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Geographical venom variations of the Southeast Asian monocled cobra (*Naja kaouthia*): venom-induced neuromuscular depression and antivenom neutralization

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ABSTRACT

The Southeast Asian monocled cobras (*Naja kaouthia*) exhibit geographical variations in their venom proteomes, especially on the composition of neurotoxins. This study compared the neuromuscular depressant activity of the venoms of *N. kaouthia* from Malaysia (NK-M), Thailand (NK-T) and Vietnam (NK-V), and the neutralization of neurotoxicity by a monospecific antivenom. On chick biventer cervicis nerve-muscle preparation, all venoms abolished the indirect twitches, with NK-T venom being the most potent (shortest t_{90}, time to 90% twitch inhibition), followed by NK-V and NK-M. Acetylcholine and carbachol failed to reverse the blockade, indicating irreversible/pseudo-irreversible post-synaptic neuromuscular blockade. KCl restored the twitches variably (NK-M preparation being the least responsive), consistent with different degree of muscle damage. The findings support that NK-T venom has the most abundant curarimimetic alpha-neurotoxins, while NK-M venom contains more tissue-damaging cytotoxins. Pre-incubation of tissue with *N. kaouthia* monovalent antivenom (NKMAV) prevented venom-induced twitch depression, with the NK-T preparation needing the largest antivenom dose. NKMAV added after the onset of neuromuscular depression could only halt the inhibitory progression but failed to restore full contraction. The findings highlight the urgency of early antivenom administration to sequester as much circulating neurotoxins as possible, thereby hastening toxin elimination from the circulation. In envenomed mice, NKMAV administered upon the first neurological sign neutralized the neurotoxic effect, with the slowest full recovery noticed in the NK-T group. This is consistent with the high abundance of neurotoxins in the NK-T venom, implying that a larger amount or repeated dosing of NKMAV may be required in NK-T envenomation.

1. Introduction

Globally, it is estimated that as high as 1,841,000 snakebite envenomings with 94,000 resultant deaths occur each year (Kasturiratne et al., 2008). The exact statistics is often underestimated in view of the persistent under-reporting of morbidity and mortality of the problem (Mohapatra et al., 2011; Warrell et al., 2013). Snakebites typically result from the defensive behavior of snakes when they are treaded upon in agricultural or rural settings, although the risk is equally shared by many hobbyists who keep venomous snakes as pets. Bites by venomous snakes potentially result in envenomation, a clinical condition marked either by local toxicity (tissue inflammation and necrosis) or systemic effect when substantial amount of toxins are absorbed and distributed into the body (WHO, 2010a). The clinical syndrome of snakebites manifests variably, with neurotoxicity and hematotoxicity being the most commonly reported. Neurotoxicity is usually associated with bites by elapid snakes such as cobras, kraits, coral snakes and sea snakes, posing a life threat that needs urgent medical care. Rapid onset of neuromuscular paralysis leads to respiratory failure and death if left untreated.

The mechanisms of neuromuscular blockade induced by snake venoms are commonly studied using *in vitro* nerve-muscle preparation from chick biventer cervicis or rat hemi-diaphragm (Barber et al., 2013; Harvey et al., 1994; Tan et al., 2015d). In elapid envenomation, the neuromuscular blockade is mainly attributed to the presence of alpha-neurotoxins and/or beta neurotoxins (Barber et al., 2013; Tan et al., 2015b). Alpha-neurotoxins are polypeptides of the three-finger toxin family that block the post-synaptic nicotinic receptors, while beta-neurotoxins are phospholipases A_2 that damage presynaptic nerve terminals causing depletion of the neurotransmitter storage (Ranawaka et al., 2013). Despite the apparently convergent function shared by

Abbreviations: ACh, acetylcholine; CCh, carbachol; CBCNM, chick biventer cervicis nerve-muscle; NK-M, *Naja kaouthia* of Malaysia; NK-T, *Naja kaouthia* of Thailand; NK-V, *Naja kaouthia* of Vietnam; KCl, potassium chloride; NKMAV, *Naja kaouthia* monovalent antivenom.

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most elapid venoms, differences in the toxicity of venoms and the therapeutic response to antivenom remain important and clinically relevant (Casewell et al., 2014; Warrell et al., 2013). This is commonly due to the wide diversity of snake venom toxins, where different isoforms within a multigene family exhibit variable amino acid sequences as adaptation for neofunctionalization propelled under accelerated evolution (Calvete et al., 2009; Kini and Doley, 2010; Tan et al., 2015a). Variations as such in venom compositions generally follow a particular trend of taxonomy and are confined to individual species. Nonetheless, in recent years substantial venom variations have been increasingly revealed even within the same species, contributed commonly by geographical factors (Lomonte et al., 2014; Tan et al., 2015c). Knowledge on this is vital to elucidate the discrepancies in the clinical effect of venoms and antivenom effectiveness, especially in situations where the treatment relies on antivenoms supplied from a single source. Moreover, in pace with the latest development of snake systematics, information on the composition of venoms from authenticated species becomes extremely valuable to the database for future references. This is highly relevant to Asiatic cobras (*Naja* sp.) which have been subjected to thorough taxonomic revision, resulting in recognition of multiple distinct species in Asia currently (Wuster, 1996).

Earlier, the scientific names of different cobras in Thailand and the surrounding countries (*Naja naja kaouthia*, *Naja naja sputatrix*, *Naja naja siamensis, Naja naja atra etc.*) were in a constant state of confusion until clarified later through phylogenetics by Wüster (Wuster, 1996). It is currently established that the distinct monocled cobra (*Naja kaouthia*) distributes across the eastern Indian subcontinent to most parts of Indochina (including the northern region of Peninsular Malaya) and south China, and is responsible for most of the envenoming cases in these regions (Chew et al., 2011; Reid, 1964; Viravan et al., 1986; Wongtongkam et al., 2005). Systemically, envenoming by this species leads to neuromuscular paralysis, while tissue necrosis (local effect) can leave survivors with permanent crippling deformity. More recently, a comparative proteomic study of *N. kaouthia* venoms from three different Southeast Asian regions (Malaysia, NK-M; Thailand, NK-T; Vietnam, NK-V) unveiled the distinctly diverse compositions among these venoms of different localities (Tan et al., 2015c), consistent with the variable clinical and lethal effects of the venoms (Leong et al., 2012; Viravan et al., 1986). This indicates the need for further investigation into the functional and mechanistic properties of the venoms sourced from different localities, as well as how they respond to antivenom that is produced from a single venom source. Considering that rapid paralysis constitutes the principal lethal syndrome in *N. kaouthia* bite, this study aimed to elucidate the mechanism of neuromuscular blockade of *N. kaouthia* venoms through the use of a chick biventer cervicis nerve-muscle preparation (CBCNM), and to study how the blockade could be reversed by the monospecific antivenom. In addition, the neurotoxicity and its reversibility were correlated *in vivo* through a challenge-rescue model in mice. It is hoped that the findings provide further insights into the venom pathophysiology of *N. kaouthia* in Southeast Asia, and serve as a guide on how antivenom use can be optimized.

2. Materials and methods

2.1. Snake venoms and antivenoms

The venom of Malaysian *N. kaouthia* was collected from specimens in the northern region of the Malayan Peninsula (NK-M, identified by author Tan CH). The venom of Thailand (NK-T) and Vietnam (NK-V) *N. kaouthia* were gifts from Professor Kavi Ratanabanangkoon of the Chulabhorn Graduate Institute, Bangkok. All the venoms were pooled samples and lyophilized products stored at −20 °C until use. The antivenom used in the studies is a product of Queen Saovabha Memorial Institute (QSMI) in Bangkok, Thailand: *N. kaouthia* monovalent antivenom (Cobra antivenin; Lyophilized; Batch no. NK00310; Exp. Date Aug. 9th, 2015). It is a purified $F(ab')_2$ obtained from antiserum of horses hyperimmunized specifically against the venom of *N. kaouthia* of Thai origin. The antivenom was reconstituted according to the product leaflet, where 10 ml of normal saline was added to one vial of the lyophilized antivenom.

2.2. Chemicals and reagents

All chemicals and reagents used in the studies are analytical grade. The acetylcholine chloride (ACh) and carbachol (CCh) were purchased from Sigma-Aldrich (USA). The ingredients used to prepare physiological salts: sodium chloride (NaCl), potassium chloride (KCl), magnesium sulfate ($MgSO_4$), monopotassium phosphate (KH_2PO_4), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$) and glucose were purchased from Merck (USA).

2.3. Animals and ethics clearance

Mice used in this study were of albino ICR strain (20–25 g) supplied by the Animal Experimental Unit, University of Malaya. Male chicks (4–10 days old) were obtained from local farm. The animal study protocol was based on the Council for International Organizations of Medical Sciences (CIOMS) guidelines on animal experimentation (Howard-Jones, 1985) and the EU Directive 2010/63/EU for animal experiments. The protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, University of Malaya (Ethics reference number: 2014-09-11/PHAR/R/TCH).

2.4. Chick biventer cervicis nerve-muscle (CBCNM) preparation

2.4.1. Experimental procedure for nerve-evoked and muscle-evoked tissue contraction

Male chicks (4–10 days old) were euthanized by isoflurane inhalation and both biventer cervicis were removed. The tissues were mounted under 1 g tension (gt) in 15 ml organ baths containing physiological solution of the following composition (mM): NaCl 118.4; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25 and glucose 11.1. The physiological salt solution was aerated with carbogen (5% CO_2 and 95% O_2) and maintained at 34 °C. The tissues were stimulated every 10 s with pulses of 0.2 ms duration at a supramaximal voltage using a Grass S5 stimulator attached to silver ring electrodes. The nerve-evoked indirect stimulation of the tissues was confirmed by the abolishment of twitches by d-tubocurarine (d-TC; 10 μM). For the direct muscle stimulation, an electrode was placed on the belly of the muscle in a fresh tissue set up. All tissue responses to indirect or direct electrical stimulation were measured *via* a force transducer (ADInstrument ML_T050/D) and recorded on a PowerLab system (ADInstruments). The change in muscle twitch tension was expressed as a percentage of the initial nerve-evoked response prior to the addition of venom (mean ± SEM). The changes in the baseline were measured from the initial baseline prior to the addition of venom. Data analyses were performed by using one-way analysis of variants (ANOVA), followed by Dunnet's multiple comparison tests (SPSS). Statistical significant is indicated when $^*p < 0.05$.

2.4.2. Neuromuscular depressant activity of N. kaouthia venoms

The responses of the tissues to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s) and potassium chloride (KCl; 40 mM for 30 s) were obtained in the absence of electrical stimulation both prior to the addition of venom and at the end of the experiment (Harvey et al., 1994). The CBCNM preparations were allowed to equilibrate for 30 min period before the experiments. Venom sample doses were left in contact with the preparations until complete twitch blockade or a maximum of 180 min period. The effect of venoms (5 μg/ml) on the direct muscle twitches (0.1 Hz, 2 ms) was examined in CBCNM