TOXINOLOGICAL, PROTEOMIC AND PHARMACOKINETIC CHARACTERIZATION OF EQUATORIAL SPITTING COBRA (*NAJA SUMATRANA*) VENOM

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ABSTRACT

Naja sumatrana, the Equatorial spitting cobra, is listed as one of the medically important species in Southeast Asia and is the common spitting cobra in Peninsula Malaysia. The aims of this study are to investigate the toxinology, proteome and pharmacokinetic characteristics of *N. sumatrana* venom, which will contribute to management of cobra envenomation. The lethality and enzymatic activities of *N. sumatrana* were compared to venoms from two other regional spitting cobras: *Naja sputatrix*, *Naja siamensis* and a non-spitting cobra *Naja kaouthia*, which also occurs in Malaysia. Previously, the three spitting cobras were considered as belonging to one species, *N. sputatrix*. Results showed that the three spitting cobra venoms possess different venom composition, but all three contain basic phospholipases A_2 and high content of polypeptide cardiotoxins. The proteome of *N. sumatrana* venom was investigated using shotgun analysis, combination of multi-dimensional chromatography and 2DE. Shotgun analysis revealed the presence of 50 individual proteins in the venom, with three finger toxins (both neurotoxins and cardiotoxins) and phospholipase A_2 constituted about 38% and 36%, respectively of the types of proteins identified. The ion exchange and reverse phase HPLC revealed the presence multiple basic venom proteins including 17 identified protein toxins (8 PLA_2, 4 neurotoxins and 5 cardiotoxins) whereas the Sephadex® G-50-2DE revealed the presence of another 11 high molecular weight proteins. Of the 17 protein toxins identified, only 7 exist in substantial amount, including 2 phospholipases A_2, 1 short and 1 long α-neurotoxin and 3 cardiotoxins. Together these seven major venom toxins constituted 87% of total venom protein and are primarily responsible for the pathophysiological action of the venom.

The pharmacokinetics of *N. sumatrana* and *N. sputatrix* venom in rabbits were also investigated. The serum toxin levels-time profile of both venoms following intravenous administration fitted a two-compartment model of pharmacokinetics with similar

pharmacokinetic parameters. The two venoms also have comparable intramuscular bioavailability in rabbits, both approximately 40%. To further understand the pharmacokinetics of venom toxin components, the pharmacokinetics of three main *N. sumatrana* venom toxins (short chain α-neurotoxin, cardiotoxin and basic phospholipase A_2) were also investigated. When toxins were injected intramuscularly, neurotoxin and cardiotoxin reached C_{max} within 30 min, which was much faster than phospholipase A_2, and the whole venom, reflecting a very rapid absorption of neurotoxin and cardiotoxin from the site of injection to systemic circulation. It was found that neurotoxin and cardiotoxin were eliminated from systemic circulation more quickly than other venom components. The neurotoxin had an intramuscular bioavailability ($F_{i.m.}$ = 81.5%) much higher than phospholipase A_2 ($F_{i.m.}$ = 68.6%) and cardiotoxin ($F_{i.m.}$ = 45.6%). The high bioavailability and short T_{max} of neurotoxin when injected intramuscularly explained why neurotoxic effect is the dominant symptom in most cobra bites. In conclusion, a comprehensive understanding of the toxinology, proteome and pharmacokinetics of *N. sumatrana* venom and its toxins provide significant insights into the overall pathophysiological actions of the venom.

ABSTRAK

Ular tedung jenis meludah, *Naja sumatrana* telah disenaraikan sebagai salah satu species yang penting dari segi perubatan di Asia Tenggara. Ia merupakan sejenis ular tedung jenis meludah yang biasa terdapat di Semenanjung Malaysia. Projek ini bertujuan untuk mengkaji sifat-sifat toksinologi, proteom dan farmakokinetik bisa ular *N. sumatrana*. Ini dapat menyumbang kepada pengurusan pembisaan ular tedung. Aktiviti-aktiviti maut dan enzim bisa ular *N. sumatrana* telah dibandingkan dengan spesis ular tedung lain di rantau ini (termasuk Malaysia) iaitu bisa daripada ular tedung jenis meludah: *Naja sputatrix*, *Naja siamensis* dan ular tedung jenis tidak meludah: *Naja kaouthia* yang juga terdapat di Malaysia. Sebelum ini, ketiga-tiga ular tedung jenis meludah tersebut dikategorikan sebagai satu species dan dinamakan sebagai *N. sputatrix*. Keputusan menunjukkan bahawa ketiga-tiga ular tedung jenis meludah mempunyai komposisi bisa yang berlainan, tetapi ketiga-tiga ular tersebut mempunyai phospholipase A_2 bersifat alkali dan kandungan kardiotoksin polipeptida yang tinggi. Proteom bisa ular *N. sumatrana* telah dikaji dengan analisis shotgun, kombinasi kromatografi pelbagai dimensi dan 2DE. Analisis shotgun menunjukkan 50 protein individu dalam bisa ular, di mana 'three finger toxins' (neurotoksin dan kardiotoksin) serta phospholipase A_2 telah merangkumi 38% dan 36% masing-masing, daripada jenis protein yang telah dikenalpasti. Kromatografi penggantian ion dan HPLC terbalik-fasa telah menunjukkan pelbagai protein bersifat alkali termasuk 17 toksin (8 PLA_2, 4 neurotoksin dan 5 kardiotoksin) manakala Sephadex® G-50-2DE menunjukkan 11 protein yang mempunyai berat molekul tinggi. Daripada 17 toksin yang dikenalpasti, hanya 7 toksin hadir dalam kuantiti yang besar. Ini termasuk 2 phospholipase A_2, 1 α-neurotoksin pendek, 1 α-neurotoksin panjang dan 3 kardiotoksin. Tujuh toksin major ini merangkumi 87% daripada jumlah protein bisa dan terutamanya terlibat dalam tindakan toksinologikal utama bisa ular.

Farmakokinetik bisa ular *N. sumatrana* dan *N. sputatrix* juga dikaji dalam arnab. Profil tahap serum toksin – masa apabila kedua-dua bisa ular tersebut disuntik secara intravena membayangkan model farmakokinetik dua-kompartmen dan mempunyai parameter-parameter farmakokinetik yang sama. Kedua-dua bisa ular ini mempunyai keterbiosediaan intraotot yang setanding, iaitu kira-kira 40%. Untuk memahami farmakokinetik komponen toksin-toksin dalam bisa ular dengan selanjutnya, farmakokinetik tiga toksin utama juga telah dikaji. Apabila, toksin disuntik secara intraotot, neurotoksin dan kardiotoksin mencapai C_{max} dalam 30 min, iaitu kadar yang lebih cepat berbanding dengan phospholipase A_2 dan bisa ular, ini menunjukkan kadar penyerapan yang cepat dari tapak suntikan ke dalam peredaran sistemik. Neurotoksin dan kardiotoksin mempunyai kadar eliminasi yang lebih cepat berbanding dengan komponen bisa yang lain. Keterbiosediaan interotot neurotoksin ($F_{i.m.}$ = 81.5%) adalah lebih tinggi berbanding dengan phospholipase A_2 ($F_{i.m.}$ = 68.6%) and kardiotoksin ($F_{i.m.}$ = 45.6%). Neurotoksin mempunyai keterbiosediaan yang tinggi serta T_{max} yang pendek apabila disuntik secara intraotot dan ini menjelaskan neurotoksiti sebagai gejala dominan dalam pembisaan ular tedung. Kesimpulannya, kefahaman yang menyeluruh terhadap toksinologi, proteom dan farmakokinetik bisa ular *N. sumatrana* dan toksin-toksinnya dapat memberikan pandangan yang mendalam mengenai tindakan keseluruhan patofisiologikal bisa ular ini.

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Michelle Yap
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sp.	Species	™	trademark
i.v.	intravenous	pI	isoelectric point
i.m.	intramuscular	kDa	kilodalton
FCA	Freund's complete adjuvant	ml	milliliter
FIA	Freund's incomplete adjuvant	mg	milligram
$F(ab')_2$	fragment antigen-binding (bivalent)	kg	kilogram
IgG	immunoglobulin G	µl	microliter
Fc	fragment crystallizable region	xg	g force
Fab	fragment antigen-binding	µg	microgram
CRISPs	cysteine-rich secretory proteins	g	gram
SVMP	snake venom metalloproteinase	µm	micrometer
3FTXs	three-finger toxins	mm	millimeter
LAAO	L-amino acid oxidase	nm	nanometer
PLA_2	phospholipase A_2	h	hour
NTX	neurotoxin	min	minute
CTX	cardiotoxin	Å	Angstrom
CDF	complement depleting factor	mA	milliampere
CVF	cobra venom factor	ng	nanogram
VNGF	venom nerve growth factor	mM	millimolar
NGF	nerve growth factor	M	molar
A	alpha	%	percent
K	kappa	w/v	weight per volume
β	beta	v/v	volume per volume
γ	gamma	°C	degree Celsius

µ	micro	Sec	seconds
®	registered	V	Volt
L	liter	Cm	centimeter
ADP	adenosine monophosphate	NPAV	Neuro Polyvalent Antivenom
mRNA	messenger ribonucleic acid	$T_{1/2\alpha}$	initial phase half-life
Tyr	tyrosine	$T_{1/2\beta}$	terminal phase half-life
Phe	phenylalanine	$V_{d,area}$	volume of distribution by area
Cys	cysteine	V_c	volume of central compartment
NaCl	sodium chloride	V_p	volume of peripheral compartment
H_2SO_4	sulphuric acid	*F*	bioavailability
HCl	hydrochloric acid	CL	systemic clearance
APS	ammonium persulfate	k_{12}	transfer rate constant from central to peripheral compartment
DTNB	dithionitrobenzoate	k_{21}	transfer rate constant from peripheral to central compartment
MES	2-(N-Morpholino) ethanesulfonic acid	C_{max}	maximum concentration
PBS	phosphate buffered saline	AUC	area under the curve
PBS-Tween	phosphate buffered saline-Tween 20	ANOVA	analysis of variance
BSA	bovine serum albumin	C.I.	confidence interval
TEMED	NN,N,N’-N’-tetramethylethylenediamine	NFU	National Formulary Unit
PVDF	polyvinylidene fluoride	ED_{50}	median effective dose
TMB	3,3',5,5'-Tetramethylbenzidine	LD_{50}	median lethal dose

HRP	horseradish Peroxidase	ADME	absorption, distribution, metabolism, elimination
EDTA	ethylenediaminetetraacetic acid	ELISA	enzyme-linked immunosorbent assay
DTT	dithiothreitol	UV	ultraviolet
IAA	iodoacetamide	Vis	visual
TFA	trifluoroacetic acid	HMW	high molecular weight
WHO	World Health Organization	MW	molecular weight
SEARO	WHO-Southeast Asia Regional Office	*m/z*	mass-to-charge ratio
NCBI	National Center for Biotechnology Information	MALDI	matrix-assisted laser desorption/ionization
CIOMS	Council for International Organizations of Medical Sciences	ESI	electrospray ionization
CID	collision-induced	TOF	time-of-flight
HPLC	high performance liquid chromatography	2DE	two-dimensional gel electrophoresis
MS	mass spectrometry	LC-MS/MS	liquid chromatography-tandem mass spectrometry
1DE	one-dimensional gel electrophoresis	S.D.	standard deviation

LIST OF APPENDICES