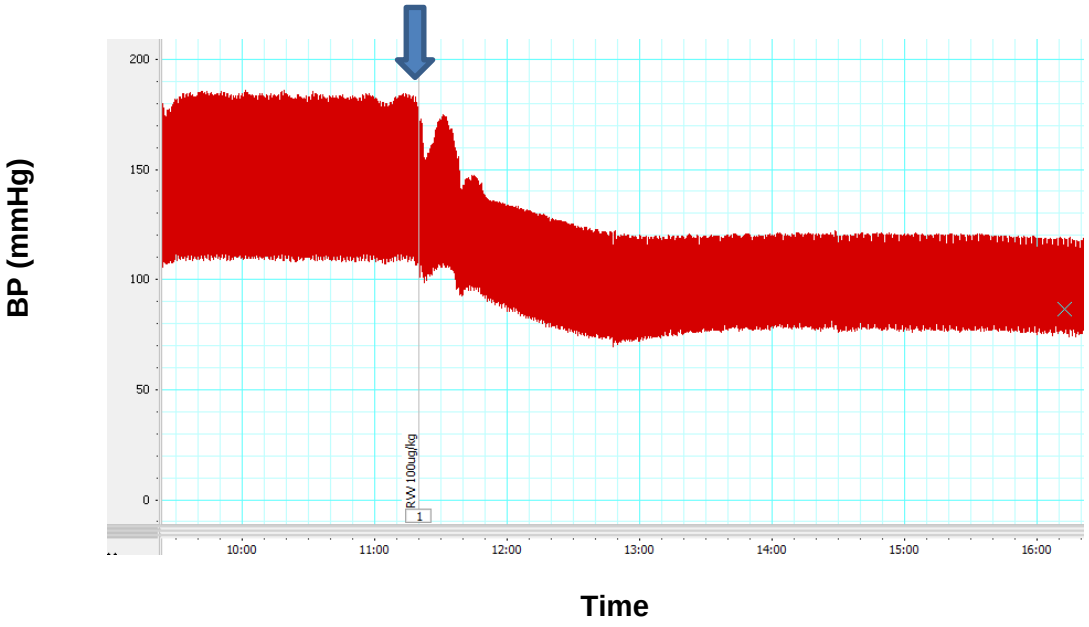


Figure 3.4: Trace showing the effects of *D. russelii* venom (100 µg/kg, i.v.) on anaesthetised rats. Venom addition indicated by arrow.

Table 3.4: Summary of effects of crude venom, recombined fractions or different reagents on blood pressure of anaesthetised rats. N ≥ 3.

Treatment	Dose (µg/kg, i.v.)	Effect on blood pressure
Crude venom	50 100	No change Prolonged hypotension
Recombined fractions	100	Prolonged hypotension
0.1M Ammonium Acetate buffer	100 µL	No change
Freeze-dried saline	100 µL	No change

Individual fractions B, C, D and E were also tested in rat mesenteric arteries mounted on a myograph. Of these, fractions C, D and E were potent vasodilators. Fractions A, F, G and H were not tested due to low/no protein content.

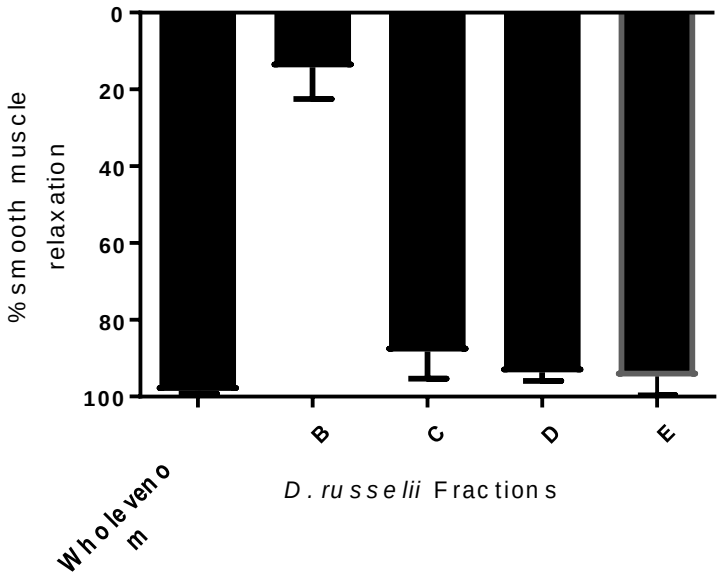


Figure 3.5: % smooth muscle relaxation of rat mesenteric arteries pre-contracted with U46619 in the presence of *D. russelii* fractions (1000 ng/mL). Each fraction was tested on 1 preparation, n = 2.

Discussion

D. russelii is a highly venomous snake found predominantly in South-East Asia. Envenoming by *D. russelii* causes a range of clinical manifestations in patients (Kasturiratne et al., 2005, Kasturiratne et al., 2008, Kularatne et al., 2014, Silva et al., 2016b). These include mild neurotoxicity, acute kidney injury, bleeding, coagulopathy and hypotension (Kularatne et al., 2014, Phillips et al., 1988, Silva et al., 2016a). The venom mainly consists of neurotoxins and myotoxins, and thus the cardiovascular effects are much less understood (Silva et al., 2016a, Silva et al., 2016b, Silva et al., 2017). In the previous manuscript, the hypotensive and vasodilatory effects of the venom were investigated in more detail and it was determined that potassium channels are involved (Kakumanu et al., 2019). The venom was then separated, using SE-HPLC, into eight fractions (A-H) of which only four fractions B, C, D and E contained a substantial amount of protein (>500 µg/mL).

All the fractions were tested in the anaesthetised rat model, alone and in different combinations (Table 3.3). The whole venom caused prolonged hypotension however none of the fractions alone affected blood pressure (Table 3.2). Since fraction E was clearly the major peak based on the size-exclusion HPLC profile, combinations of fractions with and without this fraction were tested, and still there was no prolonged hypotension. Previously, our lab has isolated and characterised fraction E from this

venom. It consists of two toxins – a potent pre-synaptic neurotoxin named U1-viperitoxin-Dr1a (Silva et al., 2017) and a weak myotoxin named U1-viperitoxin-Dr1b (Silva et al., 2016a). Interestingly, hypotension occurred when all the fractions were recombined and administrated in the rat, indicating that the toxins in *D. russelii* appear to act in a synergistic manner to cause hypotension, and that separating the toxins via HPLC and freeze-drying techniques does not seem to affect the pharmacological action of the toxins.

In the rat mesenteric arteries, *D. russelii* venom was found to be a potent vasodilator. Fractions C, D and E, but not fraction B, caused relaxation, similar to the whole venom (Figure 3.5). This was quite unexpected as the opposite was seen *in vivo* whereby none of the fractions caused a significant decrease in blood pressure. Snake venom is made up of a complex cocktail of toxins and the method by which *D. russelii* venom was separated was quite a crude method and thus it highly possible that each fraction could have had more than one type of toxin present. Although the mechanism via which the toxins caused vasorelaxation remains to be determined, based upon the findings with the whole venom it is likely that they may possess the ability to activate one of more of Kv, KCa or TPRV4 channels. Fractions A, F, G and H were not tested as there was not enough protein present in the samples. Further studies involving Reverse-Phase HPLC and other methods of purifying the toxins are required in order to determine the toxin(s) involved in vasorelaxation.

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CHAPTER 4- Vampire venom: Vasodilatory mechanisms of vampire bat (*Desmodus rotundus*) blood feeding

As previously mentioned in the Introduction (section 1.8), as part of my candidature I had the opportunity to work with a toxin derived from *Desmodus rotundus* (Vampire bat) venom. While the purpose of snake venom is predominately to aid the capture and killing of prey, vampire bat saliva contains venom that does not kill its prey. Rather it allows continuous feeding of blood (main food supply) from livestock. The venom contains anticoagulating components, such as draculin and DSPA (*Desmodus rotundus* salivary plasminogen activator), that prevent and break up blood clots. While there have been extensive studies conducted on these components, little is known about the other components of this venom. In this Chapter, the pharmacological effects of another component of *D. rotundus* venom (named vCGRP) was investigated. *In vitro* experiments were conducted using myography and rat small mesenteric arteries (as per previous Chapters). Concentration-dependent relaxation curves were constructed to vCGRP in the presence and absence of the endothelium and different receptor blockers. vCGRP is a potent vasodilator with similar amino acid sequence to human CGRP and causes relaxation of resistance-like arteries. In this Chapter, it was demonstrated that vCGRP activates CGRP1 receptors on vascular smooth muscle, and its effects are independent of the endothelium. Interestingly, similar to *D. russelii* venom, vCGRP also acts in part via the activation of potassium channels, in particular Kv channels.

This study was published as a research article in the journal, Toxins

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toxins

MDPI

Article

Vampire Venom: Vasodilatory Mechanisms of Vampire Bat (*Desmodus rotundus*) Blood Feeding

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Abstract: Animals that specialise in blood feeding have particular challenges in obtaining their meal, whereby they impair blood hemostasis by promoting anticoagulation and vasodilation in order to facilitate feeding. These convergent selection pressures have been studied in a number of lineages, ranging from fleas to leeches. However, the vampire bat (*Desmodus rotundus*) is unstudied in regards to potential vasodilatory mechanisms of their feeding secretions (which are a type of venom). This is despite the intense investigations of their anticoagulant properties which have demonstrated that *D. rotundus* venom contains strong anticoagulant and proteolytic activities which delay the formation of blood clots and interfere with the blood coagulation cascade. In this study, we identified and tested a compound from *D. rotundus* venom that is similar in size and amino acid sequence to human calcitonin gene-related peptide (CGRP) which has potent vasodilatory properties. We found that the vampire bat-derived form of CGRP (i.e., vCGRP) selectively caused endothelium-independent relaxation of pre-contracted rat small mesenteric arteries. The vasorelaxant efficacy and potency of vCGRP were similar to that of CGRP, in activating CGRP receptors and Kv channels to relax arteriole smooth muscle, which would facilitate blood meal feeding by promoting continual blood flow. Our results provide, for the first time, a detailed investigation into the identification and function of a vasodilatory peptide found in *D. rotundus* venom, which provides a basis in understanding the convergent pathways and selectivity of hematophagous venoms. These unique peptides also show excellent drug design and development potential, thus highlighting the social and economic value of venomous animals.

Keywords: vasodilatation; potassium channels; *Desmodus rotundus*; vampire bat; venom; calcitonin gene-related peptide

Key Contribution: In this study, we identified a compound from *D. rotundus* venom (vCGRP) that induces vasodilation of resistance vessels such as mesenteric arteries partly via voltage-gated potassium channels and endothelium independent mechanisms. The human form of CGRP is a potent vasodilator that acts partially via endothelium dependent and independent mechanisms.

Hence, the selectivity of vCGRP could be used for therapeutic interventions in diseases such as hypertension and diabetes.

1. Introduction

Common vampire bats (*Desmodus rotundus*) are found in Central and South America, and feed exclusively on mammalian blood [1,2]. They preferentially feed on livestock animals such as cattle [3] and produce venom components that disrupt the blood coagulation cascade, enabling a constant blood flow for feeding [4–7]. However, there are reports of rare incidents of human interactions which have led vampire bats to become more medically relevant to humans [8,9]. Outbreaks of rabies in human populations due to the vampire bats being vectors of the disease [10], have led to anti-vampire bat campaigns and culling of bat populations [11,12].

Previous studies have demonstrated that *D. rotundus* venom contains two important anticoagulant toxins: Draculin [6,7,13]; and DSPA (*Desmodus rotundas* salivary plasminogen activator) [14,15]. Draculin is a glycoprotein that irreversibly binds to factors IXa and X, and inhibits the conversion of prothrombin to thrombin [6,7,13]. This prevents fibrinogen being converted into fibrin and thus inhibits coagulation of blood during feeding [5]. DSPA components also aid in ensuring continuous blood flow by breaking up the fibrin mesh of any blood clots that are formed [16]. While there are relatively extensive studies on Draculin and DSPA, little is known about the other components of *D. rotundus* venom, with vasodilation a predicted but untested activity [15,16].

Other hematophagous animals induce anticoagulant and vasodilatory effects through the delivery of bioactive compounds, thus ensuring efficient blood flow for feeding. For example, mosquitos possess tachykinin-like peptides (sialokinins) [17,18], whilst bedbugs possess nitrosyl-hemoproteins (nitrophorins) [19,20]. In addition, sand flies contain a potent vasodilator (maxadilan) that acts via the PAC1 receptor [21,22], and horse fly disintegrins inhibit platelet aggregation like those from snake venoms [23]. Interestingly, tick prostaglandins constrict blood vessels [24]. The maintenance of blood flow during feeding is a major rate limiting step and challenge for blood feeders to overcome. Therefore, the longer they take to feed, the higher the chances the host or prey will notice, making them more vulnerable [25]. Thus, due to the similarities in feeding mechanisms between hematophagous animals, it has been postulated that vasodilators may play a key role in the venom of *D. rotundus*, targeting skin capillaries, to complement coagulation inhibition [15,16].

However, such actions have remained speculative until the current study which demonstrated selective and potent action for resistance-like arteries. Previously we showed that the transcriptome and proteinaceous products of the *D. rotundus* hematophagous secretion glands are rich in calcitonin gene related peptide variants [26], which are similar in size and amino acid sequences to CGRP but with modifications in key residues (Figure 1). CGRP is a potent vasodilator that acts via activation of CGRP1 receptors on either endothelial or smooth muscle cells [27–30]. The significance of this peptide type in relation to the obtaining of blood-meals, and the impact of residues, was tested in order to ascertain the role in securing blood-meals by *D. rotundus*. In this study, we have demonstrated that vCGRP also causes vasodilation of resistance-like arteries via similar pathways to CGRP but with greater selectivity.

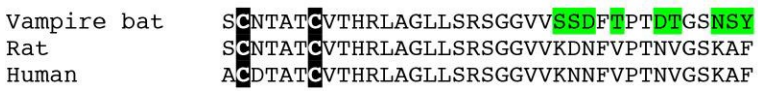

Figure 1. Alignments of vCGRP (Vampire bat), rCGRP (Rat), and hCGRP (human) with cysteines shaded in black and vampire bat specific modified residues in green.

2. Results

2.1. Vasorelaxant Responses to D. rotundus vCGRP and rCGRP

In rat small mesenteric arteries, *D. rotundus* vCGRP was a potent vasorelaxant (pEC_{50} = 9.47 ± 0.32 −logM, R_{max} = 94.6 ± 2.4%) with a potency and efficacy similar to that of rat calcitonin gene-related peptide (rCGRP; pEC_{50} = 9.16 ± 0.17 -logM R_{max} = 93.8 ± 2.6; Figure 2A). In the presence of the rat CGRP1 receptor antagonist CGRP8-37, the potency of *D. rotundus* vCGRP (Figure 2B) and rCGRP (Figure 1C) was decreased by 6-fold ($p < 0.05$) and 5-fold ($p < 0.05$) respectively, with no change in R_{max} (Figure 2B).

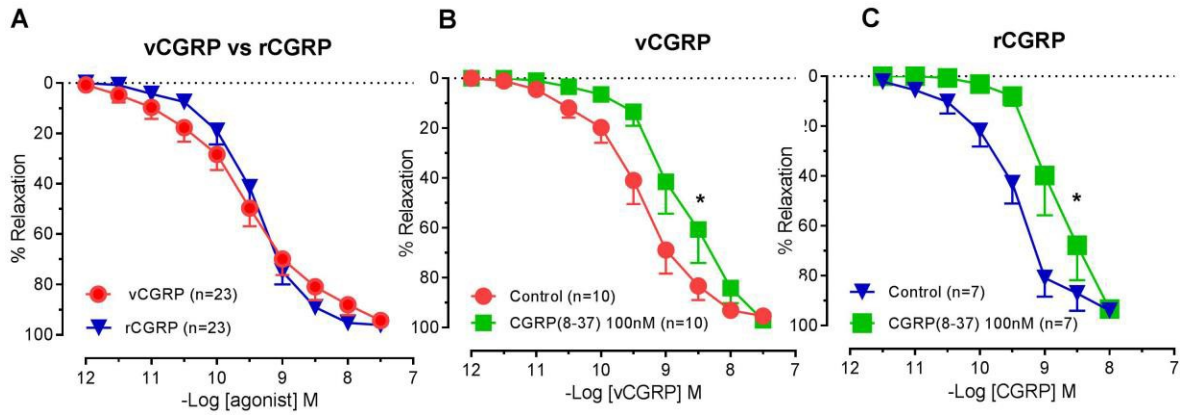

Figure 2. *D. rotundus* vCGRP causes vasodilation similar to rCGRP via CGRP1 receptors. Cumulative concentration-response curves to (**A**) *D. rotundus* vCGRP (n = 23) and rat CGRP (n = 23) alone and (**B**) *D. rotundus* vCGRP (n = 10) and (**C**) rat CGRP (n = 7) in the absence and presence of CGRP8-37 (100 nM, n = 7–10) in rat small mesenteric arteries. Values are expressed as % reversal of pre-contraction and given as mean ± SEM, where n = number of animals. * $p < 0.05$ pEC50 versus control, student's unpaired *t*-test.

2.2. Contribution of NO-SGC and Adenylate Cyclase to D. rotundus vCGRP and rCGRP Mediated Relaxation

Vasorelaxation to *D. rotundus* vCGRP was unchanged following endothelial denudation or treatment with L-NAME (100 μM) (Figure 3A). In contrast, potency to rCGRP was decreased 5-fold in the presence of L-NAME (100 μM) from 9.16 ± 0.17 to 8.62 ± 0.09 (pEC_{50} = 0.01) with no difference in maximum relaxation (Figure 3D). The presence of the soluble guanylyl cyclase inhibitor ODQ (10 μM) or the adenylyl cyclase inhibitor SQ22536 (10 μM) (Figure 3B,C,E,F) had no significant effect on *D. rotundus* vCGRP or rCGRP relaxation curves.

2.3. Contribution of Potassium Channels to D. rotundus vCGRP and rCGRP Mediated Relaxation

Raising the extracellular concentration of K^+ to 30 mM markedly attenuated the relaxant response to *D. rotundus* vCGRP (Figure 4A). Blocking voltage-dependent K^+ channels with 4-aminopyridine (1 mM) markedly attenuated *D. rotundus* vCGRP-induced relaxation, reducing the potency by approximately 30-fold ($p < 0.05$) and reducing the response at 10 nM to 53.7 ± 17.3% ($p < 0.01$). However, vasorelaxation to *D. rotundus* vCGRP was unchanged in the presence of the ATP-sensitive K^+ channel inhibitor, glibenclamide (10 μM), or the Ca^{2+} activated K^+ channel inhibitor, TEA (1 mM). Similarly, vasorelaxation to rCGRP was attenuated in the presence of 30 mM K^+ or 4-aminopyridine (1 mM) yet unchanged in the presence of TEA (1 mM) or glibenclamide (10 μM) (Figure 4B).

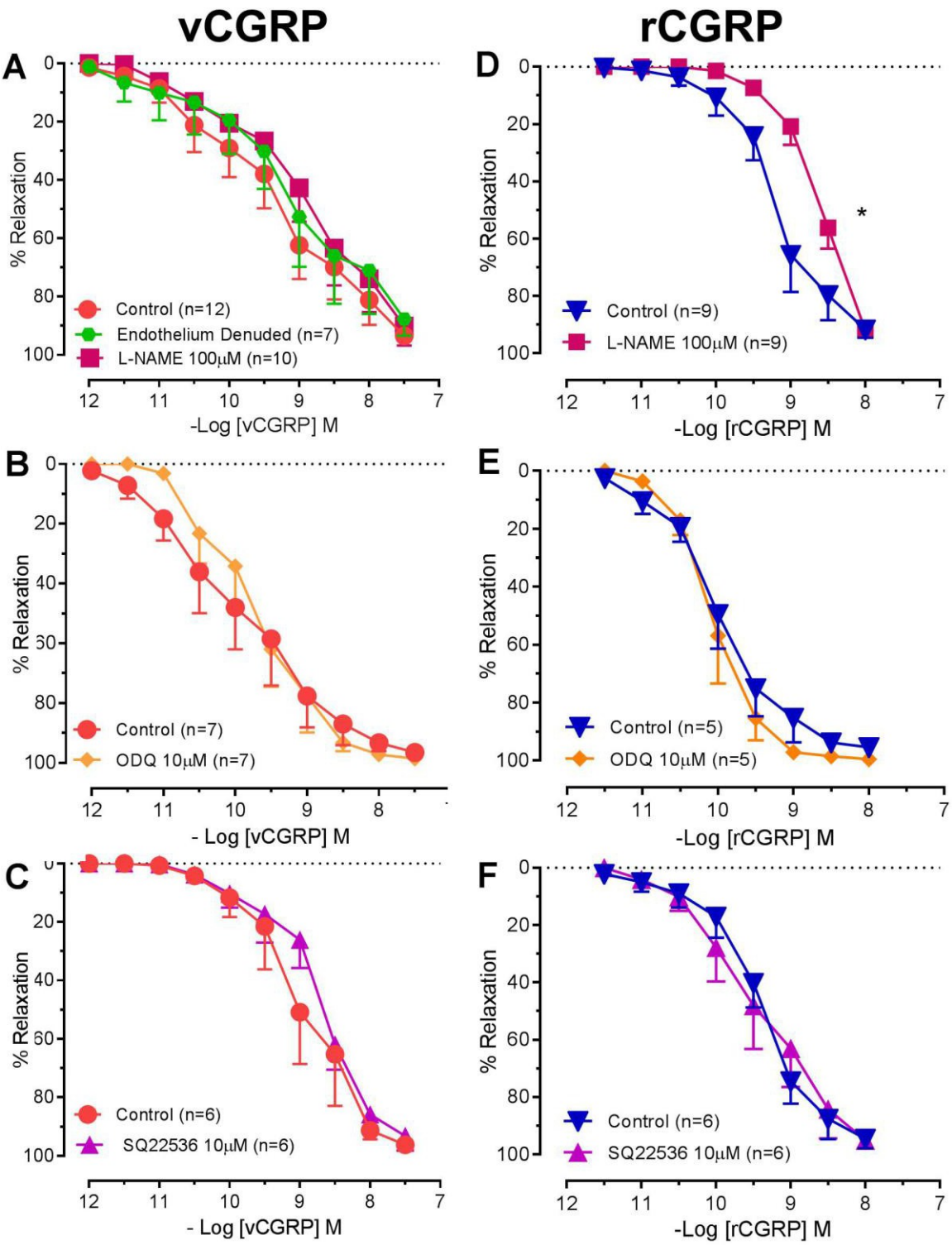

Figure 3. The soluble guanylyl cyclase or adenylyl cyclase pathways do not play a role in vasorelaxation induced by *D. rotundus* vCGRP or rCGRP. Cumulative concentration-response curves to *D. rotundus* vCGRP (**A**–**C**) or rat CGRP (**D**–**F**) in rat small mesenteric arteries in the absence (*D. rotundus* vCGRP, n = 6–12; rat CGRP, n = 5−9) or presence of either L-NAME (100 μM, n = 9–10), ODQ (10 μM, n = 5–7), SQ22536 (10 μM, n = 6) or following endothelial denudation (n = 7). Values are expressed as % reversal of pre-contraction and given as mean ± SEM, where n = number of animals. * $p < 0.05$ pEC50 versus control, student's unpaired t-test.

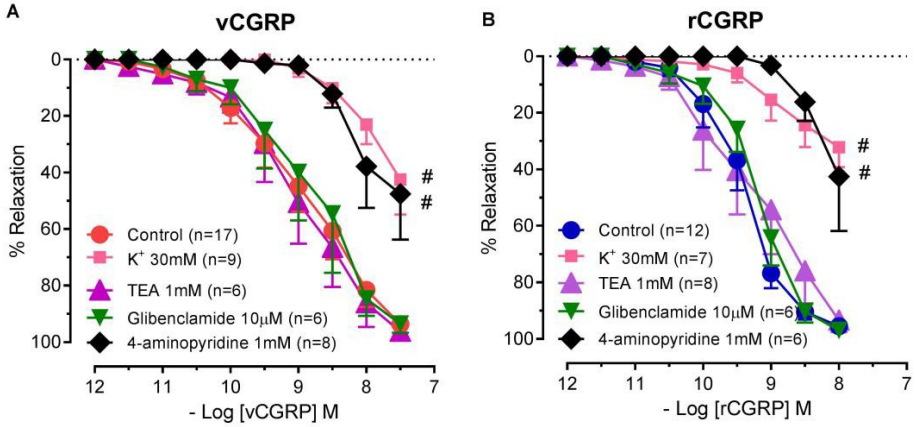

Figure 4. Voltage-gated potassium channels significantly attenuate the vasodilatory effects of *D. rotundus* vCGRP and rCGRP. Cumulative concentration-response curves to (**A**) *D. rotundus* vCGRP (n = 17) or (**B**) rat CGRP (n = 12) in rat small mesenteric arteries from rats in the absence or presence of either 30 mM K^+ (n = 7–9), TEA (1 mM, n = 6−8), glibenclamide (10 μM, n = 6) or 4-aminopyridine (1 mM, n = 6–8). Values are expressed as % reversal of pre-contraction and given as mean ± SEM, where n = number of animals. * $p < 0.05$, concentration-response curve significantly different as compared to control (2-Way ANOVA). # $p < 0.05$, response at 30 nM or 10 nM significantly different as compared to control (1-Way ANOVA, Bonferroni's post hoc).

3. Discussion

D. rotundus venom is well known to contain anticoagulating properties in order to facilitate blood feeding [26]. Indeed, a glycoprotein, Draculin, which inhibits activated coagulation factors IX (IXa) and X (Xa) has been isolated from *D. rotundus* venom [5]. In the current study, we isolated and characterised a peptide (vCGRP) from the venom, which is similar in size and amino acid sequence to CGRP found in humans and rats. CGRP is a potent vasodilator that acts via activation of CGRP1 receptors on either endothelial or smooth muscle cells [27,31]. Therefore, the aim of this study was to determine whether vCGRP also causes vasodilation via similar pathways.

We identified vCGRP as a dilator of rat small mesenteric arteries with a potency and efficacy similar to rCGRP. Importantly, like rCGRP, the vasorelaxation was attenuated by the CGRP1 receptor antagonist, CGRP8-37, indicative of an ability of the peptide to target this receptor to mediate its response though direct activation of CGRP1 receptors can be further supported by radioactive ligand binding assays in the future. Next we examined the role of endothelial cells in vasorelaxation via vCGRP. Given the vasorelaxation to vCGRP was unchanged following endothelial denudation or inhibition of nitric oxide synthase (by L-NAME), it is likely that vCGRP targets CGRP1 on vascular smooth muscle cells (VSMC) to cause endothelium-independent relaxation. In contrast, relaxation to rCGRP appeared to be, in part, dependent on endothelial-derived nitric oxide (NO) as the potency was attenuated following NOS inhibition. These findings highlight a potential point of difference with regard to CGRP derived from distinct species. Thus whilst an endothelium-dependent component of vasorelaxation to rCGRP has been observed in mesenteric [32] and retinal [33] arteries, we have demonstrated that vCGRP, like human CGRP [34], mediates relaxation via endothelium-independent mechanisms. This similarity in mechanism of action between human CGRP and vCGRP supports the notion of vCGRP becoming a potential candidate for therapeutic drug discovery.

Previous studies have also demonstrated that activation of CGRP receptors can lead to the activation of the guanylyl cyclase pathway (endothelium-dependent) or adenylyl cyclase pathway (endothelium-independent) [33–38]. However, the presence of ODQ (guanylyl cyclase inhibitor) or SQ22536 (adenylyl cyclase inhibitor), had no significant effect on rCGRP or *D. rotundus* vCGRP relaxation curves. Differences between CGRP endothelium-independent and -dependent mechanisms are related to the region, size of the vessel tested and species of CGRP. For instance, human

or rat CGRP tested in pig coronary leads to increased cAMP and causes vasorelaxation via endothelium-independent pathways [34]. However, human CGRP tested in human vessels are endothelium-dependent [28].

Therefore, we next sought to characterise the mechanism(s) via which vCGRP mediates endothelium-independent relaxation. Our finding that raising the extracellular K^+ concentration to 30 mM markedly attenuated the relaxation to vCGRP suggests that the peptide modulates relaxation of rat small mesenteric arteries in part via activation of K^+ channels. Indeed, we identified an ability of vCGRP to activate voltage-dependent K^+ channels as relaxation responses were decreased by 4-AP. This was in agreement to findings with respect to rCGRP. Neither K_{ATP} nor K_{Ca} channels appeared to be involved in relaxation to vCGRP or rCGRP as glibenclamide and TEA were without effect. Indeed, there is evidence that activation of CGRP receptors could lead to direct opening of K^+ channels, in particular Kv channels [33]. There are conflicting reports on the involvement of K_{ATP} and K_{Ca} channels in vasorelaxation, which could be related to the type of vessel studied. For instance, studies using bovine retinal arteries and rabbit mesenteric arteries report that activation of K_{ATP} channels, but not K_{Ca} channels, leads to vasorelaxation [37,39,40]. However, studies in smooth muscle cells from rat mesenteric arteries have shown CGRP directly activates BK_{Ca} channels [41]. These data further highlight that CGRP causes vasorelaxation through a variety of mechanisms which is dependent upon the species and vessel involved.

Considering the medical relevance to humans of *D. rotundus* and other vampire bat species as disease vectors for rabies [1], it is surprising that more in depth studies have not been conducted on the intricate mechanisms employed in their feeding behaviour, despite studies on other blood feeding animals such as fleas and leeches [13,26,42]. Such secretions fit within the definition of venom as 'A secretion produced in specialized cells in one animal, delivered to a target animal through the infliction of a wound and that disrupts endophysiological or biochemical processes in the receiving animal to facilitate feeding, defense or competition by/of the producing animal' [42]. As peptides used by venoms/hematophagous-secretions are modified versions of those routinely expressed in other tissues [43] future work including the other two species of vampire bat and non-hematophagous bats would be enlightening in regards to the timing of the recruitment for use in blood-feeding and the molecular diversification events. This study has opened the way for further research to investigate the pathways and intricate mechanisms of hematophagous venoms, in particular vampire bats. Therefore, we have made clear the ability of vCGRP to selectively mediate endothelium-independent vasorelaxation in part via activation of Kv channels.

This selectivity of vCGRP to target only vascular smooth cells (similar to that of human CGRP) highlights the interesting possibility that vCGRP may confer benefit in the context of cardiovascular diseases such as hypertension, heart failure and kidney diseases [44]. Further functional studies are required for vCGRP to become a therapeutic intervention with potential pharmacological applications. This research also paves the way for further evolutionary studies into hematophagous venoms.

4. Materials and Methods

Synthesis of vCGRP was accomplished using protocols previously described by us for other peptides [45].

4.1. Isolation of Rat Small Mesenteric Arteries

Male Sprague-Dawley rats (200–250 g) were euthanized via CO_2 inhalation (95% CO_2, 5% O_2) followed by exsanguination. Small mesenteric arteries (second-order branch of the superior mesenteric artery) were isolated, cut into 2 mm lengths, and mounted on 40 μm wires in small vessel myographs [46]. Vessels were maintained in physiological salt solution [composed of (in mM) 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.8 KH_2PO_4, 2.5 $CaCl_2$, 11 glucose, and 0.026 EDTA] at 37 °C and were bubbled with carbogen (95% O_2, 5% CO_2). In a subset of arteries, the endothelium was gently denuded via insertion of a 40 μm wire inside the lumen and rubbing the vessel walls. The mesenteric

arteries were allowed to equilibrate for 30 min under zero force and then a 5 mN resting tension was applied. Changes in isometric tension were recorded using Myograph Interface Model 610 M version 2.2 (DMT, Aarhus, Denmark) and PowerLab/835 (ADInstruments Inc, Bella Vista, NSW, Australia). Data was recorded with the data acquisition program Chart (V5, ADInstruments). Following a 30 min equilibration period at 5 mN, the mesenteric arteries were contracted maximally (F_{max}) using a K^+ depolarizing solution [K^+−containing physiological salt solution (KPSS); composed of (in mM) 123 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose]. The integrity of the endothelium was confirmed by relaxation to acetylcholine (ACh, 10 μM) [46] in tissues pre-contracted with the thromboxane A_2 mimetic, U46619 (1 μM) [46]. Arteries were washed with physiological salt solution and the tension allowed to return to baseline.

4.2. Vasorelaxation Experiments

Cumulative concentration-response curves to *D. rotundus* vCGRP (10^{-12}–3×10^{-8} M) or rCGRP (3×10^{-12}–10^{-8} M) [32,34,37] were constructed in vessels pre-contracted submaximally (~50% Fmax) with titrated concentration of U46619 (0.01 μM–0.2 μM). Responses to *D. rotundus* vCGRP and rCGRP were obtained in endothelium-intact mesenteric arteries in the absence or presence of either ODQ (10 μM) [47], SQ22536 (10 μM) [48], L-NAME (0.1 μM) [46], CGRP8-37 (0.1 μM) [49,50], 30 mM K^+ [33], TEA (1000 μM), 4-aminopyridine (1000 μM) [46] or glibenclamide (10 μM) [32,51]. All treatments were added for 30 min prior to precontraction with U46619. In a subset of endothelium-denuded arteries, vasorelaxation to *D. rotundus* vCGRP was also examined. Sodium nitroprusside (SNP; 10 μM) [47] was added at the end of each concentration-response curve to ensure maximum relaxation. Only one concentration-response curve to *D. rotundus* vCGRP or rCGRP was obtained in each vessel segment [47,52].

4.3. Data Analysis and Statistical Procedures

Relaxation responses were expressed as a percentage reversal of the U46619 pre-contraction. Individual relaxation curves were fitted to a sigmoidal logistic equation and pEC_{50} values (concentration of agonist resulting in a 50% relaxation) calculated and expressed as –Log $mol.L^{-1}$. Statistical comparisons between the experimental groups' mean pEC_{50} and maximum relaxation (R_{max}) values were made using a Student's unpaired *t*-test or one-way ANOVA with Bonferroni's post hoc comparison. Where pEC_{50} values could not be obtained, concentration-response curves were compared by means of a two-way ANOVA. *n* = number of artery segments from separate animals. Data represent the mean ± SEM (error bars on graph). Statistical significance was defined as * $p < 0.05$. All data analysis was performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA, USA, 2009) [46].

4.4. Reagents

Reagents and their sources were U46619 (Cayman Chemical company, Ann Arbor, Michigan, USA), SQ22356 (Tocris bioscience, Bristol, UK), ODQ, Glibenclamide, TEA, 4-aminopyridine, L-NAME, SNP, ACh, CGRP8-37 (Sigma-Aldrich, St Louis, MO, USA), and CGRP (rat) Peptide Institute, Osaka, Japan. Stock solutions of ODQ (10 mmol/L) and U46619 (1 mM) were dissolved in absolute ethanol. All subsequent dilutions of stock solutions were in distilled water. All other drugs were made up in distilled water and all dilutions were prepared fresh daily.

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CHAPTER 5- GENERAL DISCUSSION

5.1 Overview

As previously discussed (Chapter 1), snake venoms have evolved to immobilise and incapacitate prey for feeding, and as a defence mechanism against predators (Casewell et al., 2013). As such, venoms are complex mixtures of toxins with a wide range of activities targeting vital physiological processes (Fry et al., 2009). Key toxin groups including neurotoxins (Barber et al., 2013a, Chaisakul et al., 2010, Kuruppu et al., 2008), myotoxins (Gutiérrez and Lomonte, 1995, Silva et al., 2016a, Wickramaratna et al., 2003) and toxins with pro-coagulant, anticoagulant, haemolytic and local tissue necrotic activity (Gutiérrez and Lomonte, 2013a, Isbister et al., 2008, Joseph et al., 2004) which have been isolated, pharmacologically/biochemically characterised, and reasonably well studied.However, the nature and activity of the toxins affecting the cardiovascular system are less clearly understood. Thus, the focus of this thesis was to explore and demonstrate the profound cardiovascularеffects of snake venom. The work described in this thesis has provided key insights into the cardiovascular effects of some medically important snake venoms. In particular:

- *P. textilis* (Australian brown snake) and *E. ocellatus* (Carpet viper) venoms cause "rapid cardiovascular collapse" while *D. russelii* (Sri Lankan Russell's viper) and *D. siamensis* (Javanese Russell's viper) venoms cause "prolonged hypotension" *in vivo* in anaesthetised rats. We have identified key differences between these two outcomes – i.e. collapse is not prevented by commencing artificial respiration prior to venom administration, while hypotension is prevented by artificial respiration. Also, priming with low doses of venom prior to a larger dose of venom prevents collapse. However, priming does not prevent hypotension.
- *D. russelii* (Russell's viper) venom causes concentration-dependent vasorelaxation in rat small mesenteric arteries. The effect of the venom is endothelium independent and appears to be mediated by vascular smooth muscle K_v and K_{Ca}, but not K_{ATP}, channels. The venom may also be targeting TRPV4 channels.
- *D. rotundus* (Vampire bat) venom is also a potent vasodilator of rat small mesenteric arteries. However, the mechanism responsible for this activity is markedly different in comparison to *D. russelii* venom. A component (i.e. *D.r* vCGRP), which is similar in molecular weight and amino acid sequence to rat CGRP, was isolated from *D. rotundus* venom. *D.r* vCGRP targets both CGRP1 receptors and K_v channels directly on the smooth muscle to cause vasorelaxation. *D. r* vCGRP also has similar efficacy and potency to rat CGRP. Further functional and ligand

binding assays on this compound could lead to therapeutic potential for patients with endothelial dysfunction.

5.2 Blood pressure control: mechanisms

Targeting important physiological processes is a key feature of the toxins which constitute the complex mixture of components found in venoms. Given the cardiovascular system is vital for the survival of prey animals, inducing a rapid fall in blood pressure via one or more 'pathways' is advantageous to prey capture. This can lead to circulatory shock and rapid prey immobilisation, and may also aid in the diffusion of other snake venom components (Péterfi et al., 2019). As such, it is important to understand the mechanisms by which blood pressure is controlled in order to understand the effects of toxins on these processes.

Maintaining normal blood pressure is vital for all organs to function properly and is controlled by both short-term and long-term mechanisms. In general, Blood pressure = Cardiac Output (CO) x Total Peripheral Resistance (TPR), with CO = Heart Rate (HR) x Stroke Volume (SV). Based on these parameters, it is clear that in order to decrease blood pressure, toxins can affect blood vessels (i.e. TPR), and induce vasodilatation, or target the heart directly to decrease force/rate of contractions (i.e. CO). It is possible that venoms/toxins can act on either the heart or blood vessels or both. In addition, pro- and anti-coagulant toxins can also indirectly affect blood pressure. Thus, toxins that target the cardiovascular system can act via a number of different pathways to decrease blood pressure and assist in the immobilisation of prey.

The short term regulation of blood pressure is mainly controlled by the autonomic nervous system (ANS) (Stevens et al., 2016). Increases in blood pressure are detected by baroreceptors located in the aortic arch and carotid sinus. These baroreceptors send signals to the ANS which results in a decrease in heart rate, which leads to decreased cardiac output via efferent parasympathetic fibres. As seen in Figure 5.1, decreases in blood pressure are detected by the baroreceptors which then trigger a sympathetic response which causes an increase in heart rate and cardiac output, leading to increased blood pressure (Guyton et al., 1981). While this mechanism is very effective in stabilising blood pressure in the short-term, baroreceptors do not effectively maintain blood pressure in the long-term (Cowley Jr, 1992).

Long-term regulation of blood pressure involves several complex physiological mechanisms including the renin-angiotension-aldosterone system (RAAS) and anti-diuretic hormone (ADH) regulation (Cowley Jr, 1992, Guyenet, 2006). Renin, a peptide hormone found in the juxtaglomerular

apparatus in the kidney, is released in response to three different factors – (i) sympathetic stimulation, (ii) reduction of sodium-chloride delivery into the distal convoluted tubule of the kidney or (iii) decreased blood flow to the kidney. This leads to the conversion of angiotensinogen to angiotensin I, which then is converted to angiotensin II via angiotensin-converting enzyme (ACE). Angiotensin II is a potent vasoconstrictor that directly acts on the kidney to increase sodium reabsorption while also promoting the release of aldosterone. Aldosterone promotes both salt and water retention in the kidney and also increases the electrochemical gradient of sodium ions. Increased sodium and water concentration in the kidney leads to osmosis which results in decreased water excretion and thus increased blood volume which results in increased blood pressure (Guyton et al., 1981, Patel et al., 2017).

ADH is released from the hypothalamus in response to either an increase in plasma osmolarity or thirst signals. ADH stimulates sodium reabsorption from the thick ascending limb of the loop of Henle in the kidneys which increases water reabsorption which leads to increased plasma volume and decreased osmolality. It also increases the permeability of the collecting duct to water via aquaporin channels that get inserted into the apical membrane (Guyton et al., 1981, Stevens et al., 2016).

There are also other factors that can affect long-term blood pressure regulation such as natriuretic peptides (Cowley Jr, 1992). Atrial natriuretic peptides (ANP) are released in response to cardiomyocytes elongating, which occurs in response to high blood pressure. ANP is synthetised and stored in cardiac myocytes and act to promote sodium excretion by dilating the afferent arteriole of the glomerulus which increases blood flow and inhibits sodium reabsorption in the nephron. When blood pressure is low, ANP secretion is decreased accordingly which is evident of a feedback mechanism. To increase glomerular filter rate (GFR) and reduce sodium reabsorption, prostaglandins are also present. They act as local vasodilators as well as prevent excess vasoconstriction triggered by both the sympathetic nervous system and RAAS (Chen et al., 2020, Cowley Jr, 1992).

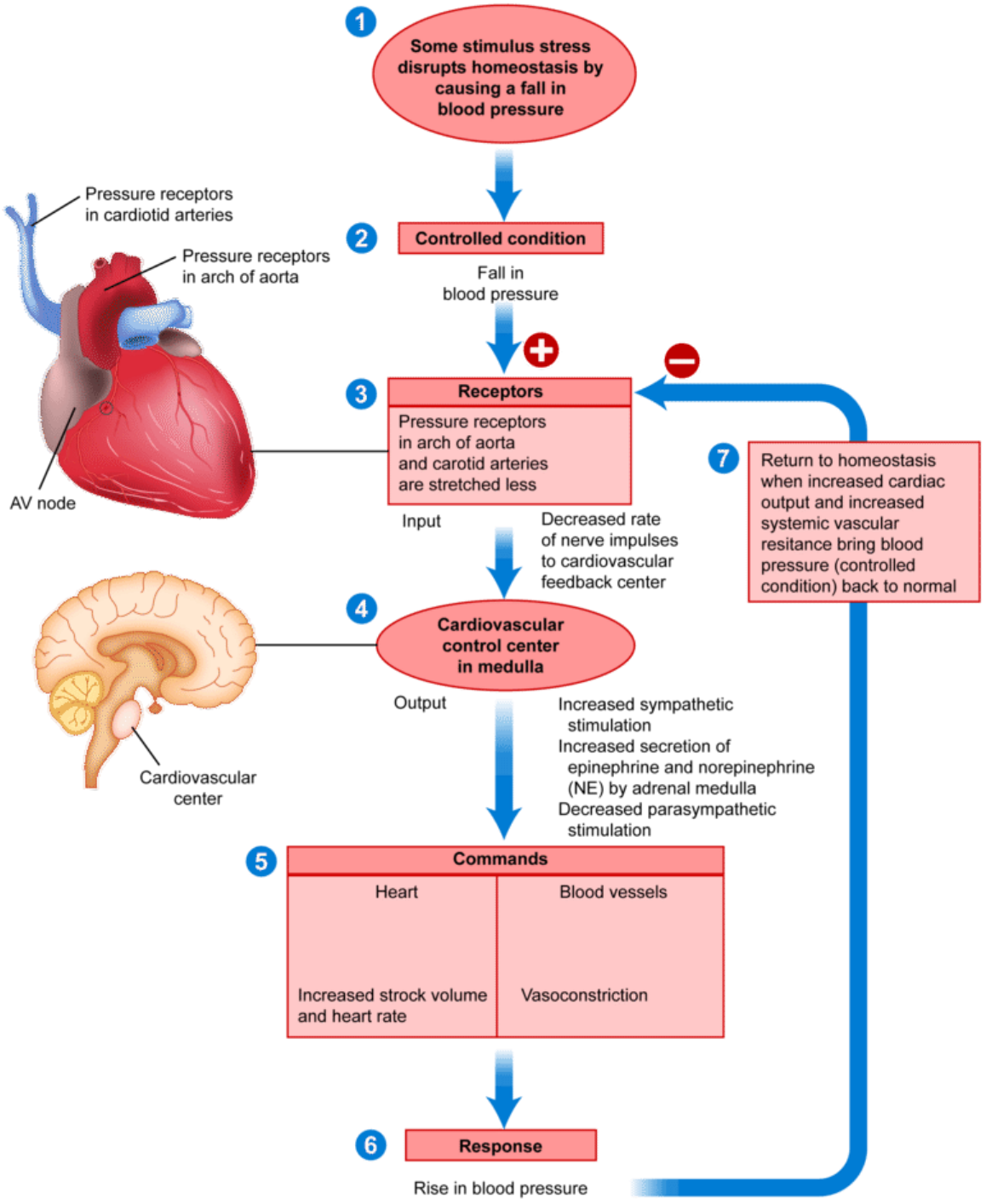

Figure 5.1: Diagram indicating the main mechanisms responsible for the control of blood pressure (Marieb and Hoehn, 2016).

5.3 Blood pressure control: potential sites of action of venoms/toxins

There are a number of cardiovascular effects associated with snake envenoming in humans, including hypotension, myocardial infarction, cardiac arrest, hypertension, brady- or tachy-cardia and atrial fibrillation (Ahuja, 1983, Koh and Kini, 2012, Moore, 1988). As previously discussed in Chapter 1, snake venom/toxins that cause hypotensive effects can be categorised into six different classes (Kininogens, Natriuretic peptides, Phospholipases A_2, Serine-Proteases, Vascular endothelial growth factor like peptides and other hypotensive components) (Latinović et al., 2020). Toxins that target blood vessels can act via different pathways including the nitric oxide (NO) pathway. For example *Crotalus oreganus abyssus* (Da Silva et al., 2011, Da Silva et al., 2012) and *Crotalus durissus cascavella* (Evangelista et al., 2008) venoms contain toxins that increase NO production which leads to vasodilatation while *Bothrops jararaca* venom contains toxins that inhibit ACE, increase NO production and lower cardiac output by inducing bradycardia and activating bradykinin receptors (Morais et al., 2011, Morais et al., 2013, Xavier et al., 2017). Snake species such as *Vipera ammodytes ammodytes* (Latinović et al., 2020), *Bothrops moojeni* (Silveira et al., 2013), *D. russelii* (O'Leary and Isbister, 2010), and *Oxyuranus scutellatus* (Chaisakul et al., 2014) contain PLA_2 components that decrease blood pressure by targeting the cyclooxygenase pathway, phospholipase pathway and inducing the release of endogenous mediators (e.g. PGI2).

Viper spp. also contain toxins such as serine-proteases that disrupt the blood coagulation pathways by activating plasminogen, activating factor V and/or disrupting the conversion of fibrinogen to fibrin (Isbister, 2009, Serrano and Maroun, 2005, Xiong and Huang, 2018). Serine-proteases present in *Lachesis muta* (Felicori et al., 2003), *Bitis arietans* (Megale et al., 2018), *Bitis gabonica rhino*ceros (Vaiyapuri et al., 2010) target the kallikerin pathway to decrease blood pressure. Toxins such as sarafatoxins and cardiotoxins are potent vasoconstrictors that directly cause cardiotoxicity (Takasaki et al., 1988).

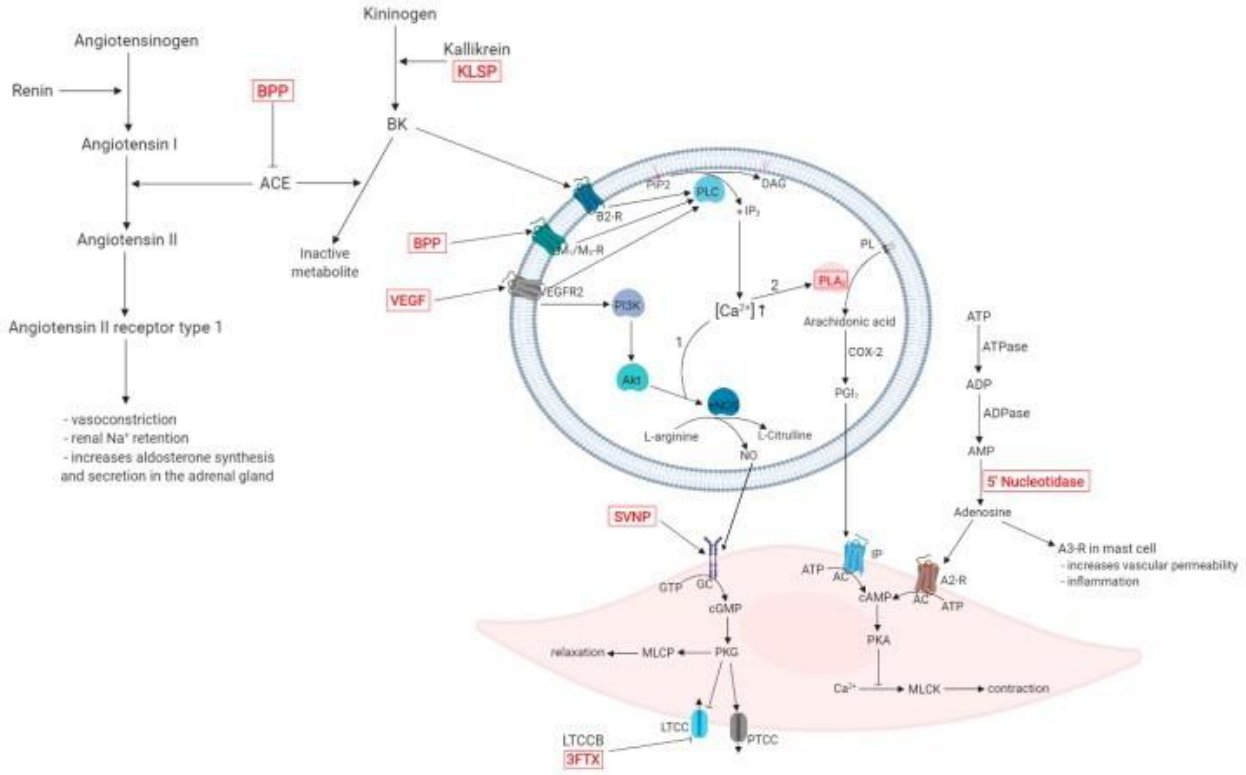

Figure 5.2: Overview of mechanisms of hypotensive effects of snake venom components. BPP (bradykinin-potentiating peptides), KLSP (kallikrein-like snake protein), VEGF (vascular endothelial growth factor), SVNP (snake venom natriuretic peptide), PLA_2 (phospholipase A_2), 3FTX (3-finger toxins) Source: (Péterfi et al., 2019)

There are many factors that can lead to venom-induced hypertension (Hodgson and Isbister, 2009). For example, sarafotoxons from burrowing vipers (*Atractaspis* spp.), which are small peptides similar to endothelins that cause vasoconstriction by binding to endothelin receptors (Kochva et al., 1982, Takasaki et al., 1988). Increasing capillary permeability protein (ICPP), isolated from blunt-nosed viper *(V. lebtina)* venom has similar structure and potency to vascular endothelial growth factor (VEGF) and causes an increase in vascular permeability (Gasmi et al., 2000). Some snake venoms have highly evolved toxins such as calciseptine, FS2 toxins, C10S2C2 and S4C8 which block L-type Ca^{2+} current, which leads to vasorelaxation (de Weille et al., 1991, Watanabe et al., 1995). Other toxins such as natriuretic peptides found in green mamba (*D. angusticeps*) venom (Schweitz et al., 1992) and bradykinin potentiating peptides (BPP's) found in *Bothrops spp.* are also potent vasorelaxants (Cintra et al., 1990, Ferreira and e Silva, 1965, Ferreira and Habermehl, 1997, Joseph et al., 2004) and lead to hypotension. BPP's inhibit both the breakdown of the endogenous vasodilator bradykinin and synthesis of the vasoconstrictor angiotensin II, resulting in a reduction of systemic blood pressure (Hodgson and Isbister, 2009). These peptides present in snake venom highlight the

complexity of snake venom and demonstrate that many different toxins can play a role in disrupting the cardiovascular system (Figure 5.2).

5.4 'Collapse' versus 'hypotension'

In this thesis, eight medically important species of snakes from a range of countries were investigated. The six exotic vipers (i.e. *B. gabonica, D. russelii, D. siamensis, C. vegrandis, E. ocellatus* and *B. arietans*) were chosen based on their high rate of mortality and/or morbidity following envenoming in humans. Their venoms have also been shown, either in animal studies or clinical reports, to affect the cardiovascular system (Chaiyabutr and Sitprija, 1999, Marsh et al., 1997, Tilmisany et al., 1986)). The two other snake species, which are both elapids, are from Australia: i.e. *P. textilis*, as it is the leading cause of death due to snake bite in Australia (via cardiovascular collapse), and *O. temporalis* which is a relatively recently described species found in Western Australia. There have been no reported cases of *O. temporalis* envenoming and very little is known about the venom.

In the current *in vivo* study, three distinct phenomena were observed. *E. ocellatus* and *P. textilis* venoms caused "cardiovascular collapse" whereas *D. russelii* and *D. siamensis* venoms caused "prolonged hypotension". *B. gabonica, C. vegrandis, B arietans* and *O. temporalis* venoms did not significantly affect the blood pressure of anaesthetised rats at the doses tested (Table 5.1). For all the snake venoms tested, we started with a standard dose that was higher than expected in envenomed patients. Healy et al (2019) reported that the average snake venom yield of 102 snakes spanning six families was between 0.15mg (in egg eating sea snake) to 571 mg in forest cobra (Healy et al., 2019). Patients envenomed with taipan venom had an average of 10 ng/ml detected in their blood (Kulawickrama et al., 2010), while patients envenomed by red-bellied black snakes were found to have a slightly higher average concentration of 19 ng/ml (Churchman et al., 2010). In patients envenomed by *D. russelli*, which injects a higher amount of venom than most snakes, the median concentration of venom found was 201 ng/ml (Isbister et al., 2015). Thus, the concentrations we used in the study (200 µg/ kg), which is approximately 3 µg/ml venom blood concentration in a 300 g rat, were much higher than what will be injected into a human during snake bite. This was to determine whether the venom caused rapid collapse, hypotension or no effect. This enabled us to separate the venoms that affected blood pressure and those that didn't. For the venoms that did affect blood pressure (i.e. *P, textilis, E. ocellatus, D. russelii and D. siamensis*), we then tested the lowest amount of venom required to observe a significant change in blood pressure. These dosages were lower than that expected in envenomed victims. This was due to either the amount of venom available for experimental use or the potency of the venom.

Previously, “cardiovascular collapse” and “hypotension” have often been used interchangeably to describe a marked decrease in blood pressure following envenoming. However, we have shown that these two phenomena have a number of key differences indicating that they are likely to be due to different mechanisms. In Chapter 2, we have defined **rapid cardiovascular collapse** as a “rapid decrease in blood pressure, often without recovery”, while **prolonged hypotension** is defined as a “slower and prolonged decrease in blood pressure, with recovery occurring in most cases” (Kakumanu et al, 2019). A defining feature of ‘rapid cardiovascular collapse” is that it is attenuated by small ‘priming’ doses of venom of the same, or different, snake species (Chaisakul et al., 2013), which demonstrates a common mechanism across species. This also indicates the preservation of certain toxins or group of toxins during evolution (Fry et al., 2009). Interestingly, this collapse response in not prevented by artificial respiration (*in vivo* model), indicating that paralysis or respiratory arrest due to neurotoxins/myotoxins are unlikely to involved in collapse. This also supports previous postulations that depletable mediators may induce collapse, which is attenuated with prior administration of smaller sub-toxic doses of venom that causes pre-release and depletion of these mediators (Chaisakul et al., 2012, Chaisakul et al., 2013, Chaisakul et al., 2015), as artificial respiration could not prevent collapse.

Table 5.1: Summary of snake venoms studied in vivo indicating hypotension/cardiovascular collapse in the presence and absence of either priming or artificial respiration.

Snake species	Cardiovascular collapse	Hypotension (prolonged)	Prevented by priming	Prevented by artificial respiration
D. russelii	-	✓	-	✓
E. ocellatus	✓	-	✓	-
B. gabonica	NA	NA	NA	NA
C. vegrandis	NA	NA	NA	NA
B. arietans	NA	NA	NA	NA
D. siamensis	-	✓	NA	NA
O. temporalis	NA	NA	NA	NA
P. textilis	✓	-	✓	-

In contrast, “prolonged hypotension” does not appear to be due to the release of depletable mediators. In order to mimic “cardiovascular collapse” a high dose of *D. russelii* venom was administrated to the *in vivo* model. Interestingly, when the rat was placed on an artificial respirator prior to venom administration, this so-called “collapse” was prevented (Kakumanu et al, 2019). *D. russelii* and *D. siamensis* venom both contain weak neurotoxins (Silva et al., 2017) that in humans do not cause life threatening paralysis. However, given that rats are much smaller than humans and have approximately

64 ml of circulating blood per kg body weight, an intravenous dose of 1mg/kg of venom will result in a very high blood concentration of 16ug/ml. This amount may be sufficient to induce respiratory paralysis as previous studies have shown that the same venom causes complete neuromuscular blockage in the chick biventer nerve-muscle preparation (Silva et al., 2017). Thus, artificial respiration is able to prevent or overcome the paralytic effects of the neurotoxins on the rat diaphragm. However, when the components of *D. russelii* venom were separated using SEC-HPLC and administrated *in vivo* in anaesthetised rats, the neurotoxic component alone did not cause a hypotensive effect (Chapter 3, Table 3.1), which indicates that the components all act in a synergistic manner to cause hypotension.

In Chapter 3, it was also demonstrated that *D. russelii* venom also caused concentration-dependent relaxation of rat small mesenteric arteries, suggesting that peripheral vasodilation contributes to the "prolonged hypotension" observed *in vivo*. In the presence of a non-selective K_{Ca} channel blocker (i.e. TEA) and voltage-gated K^+ channel inhibitor (i.e. 4-aminopyridine), the effects of the venom were markedly attenuated. Similarly, blocking large ($BK_{Ca;}$ iberiotoxin), intermediate ($IK_{Ca;}$ TRAM-34) and small ($SK_{Ca;}$ apamin) Ca^{2+}-activated K^+ channels significantly inhibited venom-mediated vasorelaxation. These findings suggest the involvement of both K_{Ca} and K_v channels in *D. russelii* venom-induced vasorelaxation (Figure 5.3). The vasorelaxant effect of the venom was also attenuated in the presence of the TRPV4 antagonist, RN1734 further supporting this concept. Whilst TRPV4 receptors are abundantly expressed on the endothelium (Baylie and Brayden, 2011), they are also present on the vascular smooth muscle (Baylie and Brayden, 2011, Inoue et al., 2006). Here activation of TRPV4 leads to Ca^{2+} influx, the generation of Ca^{2+} sparks from the sarcoplasmic reticulum and the subsequent activation of BK_{Ca} and vasorelaxation (Earley et al., 2005, Earley et al., 2009). As such, *D. russelii* venom may cause vasorelaxation of resistance arteries, in part, via activation of vascular smooth muscle TRPV4 and opening of K_{Ca}. Interestingly, denuding the endothelium did not affect the venom induced relaxation, indicating that the venom is selectively targeting K^+ channels on the vascular smooth muscle cells.

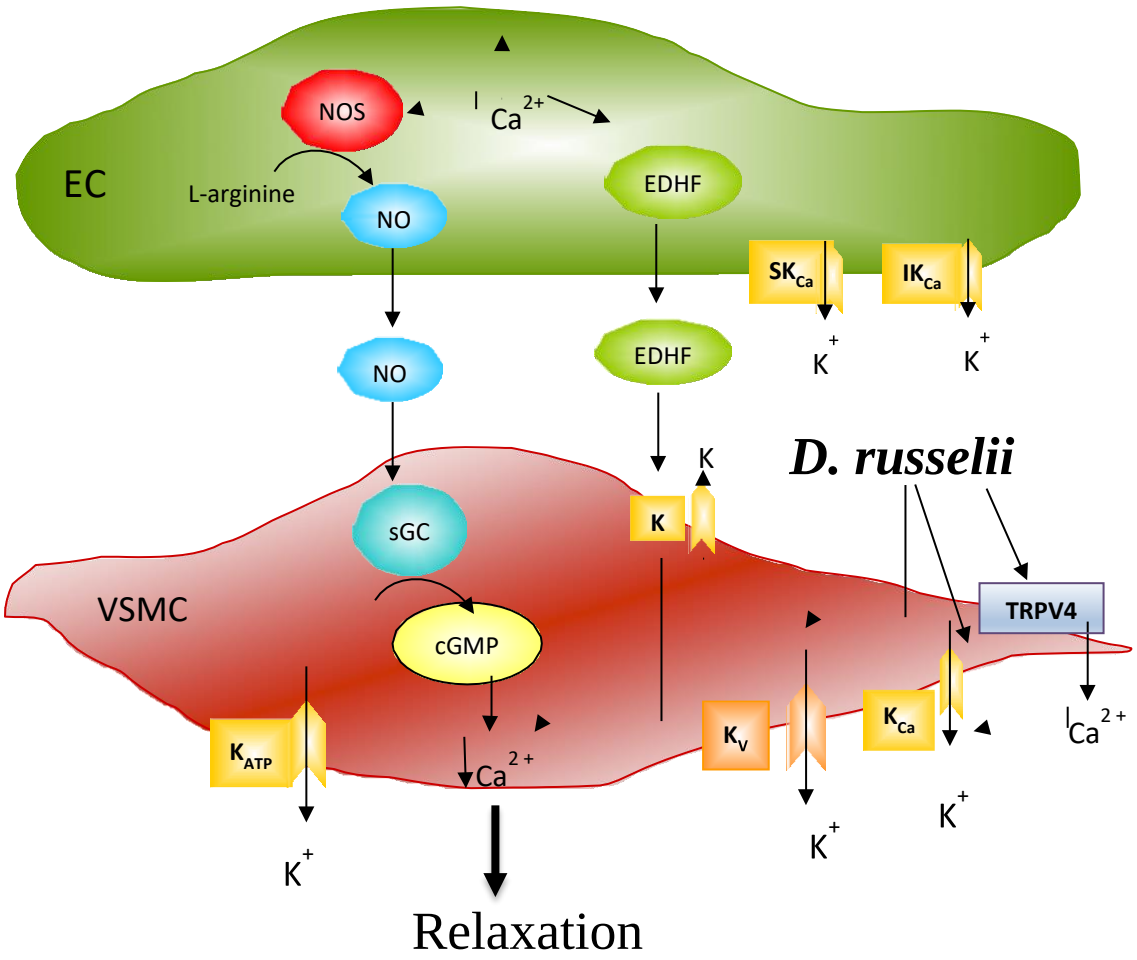

Figure 5.3: Diagram of mechanism of action of *D. russelii* venom on vascular smooth muscle cells to cause vasorelaxation. K (potassium channel), EDHF (endothelium derived hyperpolarizing factor), NO (nitric oxide) NOS (nitric oxide synthase), EC (endothelial cell), VSMC (vascular smooth muscle cell), K_{ATP} (ATP sensitive potassium channel), K_V (voltage-gated potassium channel), K_{Ca} (calcium activated potassium channel) sGC (soluble guanylate cyclase), cGMP (cyclic guanosine monophosphate), TRPV4 (Transient receptor potential cation channel subfamily V member 4).

For my final research study, we were fortunate to gain access to a synthetic peptide (venom (v)CGRP) based on a component found in vampire bat venom. vCGRP is similar in size and amino acid sequence to human CGRP. In contrast to snake venoms, the purpose of bat venom is not to kill prey, as it feeds off the blood of livestock animals (Delpietro et al., 1992). Instead, the venom has evolved to disrupt the coagulation pathway and enable constant flow of blood while the predator is undetected. *D. rotundus* venom is known to contain anticoagulating properties to aid in blood feeding. It contains a glycoprotein, Draculin, which inhibits activated coagulation factors IX (IXa) and X (Xa) (Fernandez et al., 1998). Using *in vitro* techniques, we showed that vCGRP is a dilator of rat mesenteric arteries,

with similar potency and efficacy to rat CGRP. Importantly, like rat CGRP, vCGRP also targets CGRP1 receptors to cause vasodilation as its effects were attenuated by the CGRP1 antagonist, CGRP8-37. However, surprisingly, while rat CGRP targets both endothelium and vascular smooth muscle cells, vCGRP only targets vascular smooth muscle cells as evidenced by maintained vasorelaxation following endothelial denudation or inhibition of nitric oxide synthase. Previous studies have suggested that activation of CGRP1 receptors can lead to activation of the soluable guanylyl cyclase pathway or adenylyl cyclase pathway (Boussery et al., 2005, Brain and Grant, 2004, Gray and Marshall, 1992, McNeish et al., 2012, Shoji et al., 1987, Zygmunt et al., 1995). Yet in our study, there was no significant change in the relaxation curves to rCGRP or vCGRP in the presence of ODQ (soluable guanylyl cyclase inhibitor) or SQ22536 (adenylyl cyclase inhibitor). This could be due to differences in the region, size of vessel tested and species of CGRP tested. For instance, human or rat CGRP tested in isolated pig coronary arteries leads to increased cAMP and causes vasorelaxation via endothelium-independent pathways (Shoji et al., 1987) while vasorelaxation to human CGRP, in human vessels, is endothelium-dependent (Thom et al., 1987).

Interestingly, in the presence of the K_v channel blocker aminopyridine (Figure 5.4), the effects of vCGRP and rat CGRP were markedly attenuated. Blocking K_{Ca} or K_{ATP} channels however, did not have an effect on venom-induced vasorelaxation. Previous studies have demonstrated that activation of CGRP1 can lead to direct opening of K^+ channels, especially K_v channels (Boussery et al., 2005), however there have been conflicting reports on the involvement of K_{ATP} and K_{Ca} channels, and this can be attributed to the type of vessels studied and species of CGRP (as mentioned in chapter 4). This selectivity of vCGRP to target only vascular smooth cells (similar to that of human CGRP in non-human arteries) highlights the interesting possibility that vCGRP may confer benefit in the context of cardiovascular diseases such as hypertension, heart failure and kidney diseases (Aubdool et al., 2017).

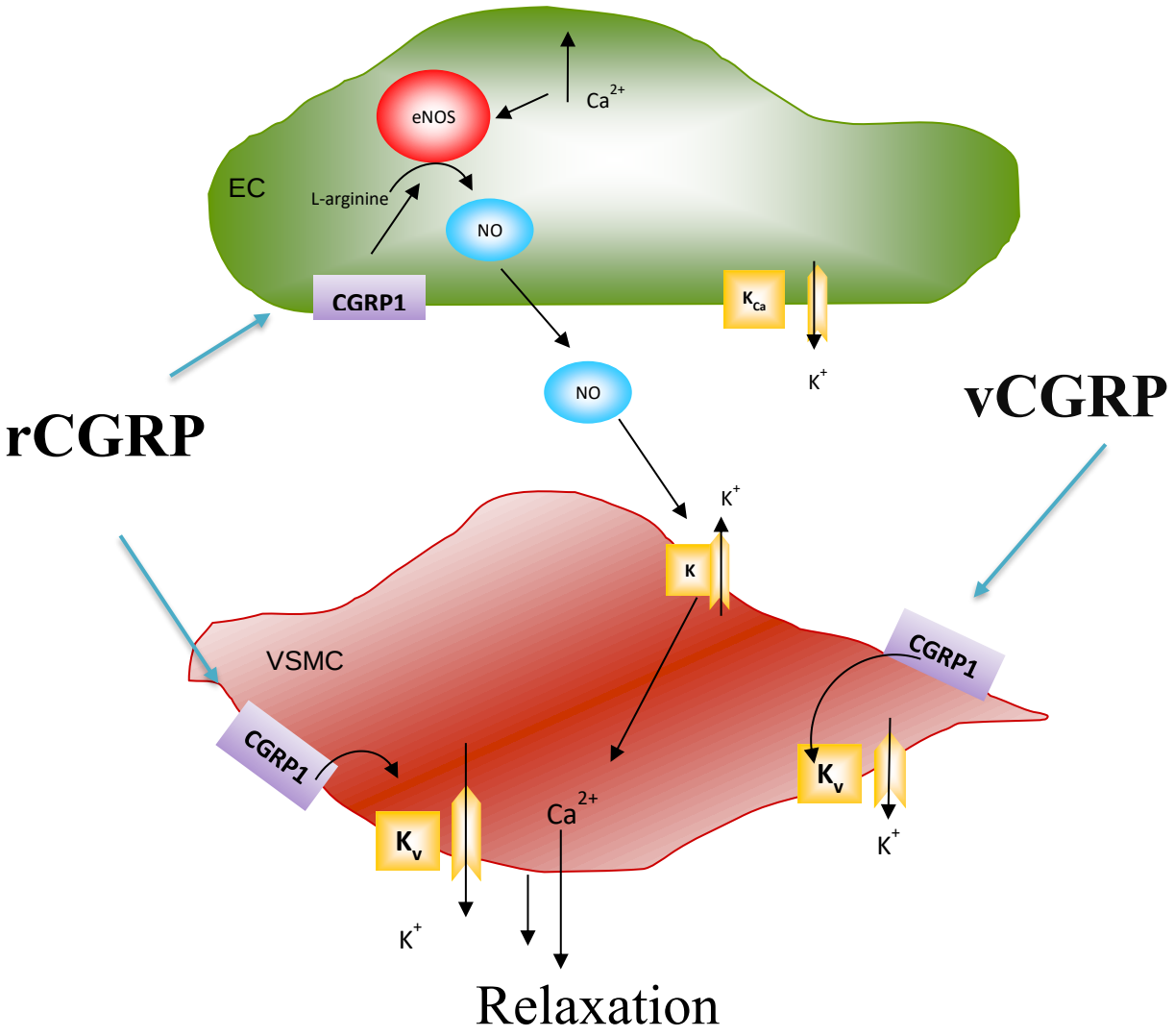

Figure 5.4: Diagram of mechanism of action of vCGRP and rCGRP on vascular smooth muscle cells to cause vasorelaxation. EC (endothelial cell), VSMC (vascular smooth muscle cell), K_V (voltage-gated potassium channel), K_{Ca} (calcium activated potassium channel), NO (nitric oxide), eNOS (endothelial nitric oxide synthase), CGRP1 (calcitonin gene-related peptidereceptor).

While *D. russelii* venom acted via potassium channels to cause vasorelaxation, *D. rotundus* venom acted via CGRP receptors. This difference in mechanism demonstrates that complexity of animal venoms. For instance, the same pathology (i.e. hypotension) from two different venoms can be due to different toxins and different mechanisms. There have been no studies that show snake venom acting via CGRP receptors to cause vasodilatation. However, studies have shown that CGRPreceptors may play a small role in *Phoneutria nigriventer* (Wandering spider) venom-induced plasma extravasation in rat skin (Costa et al., 2000) as well as pain modulation (Lauria et al., 2020). Activation of CGRP receptors by *P. nigriventer* venom can lead to hyperalgesia and release of other

pro-inflammatory cytokines (Lauria et al., 2020, Zanchet et al., 2004).

5.5 Limitations, future directions and clinical implications

There are limitations in the work described in this study. A major limitation was venom sample variabilities. Previous studies have shown that the same species of snake can have different venom composition depending on where it was found. For example, intra-specific venom variation was shown in *O. scutellatus* venom found from 13 different snakes spanning 2,000 km distance from four localities on the north-east coast of Australia (Tasoulis et al., 2020). Studies in India have also shown that *D. russelii* venom composition varied from South India and North India (Jayanthi and Gowda, 1988). More specifically, *D. russelii* venom samples from North and West India contained high acidic phospholipase activity that was not present in South Indian *D. russelii* venom samples. *P. textilis* venom from different parts of Australia was also shown to contain different amounts of procoagulants and presynaptic toxins (Skejić and Hodgson, 2013). Thus venom samples from the same species could display different experimental results due to geographical variations in venom collection.

Furthermore, in order to identify and characterise the toxins responsible for disrupting the cardiovascular system, the toxins need to be isolated. This is a lengthy process as it first requires crude separation methods of the toxins using SEC-HPLC. Next, the fractions need to be freeze-dried, tested for protein amount using biochemical assays such as BCA assay and then tested *in vivo* on rat models to determine which fractions affect blood pressure. Once those fractions are identified, further isolation and purification methods using RP-HPLC and mass spectrometry are required before testing *in vivo* once more. Once the toxin is completely isolated, then it also will require further pharmacological studies to determine mechanism of action. The information gained from isolating the toxin(s) however, will provide invaluable information that can be used both for treatment and further studies of lead compounds for therapeutic advancements.

In Chapter 3, we characterized the effects of *D. russelii* venom on resistance-like arteries. While the venom targets K^+ channels, TRPV4 channels also seems to play a role in vasorelaxation induced by the venom. Therefore, further electrophysiology experiments will be beneficial in determining whether TRPV4 is also involved in vasorelaxation.

Similarly, in Chapter 4 we identified a component of *D. rotundus* venom that caused vasorelaxtion in part via CGRP1 receptors and K^+ channels. CGRP1 knock out rat experiments as well as ligand binding assays may be useful in understanding how the toxin interacts with CGRP1 receptors, and why it selectively targets CGRP1 on the smooth muscle and not endothelial cells. This selectivity

could also be used as a therapeutical intervention for patients with endothelial dysfunction (ie. diabetes).

Another limitation of these studies is the use of rat models to determine effects of snake envenomingin humans. For instance, it was speculated that cardiovascular collapse in humans is similar to that of rodents. However, rodents are prey to snakes and thus, evolution-wise, the type and amount of toxins in snake venoms may be more tailored to immobilise and kill rodents but not humans. Numerous studies have indicated that geographical location and diet play a role in venom composition. While there are similarities between human and rat physiology, a better model could be the use of *in vitro* human cells such as cardiomyocytes and smooth muscle cells.

In regards to clinical implications, there have been cases reported where hypotension or collapse has occurred rapidly in humans after envenoming but the person has recovered before reaching the hospital. Thus, understanding the mechanism behind both these manifestations will help in providing better therapy for the patient. Similarly, hypertension due to snake envenoming is quite rare, thus understanding the physiological/pharmacological mechanism will help with better treatment strategies.

In conclusion, this thesis has primarily focussed on studying the effects and, mechanisms involved, in venoms that affect the cardiovascular system. Future work should focus on the isolation and characterisation of the different toxins present in these snake venoms. This will enable further exploration into the pharmacological characterisation and mechanisms involved in cardiovascular modulation by these toxins as well their potential as lead compounds for therapeutic interventions. Importantly, it will also help to produce more efficient treatment strategies for envenomed patients.

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