Appendix I

List of publications

1. **Das, D.,** Urs, A. N., Hiremath, V., Vishwanath, B.S. and Doley, R. **Biochemical and Biological Characterization of *Naja kaouthia* venom from North-East India and its Neutralization by Polyvalent antivenom**, *J. Venom Res*, **4**, 31-38, 2013.

2. **Das, D.,** Sharma, M., Das, H. K., Sahu, P. P. and Doley, R. **Purification and characterization of Nk-3FTx: A Three Finger Toxin from the Venom of North East Indian Monocled Cobra**, *J. Biochem. Mol. Toxicol.*, 2015.

3. Das, H., **Das D.,** Doley, R., Sahu, P.P., **Quantifying demyelination in NK venom treated nerve using its electric circuit model.** *Scientific Reports*, 6, 2016.

Other publications

1. **Das, D.,** Ansari, M. M., Namsa, N. D., Mukherjee, A. K. and Doley, R. **Assessment of antivenom potential of *Anthocephalus cadamba* leaves extract: A traditionally used medicinal plants for the treatment of snakebite patients.** *J. Bioresource*, 2015.

2. Sharma, M., **Das, D.,** Iyer, J. K., Kini, R. M., Doley, R. **Unveiling the complexities of *Daboia russelii* venom, a medically important snake of India, by tandem mass spectrometry**, *Toxicon*, 1-16, 2015.

3. Urs, A. N., Yariswamy, M., Joshi, V., Suvilesh, K.N., Sumanth, M. S., **Das, D.,** Nataraju, A., and Vishwanath, B.S., **Local and systemic toxicity of *Echis carinatus* venom: neutralization by *Cassia auriculata* L. leaf methanol extract**, *J. Nat. Medicine,* **69** (1), 111- 122, 2014.

Appendix II

Presentations in conferences and seminars

List of oral presentations

1. **Das, D.** and Doley, R., **Neurotoxic three finger toxin (3FTx) from *Naja kaouthia* with anticoagulant activity: its biochemical and biological characterization** at National seminar on Recent advances in Biotechnology research in North East India: Challenges and Prospects at Department of Molecular Biology and Biotechnology, Tezpur University, Assam, November, 2014.

2. Das, H.*, **Das, D.*,** Doley, R., Sahu, P. P., **Modelling of Human demyelinated peripheral nerve and its application in myelin repair"** at International Conference on Disease Biology and Therapeutics, December 3-4, in Institute of Advanced Study in Science and Technology (IASST), Paschim Boragaon, Guwhati, Assam, 2014. (*Contributed equally)

List of poster presentations

1. **Das, D.** and Doley, R. **Purification and partial characterization of an anticoagulant protein from Indian Monocled Cobra *(Naja kaouthia)* of North East region** in National Conference on Snakebite Management, 2nd Annual conference of Toxinological Society of India at Department of studies in Biochemistry, University of Mysore and Karnataka State Open University, Mysore, Karnataka, December, 2012.

Other posters presentations

1. **Das, D.,** Saikia, D., Kalita, R. D., Mukherjee, A. K. and Doley, R. **Green medicine for snakebite** in 1st National conference on animal, microbial, plant toxins & snakebite management (AMPTOX 2010), Biotoxins in health & disease, Kolkata, December, 2010.

Appendix III

Reprints of published papers

Journal of Venom Research ISSN: 2044-0324 J Venom Res, 2013, Vol 4, 31-38

RESEARCH ARTICLE

Biochemical and Biological Characterization of *Naja kaouthia* venom from North-East India and its Neutralization by Polyvalent antivenom

Diganta Das[α], Nanjaraj Urs[β], Vilas Hiremath[β], Bannikuppe Sannanaik Vishwanath[β] and Robin Doley[α,*]

[α]Molecular Toxinology Laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784028, Assam, India; [β]Department of Studies in Biochemistry, University of Mysore, Mysore 570006, Karnataka, India

*Correspondence to: Robin Doley, Email: doley@tezu.ernet.in, Tel: +91 37 12275412; FAX +91 37 12267005

Received: 13 June 2013; Revised: 30 October 2013; Accepted: 06 November 2013; Published: 06 November 2013

ABSTRACT

This study describes biochemical and biological properties of *Naja kaouthia* (Indian monocled cobra) venom of North-East India. The LD_{50} of the crude venom was found to be 0.148mg/kg and neurotoxicitic symptoms like paralysis of lower limbs and heavy difficulty in breathing at sub-lethal dose in mice was observed. The venom exhibited PLA_2, indirect hemolytic and myotoxic activities but showed weak proteolytic and low direct hemolytic activities. It did not exhibit any hemorrhage when injected intradermally to mice. Anticoagulant activity was prominent when recalcification, prothrombin and activated partial thrombinplastin time were tested on platelet poor plasma. Rotem analysis of whole citrated blood in presence of venom showed delay in coagulation time and clot formation time. Fibrinogen of whole citrated blood was depleted by venom when analyzed in Sonoclot. Crude venom at 10µg and after 16hr of incubation was found to degrade α chain of fibrinogen. Neutralization study showed that Indian polyvalent antivenom could neutralize some of the biochemical and biological activities as well as its fibrinogenolytic activity.

KEYWORDS: *Naja kaouthia*, haemostasis, thromboelastometry, myotoxicity, polyvalent antivenom

INTRODUCTION

Snakebite envenoming is a neglected tropical disease (WHO), which requires immediate attention. It is estimated that globally 2.5 million people are bitten by snakes each year with ~85,000 deaths (Gutierrez et al, 2010); in India, approximately 35,000 to 40,000 people die of snakebites annually (Chippaux, 1998; Kasturiratne et al, 2008). According to recent National Mortality Survey data, the incidence of snakebite cases is likely to be more than 50,000 per year in India (Mohapatra et al, 2011). However, these data may be far from the truth as most of the incidences happen in rural areas and these deaths mostly remain unreported. In India the "Big Four", *Naja naja*, *Bungarus caeruleus*, *Daboia russelii* and *Echis carinatus* are considered to be medically important snakes and are responsible for most of the deaths. Recently, it has been reported that hump-nosed pit viper (*Hypnale hypnale*) from Kerala, is capable of causing lethal envenomation (Joseph et al, 2007). Hence, in addition to the "Big Four", there might be other medically important snakes in specific geographical locations, which need attention. This is important for clinical diagnosis for treatment and for production of effective antivenoms. In India, polyvalent antivenom is raised against the "Big Four" venoms but these snakes may not be present throughout the country. Moreover, administration of this polyvalent antivenom has well documented limitations (Offerman et al, 2001; Lalloo and Theakston, 2003; Williams et al, 2007).

Naja kaouthia is recognized phenotypically with the presence of O-shaped or monocellate hood pattern. They are widely distributed in Nepal, North East India, Bangladesh, Myanmar, Thailand and Peninsular Malaysia (Whitaker, 1978; Viravan et al, 1992; Mukherjee and Maity, 2002).

According to WHO, it belongs to Category 1 of venomous snakes. The symptoms of cobra bite are general neurotoxicity leading to flaccid paralysis and death by respiratory failure, and also severe hypertension (Agarwal et al, 2006; Halesha et al, 2013). Symptoms of coagulopathy have also been reported in victims of *Naja kaouthia* of Asian origin (Khandelwal et al, 2007). The *Naja kaouthia* venom of North-East India origin has not been explored though venom of West Bengal (India) origin have been studied extensively (Mukherjee and Maity, 2002; Lalloo and Theakston, 2003; Mukherjee, 2007; Debnath et al, 2010; Sekhar and Chakrabarty, 2011). Hence, some work on biochemical and biological characterization of the *Naja kaouthia* venom and its *in vitro* neutralization by Indian polyvalent antivenom has been undertaken previously.

MATERIALS AND METHODS

Reagents and kits

$sPLA_2$ assay kit was procured from Cayman Chemical Company (MI, USA). NEOPLASTINE® CL PLUS and APTT reagent were obtained from STAGO (France). AGAPEE kit for CK/LDH analysis was purchased from AGAPPE diagnostics (Switzerland), Glass beads gbACT+ kit was obtained from Sienco, Inc. (USA). Polyvalent antivenom manufactured by Bharat Serums and Vaccines Limited (India) was purchased locally. Bovine plasma fibrinogen was obtained from Sigma-Aldrich and all other reagents used were of analytical grade and were either from Merck or Sigma-Aldrich, (USA).

Animals

Male Swiss albino mice of 40±3gm were obtained from central animal facility, University of Mysore. All animal were housed in well ventilated cages and experiments were carried out according to the Animal Ethical Committee Protocol (University of Mysore, Mysore, India, Proposal no. UOM/IAEC/25/2011).

Collection of snake venom, preparation and storage

Adult *Naja kaouthias* were captured from Jamugurihat, district Sonitpur, Assam, North-East India in the, month of May from its natural habitat and venom was extracted by allowing the snake to bite into a sterile beaker covered with para-film. The crude venom was immediately desiccated using dehydrated silica gel and stored in -20°C until further use. The permission for milking of snakes was obtained from Principal Chief Conservator of Forest (Wild Life) and Chief Wild Life Warden of Assam, India (WL/FG.27/tissue Collection/09 dated 07/10/2011).

Determination of protein content

Total protein content of *Naja kaouthia* venom was determined according to Lowry's method using BSA as standard (Lowry et al, 1951).

Phospholipase A_2 (PLA_2) activity

PLA_2 activity was assayed using $sPLA_2$ assay kit according to the manufacturer's protocol (Cayman Chemical Company, MI, USA). Briefly, in a 96-well microtitre plate, 10µl of venom (0.1mg/ml), 10µl DTNB (5, 50-dithio-bis-(2-nitrobenzoic acid)) and 5µl assay buffer were added. The reaction was initiated by adding 200µl of substrate solution (diheptanoyl Thio-PC). After gentle shaking, the optical density was measured every minute at 405nm using MultiSkan GO multi plate reader (Thermo Scientific, USA) for 10min. Assay buffer was used as blank and bee venom PLA_2 (0.01mg/ml) was used as a positive control. Tests were carried out in triplicate and mean values were taken. The activity was expressed as micromoles of diheptanoyl Thiol-PC hydrolyzed per min per mg of enzyme.

Caseinolytic assay

Digestion of casein was evaluated according to the method of Ouyang and Teng (Ouyang and Teng, 1976). Briefly, 1% (w/v) casein in 20mM Tris-Cl, pH 7.4, was incubated with various amounts of venom protein (1, 5, 10, 50 and 100µg) for 1hr at 37°C. Reaction was stopped by addition of ice cold 10% (v/v) TCA and centrifuged for 10min at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R). The digested protein in the supernatant was determined according to Lowry's method (Lowry et al, 1951). Tyrosine curve was used to determine the protease activity and one unit of protease activity is defined as *n* mole equivalent of tyrosine formed per min per ml.

LD_{50} determination

Toxicity of the venom was analyzed according to the method of Meier and Theakston (Meier and Theakston, 1986). Briefly, various amount of freshly dissolved venom (0.05 to 1mg/kg) in saline was injected intraperitoneally to eight male Swiss albino mice in a final volume of 150µl and the controls were injected with saline alone. The animals were carefully monitored for 24hr and their survival time was recorded and LD_{50} was determined.

Edema inducing activity

The procedure of Yamakawa et al, (Yamakawa et al, 1976) as modified by Vishwanath et al, (Vishwanath et al, 1988) was followed. Mice weighing 20–30gm were injected with varying amount of venom (2–15µg) in a total volume of 20µl saline into intra plantar surface of right hind foot pad. Respective left foot pad received 20µl of saline and served as vehicle. Control mice were injected with 20µl saline into intra plantar surface of both hind foot pads. After 45min the mice were anesthetized (barbitone, 30mg/kg, i.p.) before sacrifice and hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to edema is expressed as the ratio of the weight of edematous limb to the weight of vehicle (saline injected) limb x100. The amount of venom required to cause an edema ratio of 120% (20% above the basal level) is defined as minimum edema dose (MED).

Hemorrhagic activity

Hemorrhagic activity was assayed as described by Kondo et al, (Kondo et al, 1960). Various amount of venom (2–15µg) in 30µl saline were injected intradermally into mice and control mice received saline instead of venom sample. After 3hr, mice were sacrificed using anesthesia (barbitone, 30mg/kg, i.p.). The dorsal surface of the skin was removed and the inner surface was observed for hemorrhagic lesions. *E. carinatus* venom was used as positive control. The minimum hemorrhagic dose (MHD) is defined

as the concentration of venom that induce a hemorrhagic spot of 1cm diameter from the spot of injection.

***In-vivo* myotoxicity**

For myotoxicity, release of serum creatine kinase (CK) and lactate dehydrogenase (LDH) in the blood were determined using AGAPPE kit (AGAPPE diagnostics, Switzerland). Group of six male albino mice were injected (i.m) with 15µg crude venom (40µl) and control received 40µl of saline. After 3hr, mice were anesthetized and 0.5ml of blood samples was drawn using cardiac puncture. The serum obtained by centrifugation was diluted with saline at 1:20 ratio. The CK and LDH activity were measured in 10µl of plasma according to the manufacturer's protocol and were expressed in Units/liter (U/l). The results are mean ±SD of three experiments.

Collection of Blood and Platelet Poor Plasma (PPP) preparation

Fresh goat blood was collected in citrated tube (0.11M tri sodium citrate) at 1:9 ratios (citrate: blood) from local butcher's shop. Human blood was collected from healthy donors (27Yr) who had not taken any medication for last 48hr. 9ml of blood was drawn with 20 gauge 3/4" needle and immediately transferred to a plastic tube containing 1ml of 0.11M tri sodium citrate (Suntravat et al, 2010). The tubes were centrifuged at 3000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 15mins to separate the red blood cells (RBC) and platelet poor plasma (PPP) and used within 4hr of collection.

Direct and indirect hemolytic activity

The RBC pellet obtained from the blood (as described above) was washed 4–5 times and re-suspended in 0.9% (w/v) saline to a final concentration of 10% (v/v). Various amount of venom were incubated for 60min at 37°C with 150µl of 10% RBC to a final volume of 2ml with 0.9% (v/v) NaCl. The tubes were centrifugation at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 10min and the absorbance of the supernatant was measured at 540nm in a MultiSkanGO, UV-Vis spectrophotometer (Thermo Scientific, USA). The hemolysis caused by dH_2O was considered as 100%. For Indirect hemolytic, 20µl of egg yolk substrate solution was added to the reaction mixtures at the time of incubation and hemolysis was measured as described for direct hemolytic activity. The results are mean ±SD of three experiments.

Fibrinogenolytic activity

Fibrinogenolytic activity was assayed according to the method of Ouyang and Teng, using bovine fibrinogen (2mg/ml) dissolved in 50mM Tris HCl buffer, pH 7.5, 0.15M NaCl (Ouyang and Teng, 1976). To 300µl of dissolved fibrinogen, various amount of venom in 150µl of buffer was incubated for different time intervals at 37°C. The incubated mixtures were then run on a 12.5% (w/v) SDS-PAGE according to the method of Laemmeli (Laemmli, 1970). Staining was done with 0.25% (w/v) Coomassie brilliant blue R250 and destained till the protein bands were visible.

***In-vitro* coagulant assays**

Recalcification time

Recalcification time of human PPP was measured using coagulation analyzer (STAGO, France). Various amount of venom in 50µl of PBS was pre-incubated with 50µl of human PPP at 37°C for 3min and 50µl of 25mM $CaCl_2$ was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are as mean ±SD of three experiments.

Prothrombin time (PT) test

Prothrombin time was measured using PT reagent (NEOPLASTINE® CL PLUS) obtained from STAGO (France) according to the manufacturer's protocol on a coagulation analyzer (STAGO, France). Various amount of venom in 50µl of PBS was pre-incubated with 50µl of human PPP at 37°C for 1min and 100µl of PT reagent was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are mean ±SD of three experiments.

Activated partial thrombin time (APTT) test

Activated partial thrombin time was determined using APTT reagent obtained from STAGO (France) according to the manufacturer's protocol on a coagulation analyzer (STAGO, France). Various amount of venom in 50µl PBS was incubated with 50µl of human PPP and 50µl of APTT reagent for 3min at 37°C. The clot formation was initiated by adding 50µl of 25mM $CaCl_2$. The clot formation time with PBS was considered as normal clotting time. The results are mean ±SD of three experiments.

Whole citrated blood analysis

Thromboelastometry analysis

To quantify the CT (clotting time, in seconds), CFT (clot formation time, in seconds) and MCF (maximum clot firmness, in mm) of the whole citrated blood, Rotem® Analyzer (ROTEM®Pentapharm GmbH Diagnostic Division; Munich, Germany) was used. For the analysis, blood samples from healthy volunteers were collected in 0.11M tri sodium citrate at 9:1 (blood: citrate) ratio. Various amount of venom in 20µl of PBS was mixed with 20µl of 200mM $CaCl_2$, to this reaction mixture, 320µl of whole citrated blood was added and clot formation was observed over 30min. Clot formation function with only PBS was considered as control. The results are mean ±SD of three experiments.

Sonoclot analysis

A glass bead activated test tube (gbACT+ Kit obtained from Sienco, Inc, USA) was used to monitor clot detection, clot rate and platelet function (clot retraction) in a Sonoclot Coagulation and Platelet Function Analyzer (Sienco, Inc, USA). Various amount of venom in 20µl of PBS was added to 320µl of citrated human blood followed by 20µl 200mM $CaCl_2$. The head assembly of the analyzer was closed 10s after the start button was pressed. Data were acquired and analyzed with Signature Viewer software (Sienco, Inc.). The results are mean ±SD of three experiments.

Neutralization studies

For neutralization studies, various amount of polyvalent antivenom was pre-incubated with 1µg of *Naja kaouthia* venom in a final volume of 20µl for 1hr at 37°C and assays were performed as described above. The percentage inhibition was calculated by considering the activity in absence of polyvalent antivenom as 100%. The results are mean ±SD of three experiments.

RESULTS

Biological characterization

The biochemical and biological activities of the crude venom are listed in Table 1. The median lethal dose (LD_{50}) was found to be 0.148mg/kg when injected intraperitoneally to experimental mice. When sub-lethal dose of venom was injected to mice, neurotoxic symptoms like difficulty in movement; breathing and frequent drinking of water were observed followed by death after 40min. The amount of CK and LDH released after injection of 15µg of venom was found to be 6.605U/l and 26.38U/l respectively in the plasma. The CK was 10 times more than observed for the control mice (0.63U/l), however, the LDH was found to be only 3U more. The minimum edema dose (MED) of the venom was found to be 11.25µg. No direct hemolytic activity was observed up to 10µg of venom but when the amount was increased up to 100**µg**, it exhibited 1.4% RBC hemolysis. For indirect hemolytic activity, 23% hemolysis was observed for 1µg of venom. The venom showed weak proteolytic activity when tested on casein. The amount of tyrosine liberated was 0.14±0.02 moles by 100µg of venom in 1min. PLA_2 activity of the venom was 7.584µmol/min/mg when assayed using $sPLA_2$ assay kit. However no haemorraghic spot was observed when 3µg of venom was injected intradermally (Figure 1).

In-vitro coagulation activities

The venom showed anticoagulant activity in dose dependent manner. When recalcification time of human plasma was tested with 1µg venom, the plasma did not form clot up to 500s whereas the normal clotting time was 126.5sec (Figure 2). Prothrombin time increased dose dependently and at 0.1µg, clot formation was not observed up to 500sec. The APTT test on plasma did not increase significantly up to 0.1µg but when the amount was increased to 1µg venom the plasma did not form clot (Figure 2).

In Rotem® Analyzer, coagulation time (CT) for 0.1µg venom was 634±15sec and for the control it was observed to be 503±10sec. When the amount of the venom was increased to 1 and 10µg, clot formation was not observed which is depicted by a straight line (Data not shown). The clot formation time (CFT) in presence of 0.1µg of venom was recorded to be 266±10sec, whereas the CFT for control plasma was only 87±3s (Table 2). Maximum clot firmness (MCF) value at 0.1µg of venom was 61±1.3mm, whereas for the control the value was 65±2mm. However, at higher concentration of venom the blood clot did not form. Hence, the values were not measurable in the Rotem® analyzer (Table 2).

The activated clotting time (ACT) increases dose dependently and at 10µg of venom it was recorded to be 591±1.3sec in Sonoclot Coagulation and Platelet Function Analyzer. At 0.1µg venom the clot rate was similar to normal clot rate (normal range 9–35sec) but with increase in concentration, the clot rate decreased which might be due to depletion of fibrinogen. However, up to 1.0µg the platelet function was found to be normal but at 10µg the platelet function was not observed (Table 2). Lower amount of venom did not show any digestion of fibrinogen (data not shown). However, when the amount of venom was increased to 10µg, clear digestion of α chain of fibrinogen was observed after 16hr of incubation (Figure 3A).

Neutralization studies

Effect of polyvalent antivenom on some of the biochemical and biological properties of *Naja kaouthia* venom are shown in Table 3. At 1:1 ratio, the polyvalent antivenom could

Table 1. Some biochemical and biological activities of *Naja kaouthia* venom

Parameters	Activity
LD_{50}	0.148 mg/kg
PLA_2 activity assay	7.9±0.24$^{\Psi}$
Direct hemolytic assay (100µg venom)	1.4±0.51%
Indirect hemolytic assay (1µg venom)	23.0 ±3%
Caseinolytic activity (100µg venom)	0.14±0.02*
Creatine kinase (CK) (15µg i.m. injection)	6.6±0.2 U/l
Lactate dehydrogenase (LDH) (15µg i.m. injection)	26.3±2.3U/l
Minimum edema dose (MED)	11.2±0.18 µg
Haemorraghic activity (up to 15µg)	NA

*Normal CK and LDH values are 0.63 U/l and 23.39 U/l respectively; Ψµmol of diheptanoyl Thiol-PC hydrolyzed/min/mg; **n* moles of tyrosine formed/min; NA= No Activity. Results are mean ±SD (n=3)

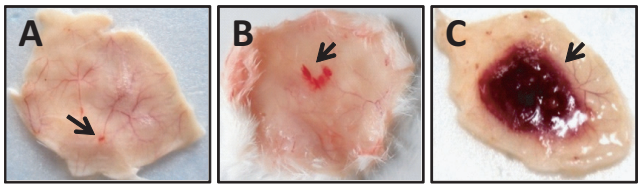

Figure 1. Haemorraghic activity of *Naja kaouthia* venom. **A.** Control (30µl of saline), **B.** *Naja kaouthia* venom (15µg), **C.** Saw scaled viper venom (3µg) (Positive control), the arrow indicates site of injection.

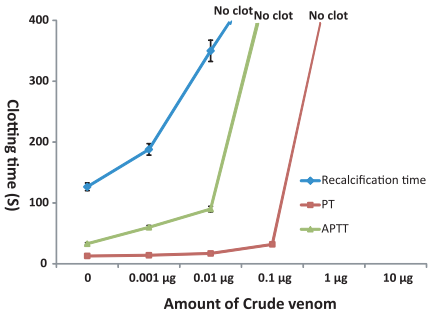

Figure 2. Dose dependent anticoagulant activity of *Naja kaouthia* venom on human plasma. Effect of crude venom on Recalcification time, Prothrombin Time test (PT) and Activated Partial Thrombin Time test (APTT). The results are mean ±SD of three experiments.

Table 2. Anticoagulant activity of *Naja kaouthia* venom on whole citrated blood. Results are expressed as mean ±SD of three experiments.

Parameters	PBS	Crude venom (µg/ml)		
		0.1	1.0	10
Thromboelastometry analysis				
Coagulation time (CT) (s)	503±10	634±15	>1200	>1200
Clot formation time (CFT) (s)	87±3	266±10	NCF	NCF
Maximum clot firmness (MCF) (mm)	65±2	61±1.3	NCF	NCF
Sonoclot analysis				
Activated clotting time (ACT) (s) (range: 128–213)	176±5.2	215±7.4	243±6.3	591±10
Clot rate (CR)(range: 9.0–35)	23±0.5	23±0.32	16±0.21	1.2±0.2
Platelet function (range: 3–5)	2.8±0.01	3.8±0.02	3.3±0.01	0

*NCF: No clot formation, the results are expressed as mean ± SD

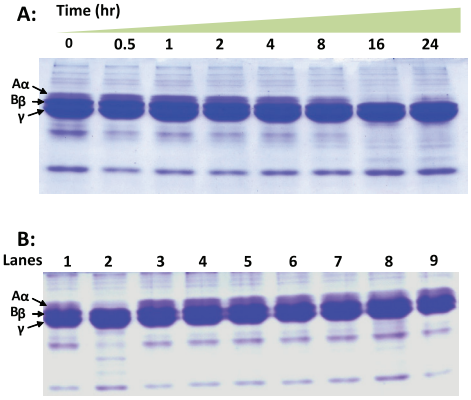

Figure 3. A. Fibrinogenolytic activity of *Naja kaouthia* venom. SDS-PAGE of bovine fibrinogen (reduced) after incubation with 10µg crude *Naja kaouthia* venom at various time intervals. **B.** Inhibition of fibrinogenolytic activity of *Naja kaouthia* by polyvalent antivenom. The venom:polyvalent antivenom (1:1, w/w) mixture was pre-incubated for 1hr at 37°C. This mixture was incubated with 300µl of fibrinogen (2mg/ml) for 24hr and aliquots were withdrawn at different time interval and fractionated in 12.5% (w/v) SDS-PAGE. **Lane 1.** Undigested fibrinogen (control). **Lane 2.** Fibrinogen incubated with only venom; **Lane 3.** After 0.5hr; **Lane 4.** 1hr; **Lane 5.** After 2hr; **Lane 6.** After 4hr; **Lane 7.** After 8hr; **Lane 8.** After 16hr; and **Lane 9.** After 24hr.

not neutralize the PLA_2 activity of the venom but at 1:100 ratios, 97.38 ± 4.8% inhibition was observed. Inhibition of the indirect hemolytic activity of venom was also observed similar to the PLA_2 activity. When the concentration of the polyvalent antivenom was increased by 100 times, indirect hemolytic activity was completely neutralized. Recalcification time of the venom was neutralized up to 49.34% at 1:1 ratio and with 10 times increase in polyvalent antivenom, 92.03% neutralization was observed. Similarly, the APTT and PT was also brought to the normal clotting time when the polyvalent antivenom was 10 times excess of the venom concentration. Moreover, degradation of α chain of fibrinogen by venom was inhibited by polyvalent antivenom at 1:1 ratio (Figure 3B).

DISCUSSION

The patho-physiological effect post-snakebite envenomation varies greatly among the various species and even within species due to variation in the venom proteins and biological activities (Glenn et al, 1983; Minton and Weinstein, 1986; Daltry et al, 1996; Saravia et al, 2002; Menezes et al, 2006). These variations affect the clinical manifestation of envenomation and require specific consideration for treatment. Hence understanding the biochemical and biological properties of snake venom from a particular geographic location is important.

The LD_{50} of the *Naja kaouthia* venom was found to be 0.148mg/kg, whereas those for cobra venoms of Thailand and Kolkata origin were reported to be 0.23mg/kg and 0.7mg/kg, respectively (Mukherjee and Maity, 2002; Leong et al, 2012). Though the route of injection was different (Kolkata origin venom given via tail vein injection) in these experiments, the lethal dose of North East origin venom was less than that of the other geographical locations suggesting it might be more lethal. However, the comparative study with indistinguishable experimental conditions would be necessary to differentiate these venoms. In mice the venom did not induce haemorraghic activity and venom of Kolkata origin is reported to be devoid of such activities. The haemorraghic is mainly caused by metalloproteases, which are abundantly found in viper venom (Kamiguti et al, 1996; Chakrabarty et al, 2000; Mukherjee, 2008). Moreover, the edema inducing activity was not found to be significant. Hence this venom might not induce inflammation and tissue damage at the site of bite. Interestingly, the venom at 100µg showed only 1.4% hemolysis of RBC, whereas at the same amount Kolkata venom activity is reported to be 39.0% (Mukherjee and Maity, 2002). The membrane damaging activity is mainly contributed by the low molecular weight proteins which might be absent in this venom. The indirect hemolytic activity of the venom in presence of the egg yolk is due to PLA_2 enzymes. The lysophospholipids and free fatty acids formed during the catalysis of phospholipids by PLA_2 enzyme exhibits this activity as they are lytic in nature (Condrea et al, 1964). The presence of various PLA_2 isoenzymes and neurotoxins in *Naja kaouthia* venom have been

Table 3. *In vitro* neutralization of whole venom activity by polyvalent antivenom

Activity	% inhibition by polyvalent antivenom		
	1:1	1:10	1:100
PLA_2 activity	0	40.0±5.0	97.38 ± 4.8
Indirect hemolytic	11.96±2.12	68.15±0.15	100
Recalcification time	49.34±5.01	92.03±3.0	96.52±2.81
PT	36.44±5.8	78.19±3.86	99±1.76
APTT	32.33±6.44	92.1±5.83	100
Fibrinogenolytic	α chain present	α chain present	α chain present

*The results are expressed as mean ± SD (n=3)
Values indicate % inhibition at each venom:antivenom (µg:µg) ratio

reported by various workers (Joubert and Taljaard, 1980; Meng et al, 2002; Qiumin et al, 2002; Doley et al, 2004). When the crude venom was analyzed for the PLA_2 activity using diheptanoyl Thiol-PC as substrate, the amount of substrate hydrolyzed product was 7.9±0.24µmol/min/mg suggesting the presence of enzymatically active PLA_2 in the venom. PLA_2 is one of the major constituent in the elapid venom, which confers multiple toxicity to the prey or victim such as membrane damaging, neurotoxicity, edema and prolongation of coagulation time (Kini and Evans, 1989; Doley et al, 2004). Hence the myotoxicity, neurotoxicity and edema induced by this venom are due to the presence of large amount of PLA_2 enzyme in the venom. The observed differences in the biochemical and biological activities in the venoms of Indian origin might be due to variation in the venom composition and content due to difference in geographical locations. Both venoms were collected during summers; however, in the present study, the ages of the snakes were unknown as they were captured from the wild. Detailed analysis of *Naja kaouthia* venoms from different locations of India need to be carried out to decipher the differences in the venom composition as well as the presence of unique toxins.

Snake venom proteins affect the haemostasis process of victim/prey either by prolonging or shortening the clotting time. Elapid venoms are anticoagulant in nature due to the presence of large amount of strong and weak anticoagulant PLA_2 enzymes. Moreover, non-enzymatic protein from elapid venom like Cardiotoxins from *Naja nigricollis crawshawii* and Hemextin A and hemextin AB complex from *Hemachatus haemachatus* venom are also reported to be anticoagulant in nature (Kini et al, 1988; Banerjee et al, 2005). The venom significantly delayed the recalcificaion time, PT and APTT of plasma under *in vitro* condition, which is due to strong anticoagulant proteins present in the venom. The plasma did not form clot at 0.01, 0.1 and 1µg concentration of venom when tested for recalcificaion time, PT and APTT, respectively. This suggests that the anticoagulant activity of the venom is most likely to affect all the pathways. Venom PLA_2 enzymes inhibit activation of FX to FXa which leads to disruption in the formation of prothrombinase complex, which is required for blood coagulation (Stefansson et al, 1990; Kerns et al, 1999; Kini, 2005). The higher amount of venom required in case of PT and APTT for non-coagulation of blood might be due to the addition of extra phospholipids during these tests; however, this needs to be verified. The venom proteins, especially the PLA_2 enzymes, hydrolyze the phospholipids which are required for the prothrombinase complex formation. The Sonoclot and Rotem analysis also demonstrated that the *Naja kaouthia* venom is anticoagulant in nature. The whole citrated blood analysis by sonoclot clearly indicated the depletion of fibrinogen in the reaction when pre-incubated with venom. The lower value of MCF by Rotem analysis indicates decreased platelet number or function, decreased fibrinogen level or fibrin polymerization disorders, or low activity of factor XIII. Recently, Nk a metaloprotease, which cleaves the α- chain, as well as a low molecular protein with fibrin(ogen)olytic activity have been reported (Wijeyewickrema et al, 2007; Debnath et al, 2010). The weak proteolytic activity towards casein and higher amount of venom and time required for complete degradation of α chain of bovine serum fibrinogen might be due to presence of these proteins in lower amount. Hence anticoagulant activity of *Naja kaouthia* might not be only due to degradation of phospholipids or α chain of fibrinogen but action of different venom proteins which might be acting enzymatically or non-enzymatically on coagulation factors and complexes.

Polyvalent antivenom is currently used by the medical practitioners for the treatment of snakebite patients in India. The Indian polyvalent antivenom is prepared using the venoms of four major poisonous snake species viz: *Naja naja, Daboia russelii, Echis carinatus* and *Bungarus caeruleus.* In most of the cases, it has been observed that the efficacy is highly reduced when antivenoms raised against venom from a particular geographic region is used to treat victims from another region (Shashidharamurthy et al, 2002; Shashidharamurthy and Kemparaju, 2007). The polyvalent antivenom could neutralize some of the biochemical and biological activity partially at 1:10 ratio (venom: polyvalent antivenom) and complete neutralization was observed when the dose of the polyvalent antivenom was increased to 10 fold. The partial inhibition might be due to the antibodies of *Naja naja* proteins present in the polyvalent antivenom, which recognizes the *Naja kaouthia* venom proteins. Present study documents that the polyvalent antivenom can neutralize some of tested biochemical and biological activities of *Naja kaouthia* venom under *in vitro* condition.

ACKNOWLEDGEMENTS

We acknowledge DBT, Govt of India, for grant under twinning programme for NER India, Tezpur University for the start-up grant and DBT, UGC and DST, New Delhi, for various grants to the department. We also thank the anonymous reviewers for their valuable comments and suggestions that have improved the manuscript.

COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

CFT; clot formation time
CT; Coagulation time
MCF; Maximum clot firmness
ACT; Activated clotting time

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Purification and Characterization of Nk-3FTx: A Three Finger Toxin from the Venom of North East Indian Monocled Cobra

Diganta Das,[1] *Maitreyee Sharma,*[1] *Hemanga Kumar Das,*[2] *Partha Pratim Sahu,*[2] *and Robin Doley*[1]

[1]Molecular Toxinology Laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur- 784028, India; E-mail: *doley@tezu.ernet.in*, *doleyr@gmail.com*

[2]Department of Electronics and Communication Engineering, Tezpur University, Tezpur 784028, India

Received 10 June 2015; revised 13 July 2015; accepted 22 July 2015

ABSTRACT: Snake venom three finger toxins (3FTxs) are a non-enzymatic family of venom proteins abundantly found in elapids. We have purified a 7579.5 ± 0.591 Da 3FTx named as Nk-3FTx from the venom of *Naja kaouthia* of North East India origin. The primary structure was determined by a combination of N-terminal sequencing and electrospray ionization liquid chromatography-mass spectrometry/mass spectrometry. Biochemical and biological characterization reveal that it is nontoxic to human cell lines and exhibit mild anticoagulant activity when tested on citrated human plasma. Nk-3FTx was found to affect the compound action potential (CAP) and nerve conduction velocity of isolated toad sciatic nerve. This is the first report of a non-conventional 3FTx from *Naja kaouthia* venom that reduces CAP for its neurotoxic effect. Further studies can be carried out to understand the mechanism of action and to explore its potential therapeutic application. © 2015 Wiley Periodicals, Inc. J Biochem. Mol. Toxicol. 00:1–12, 2015; View this article online at wileyonlinelibrary.com. DOI 10.1002/jbt.21734

KEYWORDS: ***Naja kaouthia*; Three finger toxin; Neurotoxicity; Compound action potential; Potassium channel**

INTRODUCTION

Snake venom is a complex mixture of proteins and polypeptides which are classified into various toxin families based on their structure and function. Three finger toxins (3FTxs) are one of the well characterized non-enzymatic families of snake venom proteins. They are abundantly found in elapids and hydrophiidae venom [1–3] and recently, transcripts are also reported in *Viperidae* family [4]. The amino acid sequence of 3FTx family ranges from 60 to 74 residues with eight or ten cysteine residues [5]. They are called "Three finger toxins" (3FTx) as the three loops (β stranded) project from the hydrophobic core which is connected by 4–5 disulfide bridges resembling three stretched fingers of our hand [5–7]. In non-conventional 3FTx, the fifth disulfide linkage is found in the first loop whereas in long chain α-neurotoxins and κ-neurotoxins it is present in the second loop [1, 8]. Functionally they exhibit various pharmacological effects on prey/victims. They are reported to be neurotoxic, cardiotoxic, cytotoxic, anticoagulant, myotoxic, platelet aggregation inhibition, etc. [6,9−13]. This family of protein constitutes the best example of a unique structural scaffold to support multiple biological functions as they are structurally conserved but functionally diverse. Mostly, 3FTxs exist as monomers, e.g. fulgimotoxin from green vine snake *Oxybelis fulgidus* [14], candoxin from Malayan krait *Bungarus candidus* [15], denmotoxin, from *Boiga dendrophila* (mangrove catsnake) [16], β-cardiotoxin from *Ophiophagus hannah* [17], hemachatoxin (P-type cardiotoxin) from *Hemachatus hemachatus* venom [18]. However, dimeric 3FTxs are also reported, e.g. κ-neurotoxins from *Bungarus sp.*, Hemextin AB from *hemachatus hemachatus* venom [12], haditoxin, from *Ophiophagus hannah* [19], irditoxin from *Boiga irregularis* (Brown tree snake) [20], etc.

Monocled cobra *(Naja kaouthia)* is the most common species of Asiatic cobra found in North East India [21, 22]. Phenotypically it can be distinguished by its

Correspondence to: Robin Doley.

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"O" shaped hood mark. It is one of the medically important snakes of this region as it is responsible for most of the fatal bites. *Naja kaouthia* from South East Asia have been extensively studied and are reported to have abundant 3FTxs [22]. Studies have revealed that the venom composition of these species has been found to vary among the snakes from different locations [2]. Evolutionarily, diet has been reported to play an important role for such variations [23]. Although venom of *Naja kaouthia* from other parts of India have been studied, so far only few toxins have been isolated and characterized. For example, 3FTx with antiplatelet activity, fibrinolytic activity and cytotoxicity, phospholipase A_2s (PLA_2) with strong anticoagulant activity have been reported from *Naja kaouthia* of Eastern India origin [24–27]. Recently we reported the biological and biochemical properties of crude *Naja kaouthia* venom from North East India, which is neurotoxic in nature [28]. However, not a single toxin from this venom has been reported so far. Hence, purification and characterization of a 3FTx from this snake venom has been undertaken. Studies on biochemical and biological property of individual venom proteins aid in determining the pharmacological profile of the crude venom. Moreover, the structure-function studies of individual toxin will help to develop valuable research tools or pharmaceutical leads.

MATERIALS AND METHOD

Materials

Reverse phase high performance liquid chromatography (HPLC) columns were purchased from Waters (Milford, MA, USA) and Phenomenex (Torrance, California, USA). Bupivacaine hydrochloride (BH) (sodium channel blocker) and quinine hydrochloride (QH) dihydrate (potassium channel blocker) were purchased from Sigma Aldrich (St. Louis, MO, USA), DTT from Gold Biotechnology (St. Louis, MO, USA) and Protease max from Promega (Madison, WI, USA). All other chemicals used are of analytical grade and were procured from Merck (Darmstadt, Germany) or Sigma Aldrich (St. Louis, MO, USA).

Collection of Snake Venom, Preparation and Storage

Adult *Naja kaouthia* were captured from Jamugurihat, district Sonitpur, Assam, India from wild and venom was extracted into a sterile beaker. The crude venom was immediately desiccated using dehydrated silica gel and stored at –20°C until further use. The permission for milking of snakes from Assam was obtained from Principal Chief Conservator of Forest (Wild Life) and Chief Wild Life Warden of Assam, India (WL/FG.27/tissue Collection/09 dated 07/10/2011).

Animals

Swiss albino mice of both sexes were obtained from the central animal facility, University of Mysore. The animals were housed with access to food and water at 25 ± 3°C on a 12 h light/dark cycle. The in-vivo studies were carried out as per the approved guidelines of Animal Ethical Committee of University of Mysore, Mysore, India (Proposal no. UOM/IAEC/25/2011). For neurotoxicity experiments, common Asian toads (*Duttaphrynus melanostictus*) were used. Approval for these experiments were obtained from Tezpur University Animal Ethical Committee (TUAEC) (Approval no. DoRD-Pro/TUAEC/10/56/15/Res-02).

Determination of Protein Content

Protein concentration of the crude venom and purified toxin was determined according to Lowry's method using bovine serum albumin as a standard [29].

Liquid Chromatography-Mass Spectrometry of Crude Venom

Lyophilized crude venom was dissolved in MilliQ water before loading onto a symmetry C18 (5 μ, 4.6 × 250 mm, 300 Å) (Waters, Milford, MA, USA) analytical column equilibrated with 0.1% (v/v) formic acid attached to a Accela LCQ Fleet Ion Trap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) for MS analysis. The crude mixture was eluted using a linear gradient of 80% (v/v) acetonitrile containing 0.1% formic acid at a flow rate of 50 μL/min. Electrospray ionization (ESI) mass spectrum was acquired in positive ion mode with an orifice potential of 80 V. Nitrogen was used as curtain gas with a flow rate of 0.6 L/min and as nebulizer gas with a pressure setting of 100 psi. Full scan data were acquired over the ion range from 500 to 3000 m/z with a step size of 0.1 Da. Data was analyzed by Promas for Xcaliber.

Purification of the 3FTx

Naja kaouthia venom (2 mg/mL) was subjected to reverse phase (RP)-HPLC using symmetry C18 column (5 μ, 4.6 × 250 mm, 300 Å) (Waters, Milford, MA, USA). The column was pre-equilibrated with buffer A (0.1% TFA in MilliQ water) and crude venom was fractionated using a linear gradient of buffer B (80% ACN containing 0.1% TFA) on a HPLC system (Waters, Milford, MA, USA). Elution was monitored at 215 and 280 nm and peaks were collected manually. The protein

peaks containing the 3FTx was rechromatographed on an Aeris WIDEPORE (XB-C18, 3.6 μ, 150 $\times$ 2.10 mm, 200 Å) (Phenomenex, Torrance, California, USA) to check the purity of the preparation.

Molecular Weight Determination

The molecular mass of the purified Nk-3FTx was determined by ESI mass spectrometry (ESI-MS) using an Accela LCQ fleet Ion Trap, Thermo Scientific (Waltham, MA, USA) mass spectrometry. Ion spray voltage was set at 4.4 KV and nitrogen was used as a curtain gas. The solvent used was 50% acetonitrile in 0.1% formic acid at a flow rate of 200 μL/min. Promas for Xcaliber was used to analyze and decipher the raw mass data.

N-Terminal Sequencing of the Purified Protein

N-terminal sequence was determined by automated Edman degradation process using PPSQ 31 (Shimadzu, Kyoto, Japan) with phenylthiohydantoin (PTH) derivative analyzer. Briefly, lyophilized Nk-3FTx was dissolved in 100 μL of denaturant solution (6.0 M guanidinium hydrochloride, 0.13 M Tris, 1 mM EDTA, pH-8.0) containing 0.07 M β-mercaptoethanol and incubated at 37°C for 2 h. Subsequently blocking of sulfhydryl groups was done by adding 4-vinylpyridine and incubated at room temperature for 2 h. The protein was desalted by RP-HPLC and subjected to N-terminal sequencing.

Electrospray Ionization Liquid Chromatography/Tandem Mass Spectrometry

Purified Nk-3FTx was subjected to proteolysis using trypsin with Protease Max surfactant (Promega, Madison, WI, USA) according to the manufacturer's instructions. 50 μg of Nk-3FTx dissolved in 50 μL of MilliQ water was treated with 41.5 μL of 50 mM of ammonium bicarbonate, 2 μL of 1% Protease Max and 1 μL of 0.5 M DTT were added. Incubation of the reaction mixture was carried out at 56°C for 20 min. To this 2.7 μL of 0.55 M IAA was added and incubated in dark for 15 min. Further, 1 μL of 1% Protease Max and 1.8 μL of Trypsin (1 μg/μL in 50 mM acetic acid) were added and incubated at 37°C for 3 h. The reaction was terminated adding 0.5 μL of 100% TFA and incubated at room temperature for 5 min followed by centrifugation at 12,000 rpm for 10 min.

The tryptic digested Nk-3FTx was loaded onto Accela LCQ Fleet Ion Trap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) for MS/MS analysis. 80 μL of the digested sample was loaded into a Hypersil Gold C18 column (50 $\times$ 2.1 mm, 1.9 μm, Thermo Scientific, USA) pre-equilibrated with 0.1% formic acid. Elution was carried out at a flow rate of 200 μL/min with a linear gradient of 40–80% acetonitrile in 0.1% formic acid. The ion polarity was adjusted to positive ionization and spectra were recorded in MS/MS mode. The scan range was set from 500 to 2000 m/z and for modification oxidation of methionine residues and *S*-carbamidomethylation of cysteine residues were specified. The MS/MS spectra obtained were analyzed by the software Proteome Discoverer 3.1 using Sequest program.

BIOLOGICAL ACTIVITIES

Anticoagulant Activity

Recalcification Time

Platelet poor plasma (PPP) was prepared as described by Das et al [28]. Recalcification time of human PPP was measured using coagulation analyzer (Tulip Diagnostics, Chennai, India). Nk-3FTx (0.005–5 μg) in 50 μL of 0.9% NaCl was pre-incubated with 150 μL of human PPP at 37°C for 2 min and 50 μL of 50 mM $CaCl_2$ was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are mean $\pm$ SD of three experiments.

Prothrombin Time Test

Prothrombin time was measured using PT reagent (Uniplastin) obtained from Tulip Diagnostics (India) according to the manufacturer's protocol on a coagulation analyzer (Tulip Diagnostics, Chennai, India). Various amounts of purified Nk-3FTx (0.005–5 μg) in 50 μL of 0.9% NaCl was pre-incubated with 100 μl of human PPP at 37°C for 2 min and 100 μL of PT reagent (Uniplastin) was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are mean $\pm$ SD of three experiments.

Activated Partial Thromboplastin Time

Activated partial thromboplastin time was determined using APTT reagent (Liquicelin) obtained from Tulip Diagnostics (Chennai, India) according to the manufacturer's protocol on a coagulation analyzer (Tulip Diagnostics, Chennai, India). Various amount of Nk-3FTx (0.005–5 μg) in 50 μL 0.9% NaCl was incubated with 100 μL of human PPP and 40 μL of APTT reagent for 2 min at 37°C. The clot formation was initiated by adding 50 μL of 25 mM $CaCl_2$. The clot formation time with

PBS was considered as normal clotting time. The results are mean ± SD of three experiments.

Edema Inducing Activity

The procedure of Yamakawa et al [30] as modified by Vishwanath et al, [31] was followed to assay the edema inducing activity. Studies were performed on Swiss albino mice weighing 25–30 g. Purified Nk-3FTx (5 and 10 μg) in a total volume of 20 μL saline was injected into intraplantar surface of right hind foot pad. Respective left foot pad received 20 μL of saline which served as vehicle. After 45 min the mice were anesthetized (barbitone, 30 mg/kg, intraperitoneal (i.p.)) and later sacrificed. The hind limbs were removed at the ankle joint and weighed individually. Progression and increase in weight due to edema is expressed as the ratio of the weight of edematous limb to the weight of vehicle (saline injected) limb x 100. The amount of venom required to cause an edema ratio of 120% (20% above the basal level) is defined as minimum edema dose.

Hemorrhagic Activity

Hemorrhagic activity of Nk-3FTx was evaluated as described by Kondo et al. [32]. Purified protein (5 μg and 10 μg) was dissolved in 30 μL of 0.9% sterile saline and injected intradermally (i.d.) into experimental mice (Swiss albino mice) weighing 25–30 g. After 3 h of post injection, the mice were euthanized by an i.p. injection of barbitone (30 mg/kg). Skins were then removed and fur side was reversed to measure the hemorrhagic spots. Dosages were given in triplicates and control mice received only saline.

Cytotoxicity

Exponentially growing HEK 293 (Human embryonic kidney cells) and L6 (Skeletal muscle cells) at 10^6/mL cells were incubated with various concentrations of Nk-3FTx for 24 h at 37°C with 5% CO_2 in a CO_2 incubator (Eppendorf, Hamburg, Germany). Cell count was done by vital staining with trypan blue dye and finally counting on a hemocytometer. MTT assay was performed to observe the cytotoxic effect of Nk-3FTx. 20 μL of MTT (5 mg/mL) was incubated for 3.5 h prior to the end of 24 h of incubation with Nk-3FTx at 37°C. The formazan granules formed by viable cells are dissolved in 150 μL MTT solvent and agitated for 15 min. Absorbance at 570 nm was measured using a MultiSkan Go spectrophotometer (ThermoScientific, Waltham, MA, USA). The Nk-3FTx treated cells (HEK 293) were observed under an inverted microscope (Axio Vert.A1, Zeiss, Jena, Germany), for any morphological changes.

Neurotoxicity

Preparation of Sciatic Nerve from Common Asian Toad (Duttaphrynus melanostictus)

The study was carried out with toad as an animal model. Efforts were done to minimize both animal sufferings and animal number. Sciatic nerve preparation was followed according to the method described by Katsuki et al. [33]. In brief, common Asian toads *(Duttaphrynus melanostictus)* weighing 30–35 g of either sex were decapitated and then pithed. The sciatic nerve (length 4–6 cm; 0.5–1 mm diameter) comprising the proximal and distal end was dissected from lumber plexus to the knee joint. Immediately the nerve was mounted on nerve chamber (AD Instruments, PowerLabs, New South Wales, Australia) containing Ringer's solution.

Recording of Compound Action Potential and Determination of Nerve Conduction Velocity

Dissected nerve was treated with various concentrations of Nk-3FTx (0.132–1319.4 nmol/L) for 2 min and mounted on to the nerve chamber. For measuring the compound action potential (CAP), sciatic nerve was externally stimulated with a frequency of 1 Hz and at pulses of 0.1 ms duration [33]. The nerve chamber setup comprised of two male BNC (Bayonet Neill–Concelman) connectors to three microhooks constructed of gold-plated beryllium copper used to stimulate the nerve. CAP of sciatic nerve in nerve chamber was detected at two points separated by a distance of 3 cm. Nerve end with lumber plexus of spinal cord was connected with proximal recording electrode and electrode at nerve end connecting knee joint acted as distal recording electrode. Flow of stimulus was recorded at two points to determine nerve conduction velocity (NCV) of the nerve. The procedure was completed within 20 s to avoid drying. CAP was monitored by a dual Bio Amp/stimulator and analyzed by SCOPE (Powerlabs, New South Wales, Australia). Nerve treated with BH (voltage gated sodium channel blocker) and QH dihydrate (voltage gated potassium channel blocker) were considered as control. The experiment was performed in triplicates and results shown are ±SD. NCV of the sciatic nerve was determined by the following formulae:

$$\text{NCV (m/s)} = \frac{\text{Distance between the electrodes (in meters)}}{\text{Time taken by the stimulus (in seconds)}}$$

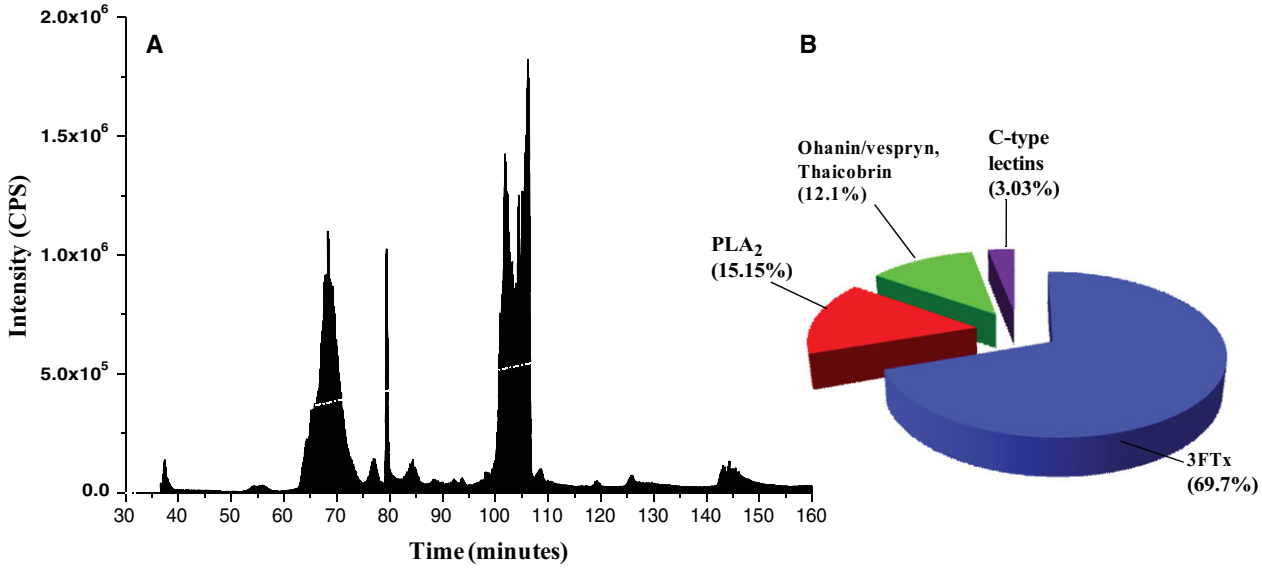

FIGURE 1. ESI-MS profile of crude *Naja kaouthia* venom. (A) Crude venom (2 mg) was loaded onto symmetry C18 column (5 μ, 4.6 $\times$ 250 mm, 300 Å) attached to the LC/MS/MS mass spectrometer. The bound proteins were eluted using a linear gradient of 80% acetonitrile in 0.1% trifluoroacetic acid (v/v) at a flow rate of 0.5 mL/min. The peak containing the protein of interest is indicated with an arrow. (B) Relative abundance of proteins present in *Naja kaouthia.* The proteins were classified into various families based on their molecular mass as obtained by LC-MS.

RESULTS

LC-MS Analysis of Crude Venom

To identify the various 3FTx toxins present in the crude venom, the whole venom was subjected to LC-MS (Figure 1). The mass of the peptides and proteins eluted at different time interval and detected by LC-MS are shown in Table 1. Based on their mass, these peptides and proteins are classified into different snake venom protein families. The masses of larger proteins could not be determined either due to its size or post-translational modifications. Relatively, 3FTx constituted the largest family of proteins (69.7%) in the venom followed by PLA_2 (15.15%), Ohanin/vespryn, thaicobrin (12.1%) and C-type lectins (3.03%) in crude venom of *Naja kaouthia* from North East India, Assam (Table 1).

Isolation and Purification of Nk-3FTx

Fractionation of the *Naja kaouthia* crude venom resulted into separation of 22 protein peaks (Figure 2A). On rechromatography of the peak containing the 3FTx using a linear gradient (40–42%) of buffer B with 0.1% trifluroacetic acid resulted into a single symmetrical protein peak suggesting the purity of the preparation (Figure 2A and B). The molecular mass of the purified protein was determined by ESI-MS and the reconstructed mass was found to be 7579.5 $\pm$ 0.591 Da (Figure 2C and D) which is in the range of 3FTx family. Hence, the purified protein was named as "Nk-3FTx" (*Naja kaouthia*-3FTx) and it constitutes about ~5% of the crude venom.

Amino Acid Sequencing

N-terminal sequencing by automated Edman degradation identified the first 33 amino acids (LTxLNxPEMFxGKFQIxRNGEKIxFKKLHQRRP) (Figure 3A). To determine the complete sequence of Nk-3FTx, the peptides were generated by trypsinization and analyzed by ESI LC-MS/MS. Total of seven peptides were obtained and its sequence were determined using Proteome Discoverer 3.1 (Table 2). The MS/MS sequences were assembled to determine the overlapping sequences and compared with *Naja naja kaouthia* CM-9a (P25679.2) (Figure 3A). We identified 55 amino acid residues of Nk-3FTx by a combination of N-terminal sequencing and ESI LC-MS/MS. However, sequence of three fragments could not be obtained as they were short peptides containing either R or K at the C-terminal end. The protein showed maximum homology to CM-9a isolated from *Naja naja kaouthia* of Thailand origin which is a non-conventional 3FTx [34] (Figure 3B). The difference in amino acid composition was found at six positions of Nk-3FTx protein sequence when compared with CM-9a. Amino acids Lys34, Asp43, Val48, Glu53, Ile54 and Lys62 in CM-9a were replaced by Phe34, Ala43, Lys48, Asp53, Val54 and Arg62 in Nk-3FTx.

TABLE 1. Molecular Mass of Various Proteins in Crude *Naja kaouthia* Venom Identified by ESI-MS

Retention Time (min)	*Mass (Da)*	*Intensity*	*Score*	*Delta Mass*	*Relative (%)*	*Total (%)*	*Assigned Protein Family*
36.63–37.57	6969.9	4.10E+04	13.93	0	100	68.53	3FTx
	7007	7.09E+03	8.15	37.1	17.31	11.86	
	6979.5	5.60E+03	4.1	9.6	13.67	9.37	
54.19–56.09	6888.4	3.73E+03	11.06	0	100	29.82	3FTx
	6853	3.06E+03	12.45	–35.4	82.05	24.47	
	6881.1	1.86E+03	6.16	–7.3	50.03	14.92	
	6978.9	1.51E+03	8.44	90.5	40.63	12.12	
63.27–70.96	7824.6	1.06E+05	16.64	0	100	50.79	3FTx
	7581.2	4.70E+04	13.04	–243.4	44.21	22.46	
	7615.3	1.15E+04	8.32	–209.3	10.77	5.47	
74.00–75.54	7110.3	2.47E+04	14.36	0	100	62.67	3FTx
	7824.7	3.43E+03	9.67	714.4	13.92	8.72	
81.17–82.41	7361.9	2.32E+04	14.9	0	100	51.77	3FTx
	7398.7	3.07E+03	6.06	36.8	13.25	6.86	
86.04–86.65	6755.6	9.79E+03	12.76	0	100	28.57	3FTx
89.09–89.69	18178.2	3.29E+03	2.94	15462.5	44.88	13.82	C–type lectins
	13480.7	3.18E+03	9.71	10765	43.38	13.36	PLA_2
90.61–91.15	7615.7	9.06E+03	8.57	0	100	38.98	3FTx
97.20–98.81	13319.9	7.54E+04	5.92	0	100	35.45	PLA_2
	13264.6	6.76E+04	6.77	–55.3	89.62	31.77	
	6739.8	2.10E+04	12.27	–6580.1	27.88	9.88	3FTx
98.88–99.22	6739.6	1.19E+05	15.61	0	100	64.73	3FTx
	6775.2	2.54E+04	1.43	35.6	21.35	13.82	
100.38–104.03	6740.6	2.49E+05	14.77	0	100	46.86	3FTx
	6839.5	2.19E+05	14.85	98.9	88.14	41.31	
	6775.4	2.13E+04	9.5	34.8	8.55	4	
	6935.9	1.68E+04	5.33	195.3	6.76	3.17	
105.69–106.61	6790.9	1.80E+04	14.08	0	100	65.7	3FTx
	6824.3	1.80E+03	8.7	33.4	9.99	6.56	
115.00–116.04	12041.7	4.78E+03	20.7	0	100	23.17	LAAO
	6789.6	4.77E+03	3.7	–5252.1	99.79	23.12	3FTx
120.45–123.14	13057.4	7.11E+03	8.87	0	100	22.52	PLA_2
	12901.4	5.10E+03	3.62	–156	71.68	16.14	Ohanin/vespryn, thaicobrin
	13095.8	1.12E+03	5.59	38.4	15.8	3.56	PLA_2
	12938.9	1.07E+03	2.85	–118.5	14.99	3.37	Ohanin/vespryn, thaicobrin
	12581.9	1.04E+03	4.52	–475.5	14.6	3.29	
136.88–141.21	12472.6	9.32E+03	5.51	0	100	36.43	Ohanin/vespryn, thaicobrin

The proteins are assigned to various snake venom protein families based on their molecular weight.

Biochemical and Biological Characterization

Purified Nk-3FTx was found to be devoid of PLA_2, direct, indirect hemolytic and antibacterial activity as compared to crude venom (Data not shown) even when the concentration was increased up to 10 μg/mL. Further, Nk-3FTx did not show any hemorrhagic or edema inducing activity on experimental mice (Data not shown). HEK 293 and L6 cell lines were treated with Nk-3FTx to evaluate its cytotoxic effect. Nk-3FTx did not display any cytotoxic effect on HEK 293 cells even up to 100 ng/mL concentration (Figure 1A in Supporting Information). However, cells treated with silver nitrate ($AgNO_3$) and crude venom exhibited altered morphology. Moreover crude venom treated cells were found to detach from surface of the culture plate (Figure 1A and B in Supporting Information). In contrast, L6 cells treated with Nk-3FTx showed moderate cytotoxicity at 100 ng/mL concentration after 24 h (Figure 1B in Supporting Information). The effect of Nk-3FTx on blood coagulation cascade was studied using human plasma. However, we observe a deviation from clotting time of treated plasma by Nk-3FTx as compared to normal clotting time (Table 3) which was not significant. There was a slight delay in the APTT, but when compared to crude venom [28], it was found to be negligible.

Neurotoxicity Studies

Treatment of Nk-3FTx to the sciatic nerve was found to decrease the CAP and NCV (Figure 4). The control nerve was found to elicit normal amplitude of

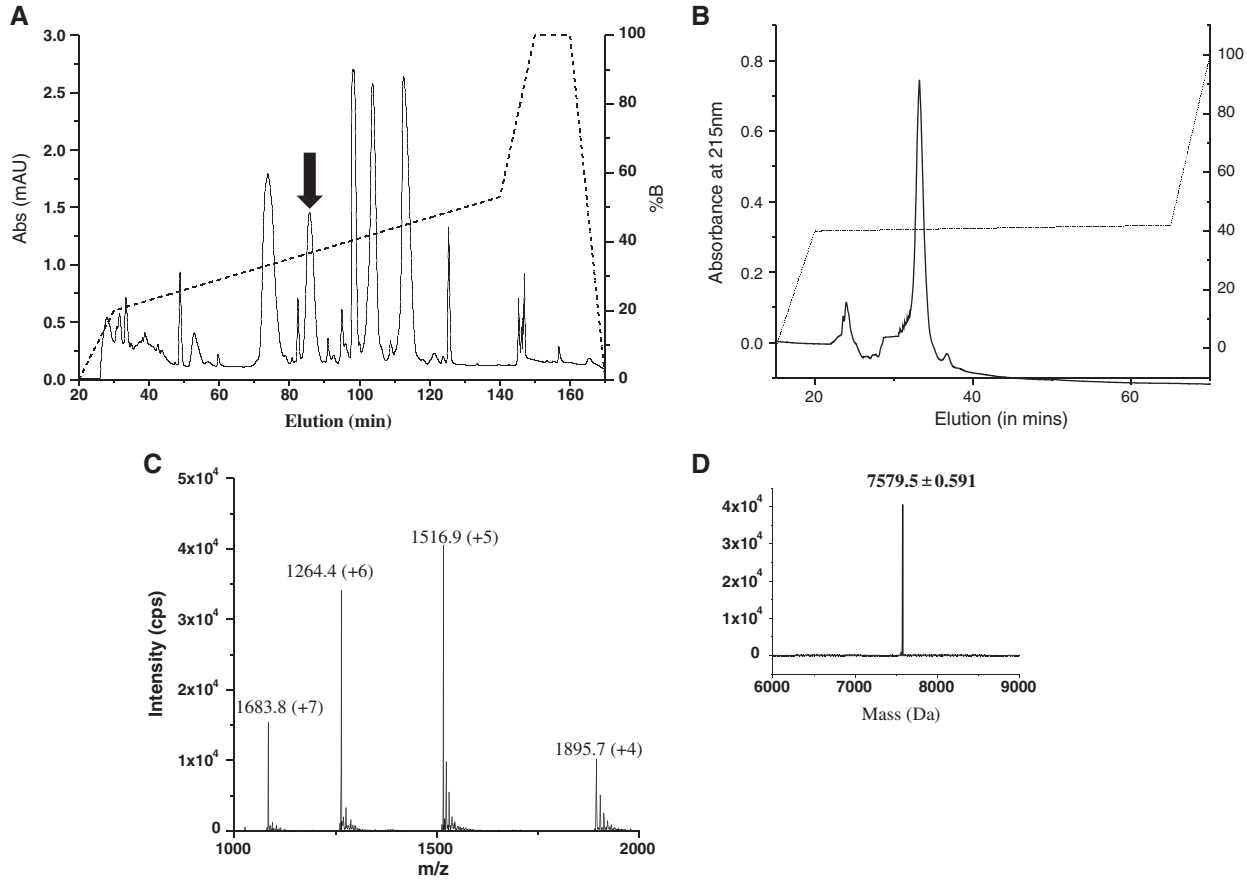

FIGURE 2. Fractionation of crude venom and isolation of Nk-3FTx. (A) Single step fractionation of crude *Naja kaouthia* venom was done on symmetry C18 column (5 μ, 4.6 × 250 mm, 300 Å). The elution was carried out with a linear gradient of 20–50% buffer B (80% acetonitrile in 0.1% trifluoroacetic acid (v/v)) at a flow rate of 1 mL/min. The Arrow indicates the protein of interest. (B) Rechromatography of the peak containing the protein of interest using a shallow gradient of 40–42%B. Rechromatography was carried out using the same column at a flow rate of 0.3 mL/min. (C) ESI/MS of the purified protein. The spectrum shows a series of multiple charged ions, corresponding to a single, homogenous peptide with a molecular weight of 7579.5 Da. (D) Reconstructed mass spectrum of the purified protein, cps = counts/s; Da = Dalton

9 mV at the distal electrode (Figure 4A); however with increasing concentration of Nk-3FTx, the CAP decreases in a dose dependent manner. At a high concentration of Nk-3FTx (1319.4 nmol/L), the CAP was 0.87 mV (Figure 4F). Similarly, a dose dependent decrease in NCV for sciatic nerve was observed with increasing concentration of Nk-3FTx (Figure 4H). To understand the effect of Nk-3FTx on voltage-gated sodium and potassium channels on the sciatic nerve specific channel blockers BH and QH were used. Both the channel blockers were tested on dissected sciatic nerve at various concentrations (0.132–1319.4 nmol/L) to determine the concentration at which saturation in the amplitude of CAP is 50%. At 13.194 nmol/L concentration both channel blockers, BH and QH inhibited the CAP to 50% as compared to nerve treated only with Ringer's solution (Data not shown). The consecutive treatment of nerve by BH and Nk-3FTx at a concentration of 13.194 nmol/L did not decrease the amplitude of CAP on sciatic nerve; (Figure 5F) however nerve treated with QH and Nk-3FTx at 13.194nmol/L was found to decrease the amplitude of CAP up to 2.3 mV (Figure 5F).

DISCUSSION

LC-MS analysis of *Naja kaouthia* venom of North East India reveals 3FTx as the major family followed by PLA_2, Ohanin/vespryn, thaicobrin and C-type lectins based on their molecular mass. This is similar to the venom of Thailand origin which is also reported to contain 3FTx as the major protein family [2]. However, variation in expression level of 3FTx and presence of unique toxins in these venoms cannot be denied. 3FTxs are structurally similar but functionally diverse group of proteins [35−37] which binds to their targets through specific amino acid residues [38]. The functional diversity in 3FTx toxin family is attributed to accelerated nonsynonymous substitutions of nucleotides in

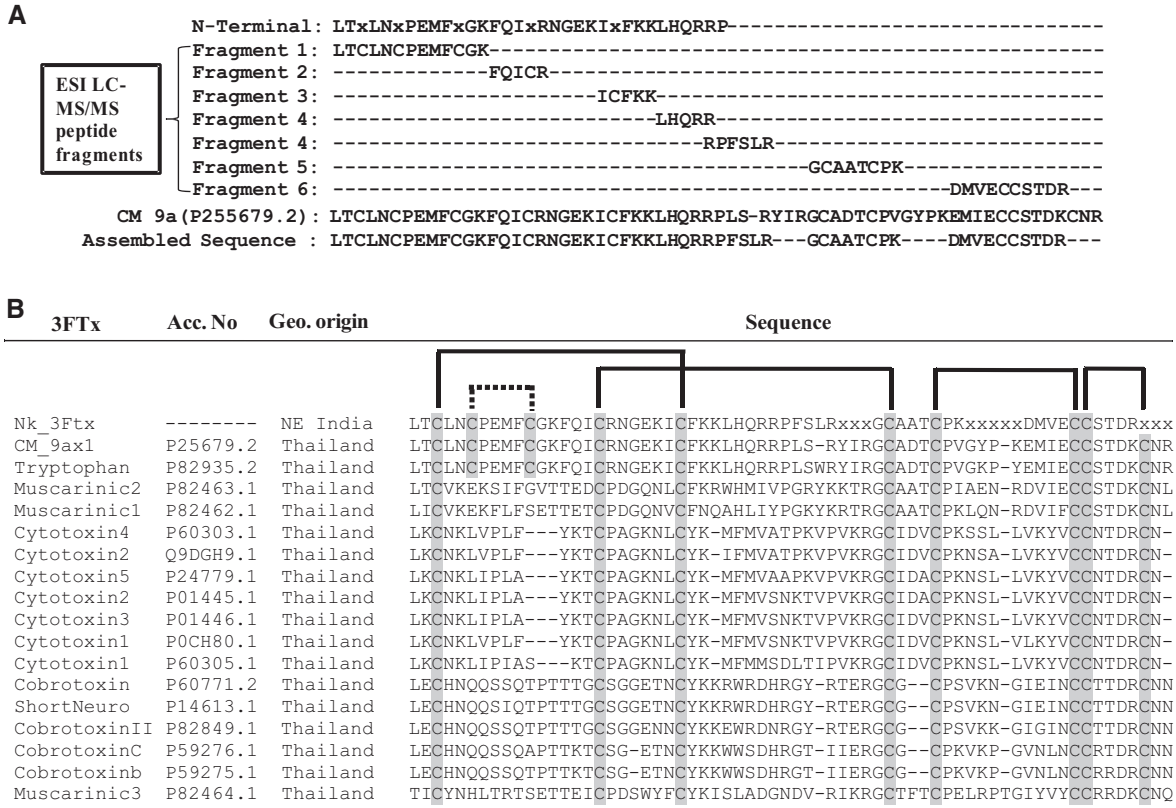

FIGURE 3. (A) Assembly of amino acid sequence of Nk-3FTx: Sequences were obtained from N-terminal sequencing and ESI LC-MS/MS of tryptic digested fragments of Nk-3FTx. The Cys residues are represented by "*x*" in N-terminal sequence. A non-conventional 3FTx, CM 9a (P25679.2) from *Naja naja kaouthia* is used as a reference to determine the overlapping sequences. (B) Multiple sequence alignment of Nk-3FTx with *Naja kaouthia* 3FTxs from different geographical locations. The linkage of Cys residues are shown in solid line and the fifth disulfide bridges are shown in dotted lines. Unidentified amino acid residues of Nk-3FTx are denoted by "*x*" and the gaps are shown by dashes.

TABLE 2. MS/MS Sequences of the Peptides Obtained from ESI LC-MS/MS

MS/MS Sequences	*Protein Group Accessions*	*Modifications*	*Charge*	*MH+ (Da)*	*ΔM (ppm)*
LTcLNCPEMFcGK	14195693	C3; C11	2	1573.01348	219.74
LTcLNcPEmFcGK	14195693	C3; C6; M9; C11	2	1646.22466	328.30
LTcLNcPEMFcGKFQIcR	14195693	C3; 6; C11; C17	2	2334.52300	210.27
LTcLNcPEMFcGK	14195693	C3; 6; C11	2	1629.66802	–13.05
FQIcR	14195693	C4	1	723.48621	173.49
LTcLNCPEMFCGK	14195693	C3	2	1515.38701	–171.14
IcFKK	14195693	C2	1	695.36096	–43.09
LTcLNcPEmFCGK	14195693	C3; 6; M9	3	1588.06308	–377.60
IcFK	14195693	C2	1	567.35712	107.80
RPSLR	14195693	M2; C12	1	14.25	0
GcAAcPK	290560289	C2; C6	1	735.7	60.39
DMVEccSTDR	290560289	C5; C6	2	1272.98076	404.85

Sequence were identified by the Proteome Discoverer with the Sequest program. Oxidation of methionine residues and S-carbamidomethylation of cysteine residues are shown in the table as modifications.

the protein coding region especially in the loops [4]. Sequence alignment of Nk-3FTx with other 3FTx reported from the venom of *Naja kaouthia* showed high similarity despite being partially sequenced with few amino acid substitutions. Nk-3FTx showed maximum similarity with CM-9a, but the substitutions in the amino acid might lead to functional differences with CM-9a.

Cytotoxic 3FTx have been reported to have a spatial distribution of positively charged or continuous hydrophobic domains (~40% of the total molecular surface) which interacts with the cell surface

TABLE 3. Effect of Nk-3FTx on clotting time of human plasma

	Normal Clotting Time	*0.005 μg/mL*	*0.05 μg/mL*	*0.5 μg/mL*	*5.0 μg/mL*
Re-calcification time (in s)	114.7 ± 2.2	115.6 ± 0.603	116.1 ± 0.87	120.7 ± 1.44	147.6 ± 4.01
PT (in s)	21.25 ± 0.64	21.1 ± 0.38	21.7 ± 0.4	22 ± 0.3	23.75 ± 0.07
APTT (in s)	42.1 ± 1.93	42 ± 0.35	43.15 ± 8.7	47.1 ± 1.4	68.5 ± 3.45

Clotting time of plasma in presence of Tris buffer was considered as normal clotting time.
Values represented are mean ± SD of three independent experiments. PT, prothrombin time; APTT, activated partial thromboplastin time.

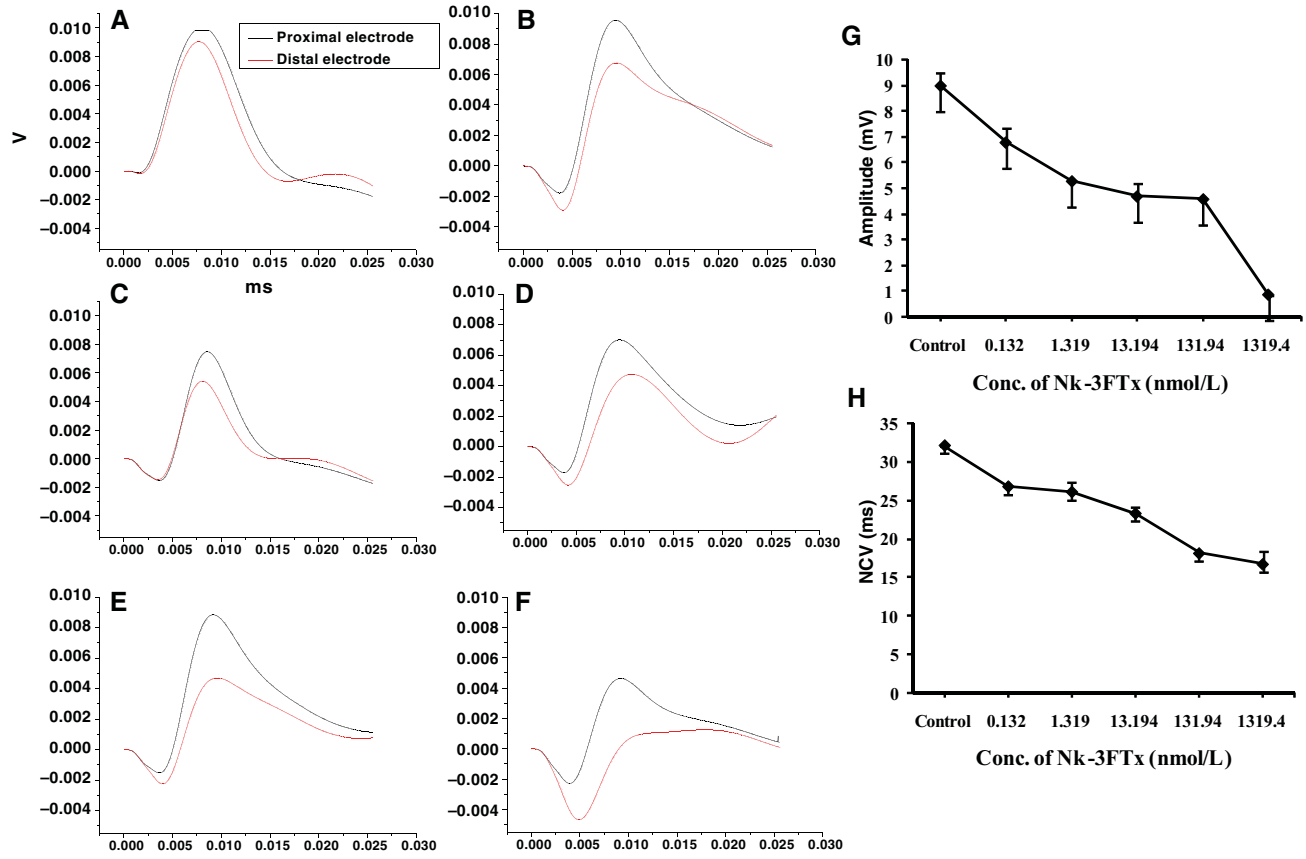

FIGURE 4. Effect of Nk-3FTx on isolated toad sciatic nerve. (A–F) Representative plot of CAP of isolated sciatic nerve pre-treated with various concentration of Nk-3FTx (A) Nerve treated with Ringer's solution as control; (B) 0.132 nmol/L of Nk-3FTx; (C) 1.319 nmol/L of Nk-3FTx; (D) 13.194 nmol/L of Nk-3FTx; (E) 131.194 nmol/L of Nk-3FTx; (F) 1319.4 nmol/L of Nk-3FTx. (G) Dose-dependent effect of Nk-3FTx on the amplitude of distal electrode on sciatic nerve. (H) Dose-dependent effect of Nk-3FTx on NCV (nerve conduction velocity) of the sciatic nerve. CAP and NCV of the sciatic nerve in absence of Nk-3FTx were considered as control. The values are ±SD of 3 independent experiments.

receptors followed by internalization to exhibit cytotoxicity [39, 40]. Any minor change in the distribution of positively charged residues leads to significant changes in its activity [40]. The noncytotoxic nature of Nk-3FTx could be due to absence of such patches of hydrophobic amino acid residues on its surface.

Opening of Na^+ channel leads to depolarization while the opening of K^+ channel leads to repolarization of the nerve during nerve transmission [41−43]. When CAP of treated sciatic nerve was recorded, changes in the recordings of distal electrodes were observed which suggest effect on any of the voltage-gated ion channels present on the nerve axon. When equimolar concentration of Na^+ channel blocker and Nk-3FTx were treated to sciatic nerve, the waveform obtained did not deviate from the nerve treated with only Na^+ channel blocker. However, when the equimolar concentration of K^+ channel blocker and Nk-3FTx were treated, to sciatic nerve it reduced the amplitude significantly and also the repolarization was delayed. This might be due to binding of Nk-3FTx to voltage-gated K^+ channels. Any effect on depolarization and repolarization of nerve would delay the signal transmission which can be correlated to neurotoxic activity [43, 44].

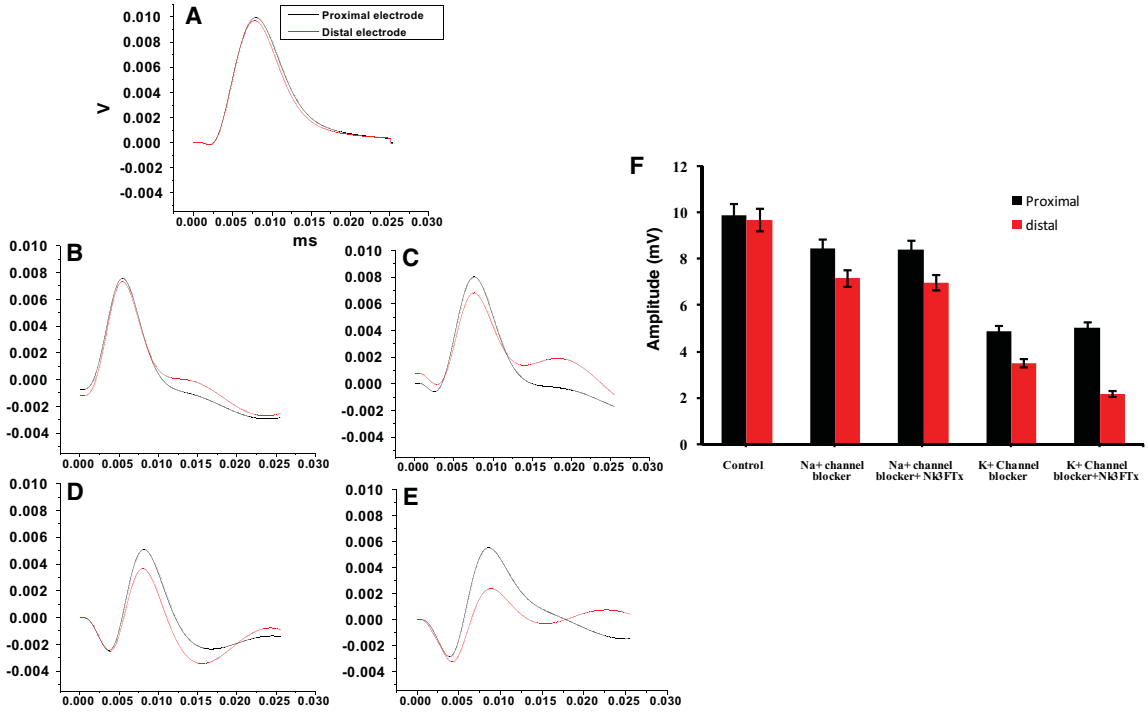

FIGURE 5. Determination of channel targeting by Nk-3FTx using specific channel blockers. (A–E) Representative plot of CAP of sciatic nerve pre-treated with specific channel blockers and Nk-3FTx (A) Nerve treated with Ringer's solution as control; (B) 13.194 nmol/L of BH (sodium channel blocker); (C) 13.194 nmol/L of Nk-3FTx; (D) 13.194 nmol/L of QH dihydrate (potassium channel blocker); (E) 131.194 nmol/L of Nk-3FTx; (F) Effect of channel blockers and Nk-3FTx on CAP of sciatic nerve. CAP of sciatic nerve in the absence of Nk-3FTx was considered as control. The values are ±SD of 3 independent experiments.

The inhibition of voltage-gated potassium channels by Charybdotoxin (ChTx), a scorpion toxin, is reported to be through a dyad consisting of positively charged Lys residue at 27 position and hydrophobic residue (Tyr36) at the C-terminal end [45]. Structure-function studies have revealed that Lys residue is critical for binding to K^+ channel [45, 46]. In Nk-3FTx, at 27th position Lys residue is present but the 36th position is occupied by another Lys. Further mutation of Lys at 31 and 32 position of ChTx has been found to decreases the binding affinity to wild type IKCa1 channel and Kv 1.3 potassium channel by ~47 fold and ~350 fold [46]. However in Nk-3FTx Arg is present at 31st and 32nd positions rather than Lys. Though there is a substitution of Lys to Arg, it might be involved in interacting with the potassium channel similar to ChTx and inhibit the CAP. Thus Nk-3FTx might be binding to K^+ channel for exhibiting its neurotoxic effect.

ACKNOWLEDGMENTS

This work was supported by a grant (BT/43/NE/TBP/2010) to RD by DBT, Govt. of India under the twinning Program to NER Region. Authors acknowledge the Forest Department, Govt. of Assam, India, for granting permission to collect venom samples. Grants from DBT, DST, and UGC to the Department of Molecular Biology and Biotechnology, Tezpur University are also highly acknowledged.

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SCIENTIFIC REPORTS

OPEN

Quantifying Demyelination in NK venom treated nerve using its electric circuit model

Received: 24 September 2015
Accepted: 10 February 2016
Published: 02 March 2016

H. K. Das[1], D. Das[2], R. Doley[2] & P. P. Sahu[1]

Reduction of myelin in peripheral nerve causes critical demyelinating diseases such as chronic inflammatory demyelinating polyneuropathy, Guillain-Barre syndrome, etc. Clinical monitoring of these diseases requires rapid and non-invasive quantification of demyelination. Here we have developed formulation of nerve conduction velocity (NCV) in terms of demyelination considering electric circuit model of a nerve having bundle of axons for its quantification from NCV measurements. This approach has been validated and demonstrated with toad nerve model treated with crude *Naja kaouthia* (NK) venom and also shows the effect of Phospholipase A_2 and three finger neurotoxin from NK-venom on peripheral nerve. This opens future scope for non-invasive clinical measurement of demyelination.

Speedy and efficient transmission of action potential sequences carrying neuro-signals depends on axon membrane sheath made by myelin[1,2]. Demyelination in nerve axon of peripheral nerve results in reduction of nerve conduction velocity (NCV) occurred in most of neuro diseases such as chronic inflammatory demyelinating polyneuropathy (CIDP)[3,4], Guillain-Barre syndrome (GBS)[5,6] etc. In fact, Node of Ranvier (NoR) is distorted by demyelination leading to slow movement of Na^+/K^+ ions. Clinical analysis of these diseases requires quantification of demyelination. In this direction, invasive measurement of demyelin/myelin thickness using SEM imaging and nerve biopsy is a destructive and clinically critical and harmful for the patients. Moreover, these techniques have risk for site selection of the damaged nerve and may cause discomfort and infection after surgery for nerve testing[7,8]. Hodgkin and Huxley first demonstrated quantitative description of axonal membrane currents for giant axon of a squid with the use of electrical circuit model developed by them[9]. Later, experiments were performed on toad model with consideration of node of Ranvier to prove that the membrane currents are dependent on Na^+, K^+ ions and other Ca^{++} ions (contributing leakage current). As seen in previous studies[10–12], snake venom especially *Naja kaouthia* venom causes degradation of myelin sheath due to having high percentage of phospholipase A_2 (PLA_2), neurotoxins and cytotoxins[13–16]. In fact, NK-PLA_2 belonging to lypolytic enzyme family catalyzes the hydrolysis of fatty acid esters at position 2 of 1, 2 diacyl-sn-phosphoglycerides producing lysophospholipids and free fatty acids[17]. So *Naja kaouthia* venom is one of the strong candidates for demyelination of myelin sheath due to the presence of NK-PLA_2. Previous studies show that PLA_2 also causes Alzheimer's disease[18], Multiple Scelerosis (MS)[19,20], Epilepsy[21] due to motor dysfunction. In this direction the development of toad nerve model has revolutionized the field of neuroscience especially for human peripheral clinical treatment[22,23]. Moreover, the membrane transport and activity of neurotransmitter are better examined on isolated toad nerve having bundle of axons[24,25].

Electric model of a demyelinated nerve. A rigorous theory of electrical circuit model of a demyelinated nerve was made by previous authors[26–28]. Since frog nerve consists of bundle of axons (Fig. 1(a,b)), H-H electrical circuit model for giant nerve is modified considering bundle of axons as shown in Fig. 1(b,c). The ephaptic interactions between action potential impulses of parallel axons in a bundle is demonstrated to describe NoR misalignment among axons (Fig. 1(a,d)). The nerve conduction velocity with incorporation of demyelinating factor γ, alignment parameter A and an ephaptic coupling constant α among axon-1 surrounded by six axons derived as

[1]Department of Electronics and Communication Engg., Tezpur University, Tezpur-784028, Assam, India. [2]Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, Assam, India. Correspondence and requests for materials should be addressed to P.P.S. (email: ppstezu@gmail.com)

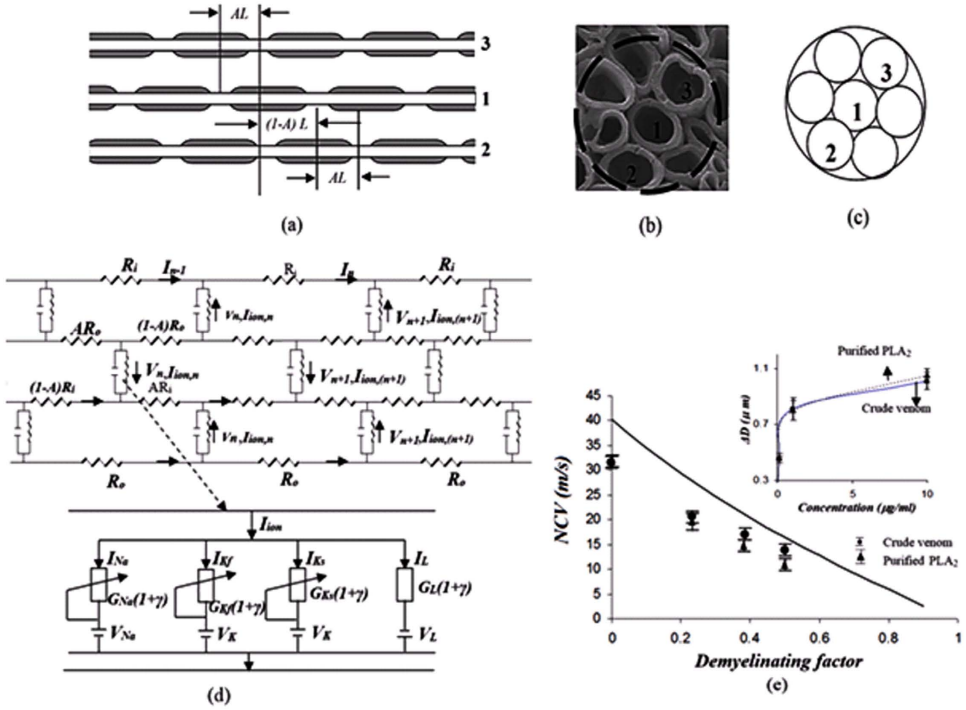

Figure 1. A demyelinated nerve consists of bundle of axons. (**a**) a bundle of nerve denoting three nerves – nerve 1, nerve 2 and nerve 3 respectively where the Node of Ranvier of axons are misaligned by alignment factor A (where $\frac{1}{2} \leq A \leq 1$). A = 1 indicates that two axons are aligned exactly, and $A = \frac{1}{2}$ indicates that two axons are evenly staggered. "L" is the internodal length i.e., the distance between two internodes in the nerve. (**b**) Typical SEM image of normal nerve having bundle of axons in which axon 1 is surrounded by six axons. (**c**) Correspondingly to SEM image we consider axon 1 is surrounded by six axons of equal diameter. We consider only ephaptic interactions of six surrounded axons on neuro conduction in axon 1. (**d**) Electric circuit model corresponds to bundle of axons in a nerve shown in the figure. (**e**) Nerve conduction velocity (NCV in m/s) versus demyelinating factor is obtained by using equation (1). NCV is formulated by using electric circuit model of a nerve having bundle of axons misali gned by alignment factor, $A = \frac{1}{2}$. The NCV of toad sciatic nerve decreases with increase of demyelination. The experimental points of black dots are obtained from conduction of action potential (latency between proximal and distal action potential) in sciatic nerve of frog model demyelinated by *Naja kaouthia* crude venom with concen tration 0.1, 1.0 and 10 μg/ml. Similarly experimental points of triangle are obtained from sciatic nerves treated with NK-PLA_2 (purified from crude venom). The inset of Fig. 1(e) shows demyelination ΔD in μm versus concentration of crude venom/NK-PLA_2 (ΔD is the difference between normal nerve thickness and demyelinated nerve, obtained from SEM images shown in Fig. 2). The solid line and dashed line in inset figure are drawn by using experimental points with minimum deviation. The lines are almost close to each other proving NK-PLA_2 mainly responsible for demyelination of the nerve of toad model.

$$
\begin{aligned}
V_c \;=\; & \sqrt{\frac{G_{Na}(1+\gamma)(1-\alpha)}{R_o(N-1)A(1-\gamma)C^2(1+\gamma)^2}}\left(\frac{V_{Na}-2V_{th}^{Na}}{\sqrt{2V_{Na}}}\right)(1-\gamma) \\
& + \sqrt{\frac{G_{Kf}(1+\gamma)(1-\alpha)}{R_o(N-1)A(1-\gamma)C^2(1+\gamma)^2}}\left(\frac{V_K-2V_{th}^{Kf}}{\sqrt{2V_K}}\right)(1-\gamma) \\
& + \sqrt{\frac{G_{Ks}(1+\gamma)(1-\alpha)}{R_o(N-1)A(1-\gamma)C^2(1+\gamma)^2}}\left(\frac{V_K-2V_{th}^{Ks}}{\sqrt{2V_K}}\right)(1-\gamma) \\
& + \sqrt{\frac{G_{L}(1+\gamma)(1-\alpha)}{R_o(N-1)A(1-\gamma)C^2(1+\gamma)^2}}\left(\frac{V_L-2V_{th}^{L}}{\sqrt{2V_L}}\right)(1-\gamma)
\end{aligned}
\tag{1}
$$

where, N = number of nerves surrounding a demyelinated nerve (here N is considered to be six), as shown in Fig. 1(b,c), $R = \frac{R_o(N-1)A(1-\gamma)}{(1-\alpha)}, \alpha = \frac{R_i}{R_i + R_o A(N-1)(1-\gamma)}$,

γ = demyelination factor which is defined as the ratio of change in myelin thickness due to demyelination to the actual amount of myelin thickness of the nerve. The effect of demyelination factor upon resistance, capacitance and conductance in the circuit has already been discussed in our previous work[29,30]. The total resistance in

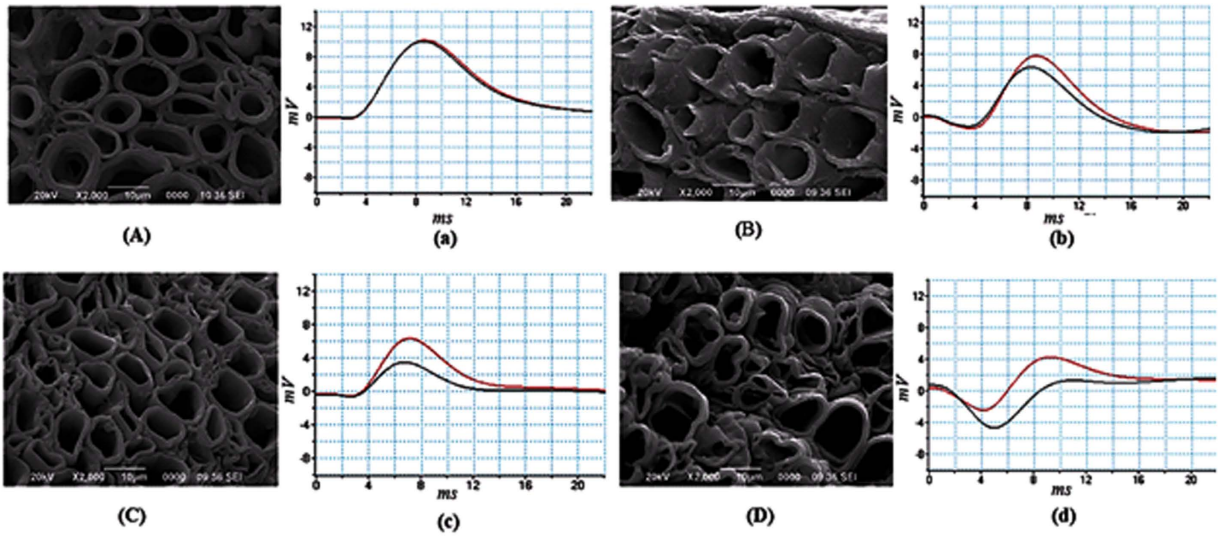

Figure 2. Effect of crude venom with different concentration on sciatic nerves of toad and their nerve conduction signals (proximal and distal action potential) with corresponding SEM images of their sciatic nerves. We have repeated the experiments for six times and estimated results statistically (as shown in supplementary table S1 and supplementary Fig. S1(a)). (**A**) SEM image of normal sciatic nerves with myelin thickness of $1.79 \pm 0.23\,\mu m$ at $\gamma = 0$ (normal). (a) Corresponding neuro conduction signal obtained by AD instrument (Powelabs, Australia) consists of proximal and distal action potential. The NCV is determined by using distance between two electrodes and divided by latency between proximal and distal action potential (shift between peaks of proximal and distal action potential) as 32.1 ± 1.71 m/s, which is very close to normal value of toad nerve. (**B**) SEM image of toad sciatic nerve treated with 0.1 μg/ml crude NK-venom shows slight reduction of myelin sheath, the thickness measured is $1.22 \pm 0.15\,\mu m$ corresponding to demyelinating factor, $\gamma = 0.32$ (which represents 32% reduction of myelin thickness). (b) Corresponding neuro conduction signal provides NCV of 21.95 ± 0.99 m/s (determined from latency between proximal and distal action potential peaks) which is slight less than the normal value. (**C**) SEM image of toad sciatic nerve treated with 1 μg/ml crude NK-venom shows more reduction of myelin thickness of $1.00 \pm 0.9\,\mu m$ (i.e., more demyelination) with $\gamma = 0.44$ (which indicates 44% reduction of myelin thickness). (c) NCV is determined from neuro conduction signal as 18.33 ± 1.2 m/s which is less than normal value. (**D**) SEM image of toad sciatic nerve treated with 10 μg/ml crude NK-venom shows reduction of myelin sheath, the myelin thickness of which is found to be $0.91 \pm 0.08\,\mu m$ corresponding to demyelinating factor, $\gamma = 0.49$ (which shows 49% reduction of myelin thickness). (d) NCV is estimated from latency of neuro conduction signal as 14.28 ± 0.85 m/s which is far below normal value.

the circuit is mainly divided into internal resistance (R_i) and external resistance (R_o). The I_n and V_n are the ionic current and voltage at node n. The total ionic current $I_{ion,n}$ at node n consists of current contributed by sodium ion as ($I_{Na,n}$), fast potassium as ($I_{Kf,n}$), slow potassium as ($I_{Ks,n}$) and also leakage current contributed by mainly calcium and chlorine as ($I_{L,n}$), through demyelinated ionic resistances $1/G_{Na}(1+\gamma)$, $1/G_{Kf}(1+\gamma)$, $1/G_{Ks}(1+\gamma)$ and $1/G_L(1+\gamma)$ respectively, where G_{Na}, G_{Kf}, G_{Ks} and G_L are ionic conductance of sodium ion, fast potassium, slow potassium and leakage ions (mainly calcium, chlorine etc.). The voltage V_n at node n depends on V_{Na}, V_{Kf}, V_{Ks} and V_L, Nernst (diffusion) potentials of sodium, fast potassium, slow potassium and leakage ions at which corresponding currents return to be zero. V_{th}^{Na}, V_{th}^{Kf}, V_{th}^{Ks} and V_{th}^{L} are threshold voltages of sodium, fast potassium, slow potassium and leakage currents respectively at which sodium current, fast potassium, slow potassium and leakage currents start to flow at active node as shown in Fig. 1(d). The values of ionic conductance, Nernst potential and threshold voltage of sodium, slow potassium, fast potassium and leakage ions of frog are obtained from previous works[29,30]. The variation of NCV is determined by using equation (1), decreases with increase of demyelinating factor (Fig. 1(e)). To validate our formulation of neuro-signal conduction, the experiments were performed on toad nerves treated with crude venom of *Naja kaouthia* (NK) for six times and the decrease of myelin sheath (demyelination) with increase of concentration of crude venom was observed every time (Fig. 1(e)).

Results

For experimental estimation of demyelination, sciatic nerves were isolated from toad and its storage was made in Ringer's solution. During experiments on nerve conduction by using AD instruments, the sciatic nerves were kept moist in nerve chamber using Ringer's solution. The reduction of myelin thickness (i.e., demyelination) with increase of crude venom concentration was confirmed by SEM images in Fig. 2(A–D). We have obtained experimentally propagation of action potential stimuli applied on sciatic nerves treated with different crude venom concentration as shown in Fig. 2(a–d). The nerve conduction signals obtained from proximal and distal action potential nerve treated with 0.1 μg/ml, 1.0 μg/ml and 10 μg/ml crude venom concentration shows reduction of nerve conduction velocity (NCV) due to demyelination of axons in the nerve (Fig. 2). It also shows the reduction of action potential amplitude with crude venom concentration. The experimental values of NCV in Fig. 1(e)

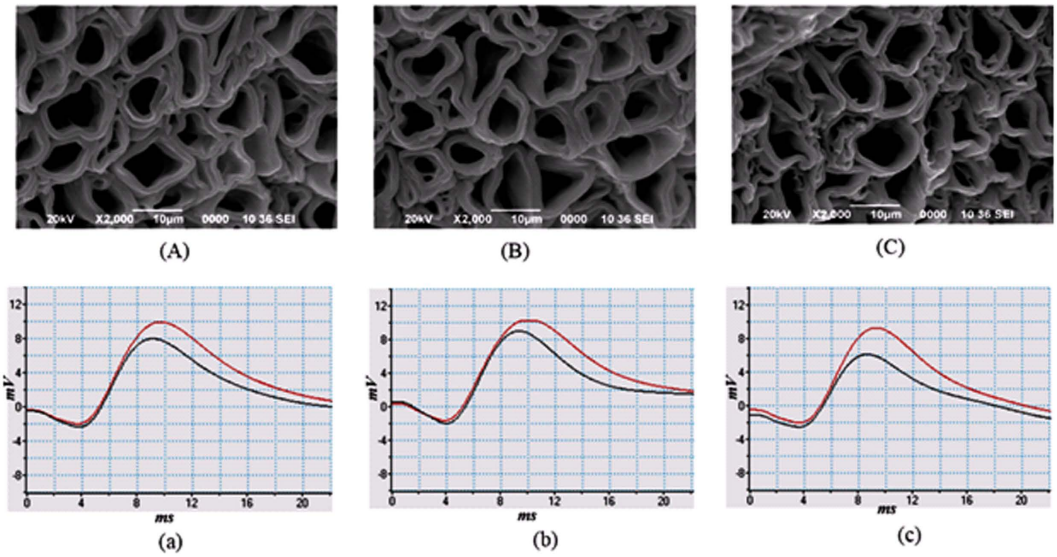

Figure 3. Effect of purified NK-PLA$_2$ with different concentration on sciatic nerves of toad and their nerve conduction signals (proximal and distal action potential) with corresponding SEM images of their sciatic nerves. We have performed the experiments repeatedly and the myelin thickness with its demyelinating factor is estimated statistically (as shown in supplementary table S1 and supplementary Fig. S1(b)). (**A**) SEM image of sciatic nerve with 0.1 μg/ml NK-PLA$_2$ with myelin thickness of 1.4 ± 0.29 μm with $\gamma = 0.21$ (which shows 21% reduction of myelin thickness). (a) Corresponding neuro conduction signal obtained by AD instrument and NCV is obtained from distance between two electrodes divided by latency between proximal and distal action potential (shifts between peaks of proximal and distal action potential). (**B**) SEM image of toad sciatic nerve treated with 1 μg/ml NK-PLA$_2$ shows a reduced thickness of myelin of 1.12 ± 0.34 μm corresponding to demyelinating factor, $\gamma = 0.37$ (which represents 37% reduction of myelin thickness). (b) Corresponding neuro conduction signal. (**C**) SEM image of toad sciatic nerve treated with NK-PLA$_2$ concentration of 10 μg/ml with more demyelination, the myelin thickness being 0.89 ± 0.15 μm, the demyelinating factor being $\gamma = 0.5$(which indicates 50% reduction of myelin thickness). (c) Corresponding neuro conduction signal at 10 μg/ml of NK-PLA$_2$. The SEM images shows increase of demyelination with increase in purified NK-PLA$_2$ concentration, while the neuro conduction signals confirms the reduction of NCV as the concentration of purified NK-PLA$_2$ increases.

matches well with theoretical values of NCV which was obtained by using the formulation (equation 1) using MATLAB and these values of NCV may used to predict the amount of demyelination as shown in the curve of the figure. From the SEM images (Fig. 2(B–D)), the demyelination factor γ of sciatic nerve treated with 0.1 μg/ml, 1.0 μg/ml and 10 μg/ml crude venom concentration are estimated as ~0.21, 0.37 and 0.5 respectively (as shown in supplementary table S1 and supplementary Fig. S1(a)) which are close to those obtained from NCV results of Fig. 1(e). Estimation of demyelination from NCV formulation will avoid the invasive difficulties of SEM imaging and nerve biopsy. The rate of increase of demyelination (ΔD) with crude venom concentration is very fast up to 1 μg/ml and it becomes saturated after that (inset of Fig. 1(e)). As seen in Fig. 3, NK-PLA$_2$ present in crude venom is mainly responsible for demyelination as per the following reaction of NK-PLA$_2$ with phospholipids of myelin sheath.

$$\text{NK-PLA}_2 + \text{Myelin} \xrightarrow[\text{Ca}^{2++}\text{ ion as cofactor}]{\text{Hydrolysis}} \text{Lysophospholipids} + \text{free fatty acids} \quad (2)$$

The rate of reaction increases with increase of NK-PLA$_2$ molecules (as NK-PLA$_2$ concentration increases with crude venom concentration/dose) and as a result more demyelination takes place with increase in concentration of NK-PLA$_2$ (Fig. 3). The nerve conduction experiment of NK-PLA$_2$ (purified from NK crude venom) treated sciatic nerves shows reduction of NCV due to demyelination in which the variation of demyelination thickness (ΔD) with NK-PLA$_2$ concentration for NK-PLA$_2$ treated nerve is almost close to that of crude venom treated nerve proving major contribution of demyelination by NK-PLA$_2$ (Fig. 1(e)). The demyelination factor γ of sciatic nerve (treated with 0.1 μg/ml, 1.0 μg/ml and 10 μg/ml NK-PLA$_2$ concentration) estimated from the SEM images (Fig. 3(A–C), are also almost close to those obtained from NCV results of Fig. 1(e) as shown in supplementary table S1 and supplementary Fig. S1(b).

The similar results are obtained after repeated nerve conduction experiments of normal sciatic nerve and nerves demyelinated with both crude venom and NK-PLA$_2$ and NCV are estimated with $\pm$SD (standard deviation) statistically to minimize the percentage of error as per standard method[31]. The theoretical curve of NCV versus demyelination factor (Fig. 1(e)) validated with nerve conduction experiments, establish a hypothesis to quantify demyelination in the patients and the demyelination of nerve is obtained from known NCV using

demyelination factor as shown in the curve. This hypothesis may help the medical experts to estimate the quantity of demyelination from the range of NCV of the patients.

Reduction of amplitude of NC signal. The Nerve conduction signals in Fig. 4(e–g) shows decrease of amplitude of wave due to both Na^+ and K^+ channel blocking in crude venom treated toad nerve. The weak three finger neurotoxin (3FT-neurotoxin) which constitutes a major percentage of cobra venom is mainly responsible for channel blockade activity in nerve axon[15,32–36]. 3FT-neurotoxin binds the neural Ach receptors causing blocking of transmission of both Na^+ and K^+ ions through the ion gated channels (Fig. 4(a–d)). The blockade increases with increase of crude venom concentration/dose due to increase in 3FTx concentration. The nerve conduction experiments of 3FT-neurotoxin (purified from crude venom) treated nerve confirms reduction of action potential due to blocking of channel[37]. There is no change of value of latency between peak of proximal and peak of distal action potential even with increase of 3FTx concentration proving no reduction of myelin thickness of sciatic nerve treated with 3FTx and it is confirmed from SEM image (Fig. 4(h)).

Discussion

Although we have performed experiments on sciatic nerve isolated from toad our findings *may* open a non-invasive clinical way to quantify demyelination of a peripheral nerve from NCV measurements. The observation shown here is emerging experimental evidences for demyelination and channel blockade using NK crude venom. Our electric circuit model for a nerve consisting of six axons matches with experimental results of demyelination by using NK crude venom. Our results may also provide demyelination analysis of different critical neuro-diseases such as CIDP, GBS etc. Although our experiments are limited to peripheral sciatic nerve, our model may also quantify demyelination of central nervous system (CNS) including motor nerve from nerve conduction velocity. So, future studies will be needed to determine the role of crude venom in CNS demyelination. The result may extend to support non-invasive clinical analysis of CNS demyelinating diseases like multiple sclerosis, Alzheimer's disease, Epilepsy, etc. In our works, we have also demonstrated channel blocking in sciatic nerve using 3FTx weak neurotoxin (Fig. 4). It may lead a way to quantify Na^+ and K^+ channel blocking, due to the binding of 3FTx with nACh receptors.

Materials and Methods

Ethics Statement. Common Asian toads (*Duttaphrynus melanostictus*) were used for neurotoxicity studies. All animal experiments were performed in accordance with the guidelines from the Defence Research Laboratory, Tezpur, Assam (India) under Registration Number 1227/bc/07/CPCSEA and approved by Tezpur University Animal Ethical Committee (TUAEC) (Approval no: DORD-Pro/TUAEC/10-56/14/Res-06). Efforts were made to minimize the number as well as sufferings of animals during the experiments.

Animals. For validation of NCV in a demyelinated nerve, we have extracted sciatic nerve from common Asian toad (*D. melanostictus*). Further, we have attempted to see the variation of demyelination in the sciatic nerve using different concentration of snake venom. We have taken 48 toads of same age (~1–2 months) for validation of demyelination measurements from nerve conduction velocity and divided into four groups (A, B, C and D). The sciatic nerves of toads of group A is not treated with crude venom whereas the sciatic nerves of B,C and D groups are treated with *Naja kaouthia* venoms of 0.1 μg/ml, 1 μg/ml and 10 μg/ml respectively.

Collection of snake venom. Crude venom from *Naja kaouthia* of North East India was collected from the wild and stored as explained in Das *et al.*[38]. The permission for milking of snakes from Assam was obtained from Principal Chief Conservator of Forest (Wild Life) and Chief Wild Life Warden of Assam, India (WL/FG.27/tissue Collection/09 dated 07/10/2011).

Determination of protein content. Protein concentration of the crude venom and purified toxin was determined according to Lowry's method using BSA as a standard[39].

Partial purification of fraction containing Phospholipase A_2 (PLA_2) and three finger toxin (3FTx). PLA_2 and three finger toxin was purified as described in Das *et al.*[37]. Briefly, crude venom was subjected to single step fractionation on RP-HPLC using symmetry C18 column (5 μ, 4.6 × 250 mm, 300 Å) (Waters, USA). Fractionation was carried out in a linear gradient of buffer B (80% ACN containing 0.1%TFA) in a pre-equilibrated column with buffer A (0.1% TFA in milli Q water) on a HPLC system (Waters, USA). Eluted protein peaks were monitored at 215 and 280 nm and collected manually. Considering the retention time of the protein peaks likely to contain PLA_2 and 3FTxs were isolated.

Phospholipase A_2 activity assay. PLA_2 activities of the crude venom as well as the fractionated peaks were determined according to Doley and Mukherjee[40] using egg yolk as a substrate. One unit of PLA_2 activity was defined as the amount of protein which produces a decrease in 0.01 absorbance in 10 mins at 740 nm.

Preparation of Sciatic nerve. The method as described by Katsuki *et al.* was followed for sciatic nerve preparation[31]. In brief, common Asian toads *(Duttaphrynus melanostictus)* weighing 30–35 gm of either sex were decapitated and then pithed. Sciatic nerve of length 4–6 cm and 0.5–1 mm diameter was dissected from lumber plexus to the knee joint. Throughout the procedure the nerve was continuously flooded with Ringer's solution. Finally, the dissected nerve was treated with venom sample and mounted on nerve chamber (AD Instruments, Powerlabs, Australia) containing Ringer's solution.

Recording of compound action potential (CAP) and determination of nerve conduction velocity (NCV). Various concentrations of crude NK venom and partially purified PLA_2 from the crude venom (0.1 μg/μl,

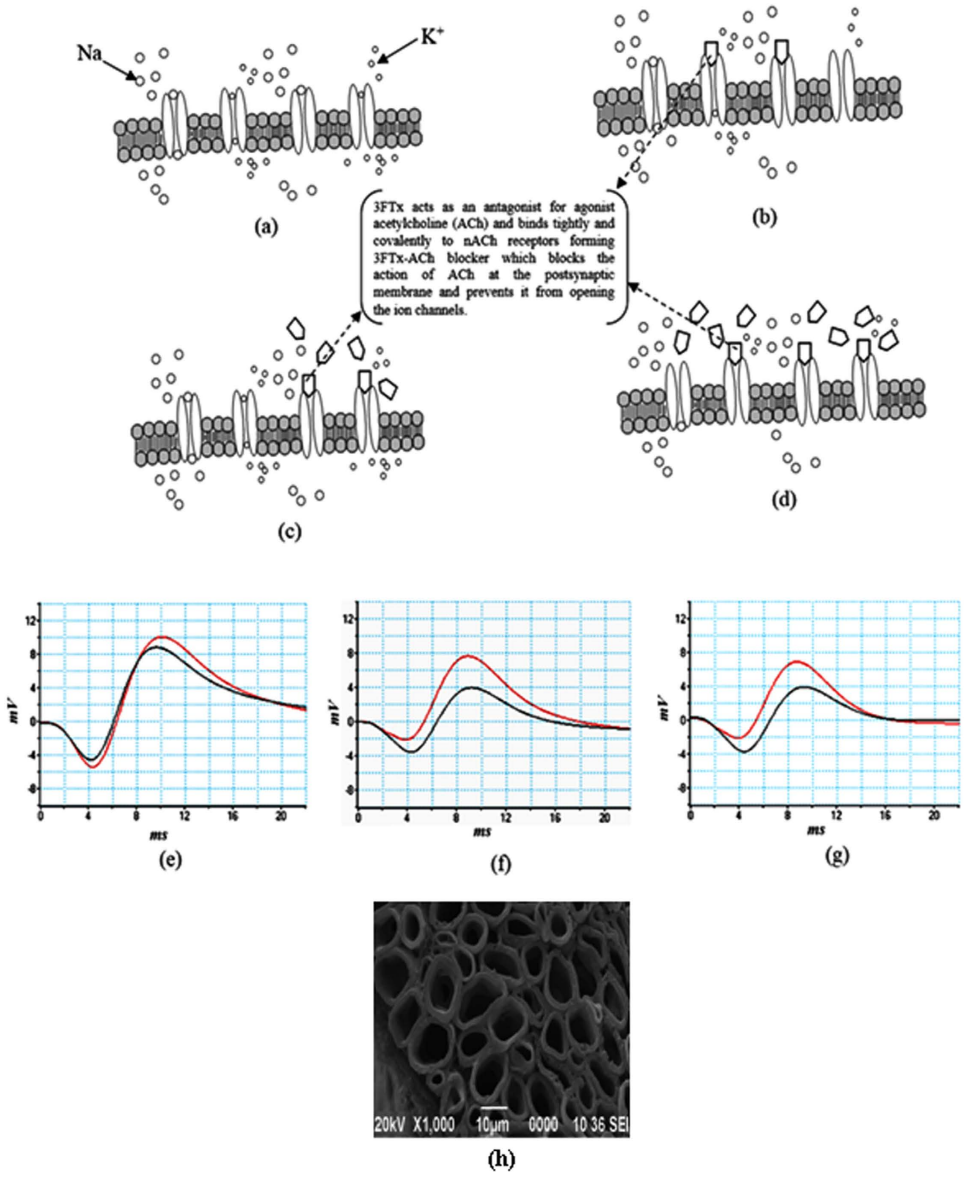

Figure 4. Mechanism of channel blocking in cell membrane. We have performed channel blocking of sciatic nerve using 3FTx neurotoxin for 5–6 times and the results obtained from repeated experiments are almost same. We have shown the results which are estimated statistically. (**a**) transportation of Na^+ into cell through cell membrane and K^+ transportation leaving from cell through membrane and provides saltatory movement of the nerve impulse (action potential). (**b**) Shows less blocking of Na^+ and K^+ channel by NK crude venom in which 3FT weak neurotoxin binds with acetylcholine receptors (AchR) and then blocks channel. Number of blocking is small if crude venom concentration is less. (**c**) Represents more blocking if crude venom concentration becomes more because of presence of more 3FT neurotoxin. (**d**) Represents most of channels are blocked if crude venom concentration is very high (more than 10 µg/ml). (**e**) Neuro conduction signal (proximal and distal action potential) obtained by using AD instruments. There is a reduction of peak of distal action potential with respect to proximal action potential showing Na^+ and K^+ channel blocking. (**f**) Neuro conduction signal of 1 µg/ml 3FTx-weak neurotoxin treated sciatic nerve shows a sharp reduction of peak of action potential. (**g**) Neuro conduction signal of 10 µg/ml 3FTx neurotoxin shows almost same sharp reduction of distal action potential peak. As in 1 µg/ml 3FTx treated sciatic nerve, most of channels are blocked (Fig. 4(d)), same reduction of distal action potential was observed even after treatment of 10% 3FTx treated sciatic nerve. (**h**) SEM image of toad sciatic nerve treated with 10 µg/ml 3FTx shows no reduction of myelin sheath even at high concentration. The myelin thickness of which is found to be 1.74 ± 0.18 µm, which is similar to normal sciatic nerve.

1 µg/µl and 10 µg/µl) was treated *in-vitro* to the dissected nerve for 2 mins. Standard techniques for extracellular recordings were followed. CAP was measured in a nerve chamber (AD Instruments, Powerlabs, Australia) equipped with 15 stainless steel electrodes as shown in Fig. 5. A dual Bio Amp/stimulator was used to obtain and

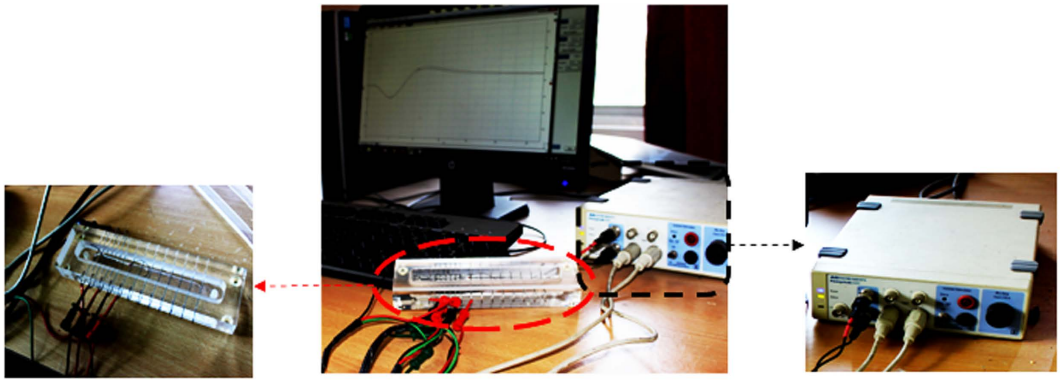

Figure 5. A schematic picture of the recording set up. AD Instrument consists of a dual Bio Amp/stimulator and a nerve chamber. The dual Bio Amp/stimulator is used to obtain and record CAP and NCV. The nerve chamber is equipped with 15 stainless steel electrodes and the dissected nerve is mounted on it filled with Ringer's solution. The setup was comprised of two male BNC (Bayonet Neill–Concelman) connectors to three micro-hooks constructed of gold-plated beryllium copper which was used to stimulate the nerve. CAP was analyzed by SCOPE (Powerlabs, Australia).

record CAP. In brief, the dissected sciatic nerve was externally stimulated with a frequency of 1 Hz where pulses at 0.1 ms duration were used to determine the CAP[31]. The setup was comprised of two male BNC (Bayonet Neill–Concelman) connectors to three micro-hooks constructed of gold-plated beryllium copper which was used to stimulate the nerve. The electrodes for proximal and distal stimulus recording were placed at a distance of 3 cm. Nerve end with lumber plexus of spinal cord was connected with proximal recording electrode and electrode at nerve end connecting knee joint acted as distal recording electrode. Each experiment for recording of CAP was completed within 20 s of timeframe to avoid drying of the dissected nerve. CAP was analyzed by SCOPE (Powerlabs, Australia). Nerve only treated with Ringer's solution was considered as control. The experiments were performed six times and ±SD was estimated statistically to minimize the percentage of error as per standard method[31]. The NCV was calculated using the latency and distance data from the signal.

SEM imaging of sciatic nerve treated with different concentration of venom. Sciatic nerves were subjected for SEM analysis to check the effect of crude venom on morphology of the isolated nerve. Untreated nerve is served as control for the analysis. Sciatic nerve preparations were incubated for 15 mins with various concentrations of crude venom (0.1 mg/ml–10 mg/ml) and primary fixation was done using 2.5% gluteraldehyde for 4 hr. Further the nerve was subjected for secondary fixation using 1% OsO_4 (Osmium tetroxide) for 4 hr for better penetration. Cross section of sciatic nerve was made by slicing at a length of 10 mm using glass cutter in a microtome maintaining uniformity. The sliced nerve segments were further observed under SEM for the structural change. Demyelination or reduction of myelin thickness considering 6–7 nerves in a bundle was quantified with the use of measuring scale installed in the SEM.

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Acknowledgements

This work was supported by a grant to Das, H. K. by UGC. The author acknowledge UGC-RGNF for granting the fund (F1-17.1/2012-13/RGNF-2012-13-SC-ASS-14921). The authors are thankful to Mr. Ratan Baruah, Dept. of Physics, Tezpur University, for his help during the SEM images. The authors are also thankful to Dr. P. Chattopadhyay, Division of Pharmaceutical Technology, Defence Research Laboratory, DRDO, Tezpur, Assam, India, for his guide in performing the animal experiments.

Author Contributions

H.K.D., D.D., R.D. and P.P.S. contributed equally in performing the animal experiments. H.K.D. and P.P.S. wrote the main manuscript text and prepared Figures 1–4. D.D. and R.D. thoroughly reviewed the manuscript.

Additional Information

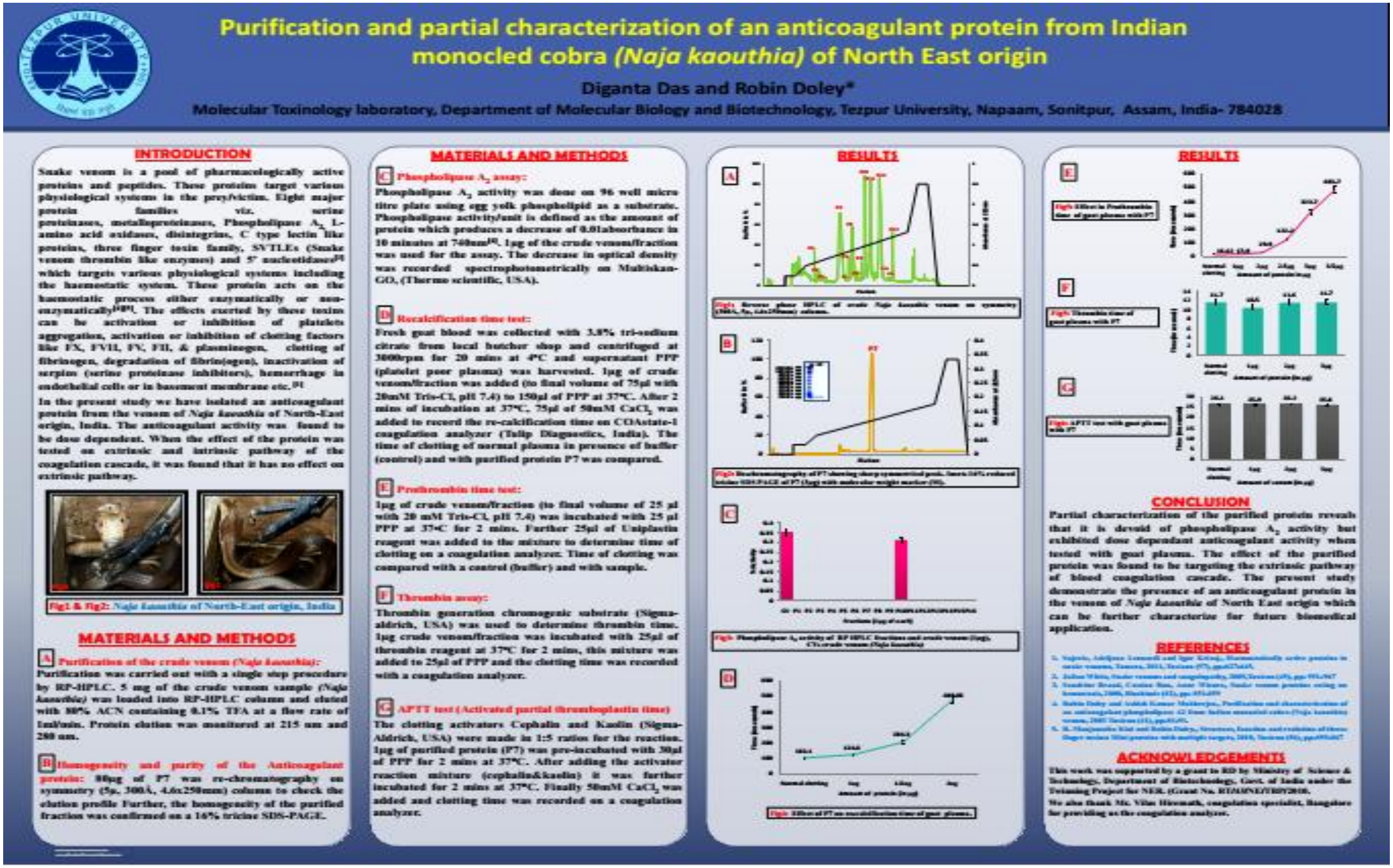
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Das, H. K. *et al.* Quantifying Demyelination in NK venom treated nerve using its electric circuit model. *Sci. Rep.* **6**, 22385; doi: 10.1038/srep22385 (2016).

Appendix IV

Reprints of poster presented


Purification and partial characterization of an anticoagulant protein from Indian monocled cobra (Naja kaouthia) of North East origin
Diganta Das and Robin Doley*
Molecular Toxinology laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Napaam, Sonitpur, Assam, India- 784028
INTRODUCTION
Snake venom is a pool of pharmacologically active proteins and peptides. These proteins target various physiological systems in the prey/victim. Eight major protein families viz. serine proteinases, metalloproteinases, Phospholipase A2, L-amino acid oxidases, disintegrins, C type lectin like proteins, three finger toxin family, SVTLEs (Snake venom thrombin like enzymes) and 5' nucleotidases which targets various physiological systems including the haemostatic system. These protein acts on the haemostatic process either enzymatically or non-enzymatically. The effects exerted by these toxins can be activation or inhibition of platelets aggregation, activation or inhibition of clotting factors like FX, FVII, FV, FII, & plasminogen, clotting of fibrinogen, degradation of fibrin(ogen), inactivation of serpins (serine proteinase inhibitors), hemorrhage in endothelial cells or in basement membrane etc.
In the present study we have isolated an anticoagulant protein from the venom of Naja kaouthia of North-East origin, India. The anticoagulant activity was found to be dose dependent. When the effect of the protein was tested on extrinsic and intrinsic pathway of the coagulation cascade, it was found that it has no effect on extrinsic pathway.
Fig1 & Fig2: Naja kaouthia of North-East origin, India
MATERIALS AND METHODS
A Purification of the crude venom (Naja kaouthia):
Purification was carried out with a single step procedure by RP-HPLC. 5 mg of the crude venom sample (Naja kaouthia) was loaded into RP-HPLC column and eluted with 80% ACN containing 0.1% TFA at a flow rate of 1ml/min. Protein elution was monitored at 215 nm and 280 nm.
B Homogeneity and purity of the Anticoagulant protein: 80μg of P7 was re-chromatography on symmetry (5μ, 300Å, 4.6x250mm) column to check the elution profile Further, the homogeneity of the purified fraction was confirmed on a 16% tricine SDS-PAGE.
MATERIALS AND METHODS
C Phospholipase A2 assay:
Phospholipase A2 activity was done on 96 well micro titre plate using egg yolk phospholipid as a substrate. Phospholipase activity/unit is defined as the amount of protein which produces a decrease of 0.01absorbance in 10 minutes at 740nm. 1μg of the crude venom/fraction was used for the assay. The decrease in optical density was recorded spectrophotometrically on Multiskan-GO, (Thermo scientific, USA).
D Recalcification time test:
Fresh goat blood was collected with 3.8% tri-sodium citrate from local butcher shop and centrifuged at 3000rpm for 20 mins at 4°C and supernatant PPP (platelet poor plasma) was harvested. 1μg of crude venom/fraction was added (to final volume of 75μl with 20mM Tris-Cl, pH 7.4) to 150μl of PPP at 37°C. After 2 mins of incubation at 37°C, 75μl of 50mM CaCl2 was added to record the re-calcification time on COAstate-1 coagulation analyzer (Tulip Diagnostics, India). The time of clotting of normal plasma in presence of buffer (control) and with purified protein P7 was compared.
E Prothrombin time test:
1μg of crude venom/fraction (to final volume of 25 μl with 20 mM Tris-Cl, pH 7.4) was incubated with 25 μl PPP at 37°C for 2 mins. Further 25μl of Uniplastin reagent was added to the mixture to determine time of clotting on a coagulation analyzer. Time of clotting was compared with a control (buffer) and with sample.
F Thrombin assay:
Thrombin generation chromogenic substrate (Sigma-aldrich, USA) was used to determine thrombin time. 1μg crude venom/fraction was incubated with 25μl of thrombin reagent at 37°C for 2 mins, this mixture was added to 25μl of PPP and the clotting time was recorded with a coagulation analyzer.
G APTT test (Activated partial thromboplastin time)
The clotting activators Cephalin and Kaolin (Sigma-Aldrich, USA) were made in 1:5 ratios for the reaction. 1μg of purified protein (P7) was pre-incubated with 30μl of PPP for 2 mins at 37°C. After adding the activator reaction mixture (cephalin&kaolin) it was further incubated for 2 mins at 37°C. Finally 50mM CaCl2 was added and clotting time was recorded on a coagulation analyzer.
RESULTS
A
B
C
D
RESULTS
E
F
G
CONCLUSION
Partial characterization of the purified protein reveals that it is devoid of phospholipase A2 activity but exhibited dose dependant anticoagulant activity when tested with goat plasma. The effect of the purified protein was found to be targeting the extrinsic pathway of blood coagulation cascade. The present study demonstrate the presence of an anticoagulant protein in the venom of Naja kaouthia of North East origin which can be further characterize for future biomedical application.
REFERENCES
ACKNOWLEDGEMENTS
This work was supported by a grant to RD by Ministry of Science & Technology, Department of Biotechnology, Govt. of India under the Twinning Project for NER.
We also thank Mr. Vilas Hiremath, coagulation specialist, Bangalore for providing us the coagulation analyzer.

Appendix V

Permissions and ethical approvals

GOVERNMENT OF ASSAM
OFFICE OF THE PRINCIPAL CHIEF CONSERVATOR OF FORESTS::WILDLIFE:
BASISTHA:: GUWAHATI-29.

Corrigendum

No. WL/FG.27/Tissue collection/09, Dt..15./11/2011.

Please read the "year 2011-12 in place of 2010-2011"in the Office order No. 450, dt. 1-1-2011 and memo No. WL/FG.27/Tissue collection/09, dt. 7-10-2011.

Principal Chief Conservator of Forests (Wildlife), Assam.

Memo No. WL/FG.27/Tissue collection/09, dt..15...../11/2011.

Copy to:-

1) The Divisional Forest Officers, all Wildlife divisions of Assam.
2) The Divisional Forest Officers, all (T) divisions of Assam.
3) Dr. Robin Doley, Asstt. Prof., Deptt. of Molecular Biology and Biotechnology, Tezpur University, Naapam, Tezpur for information.

Principal Chief Conservator of Forests (Wildlife), Assam.

GOVERNMENT OF ASSAM
OFFICE OF THE PRINCIPAL CHIEF CONSERVATOR OF FORESTS:: WILDLIFE
BASISTHA:: GUWAHATI-29.

O.O.No. 450 Dt. 01/10/11

On submission of the undertaking to abide all the stipulations laid down and communicated vide this Office letter No. WL/FG.27/ Tissue Collection/09 dtd. 18.08.11 (copy enclosed), fulfilling provisions of the clause 10 and having deposited an amount of Rs. 10,000/- vide the Union Bank of India deposit No. EM/COM/A026337 dtd. 26-09-2011 in the form of "fixed deposit" pledged In favour of the Chief Wildlife Warden, Assam, Guwhati-29, towards security deposit and special purpose permit fee Rs. 1000/- vide receipt No.9967 dtd.1.10.11, permission under section 12 of the Wildlife (Protection) Act, 1972 is hereby accorded to Dr. Robin Doley to collect snake venom samples from Assam during 2010-2011.

Encl: As stated.

PCCF (WL) & Chief Wildlife Warden, Assam.

WL/FG.27/ Tissue Collection/09, Dt. 07/10/11.

Copy for information and necessary action to:
1. The DFOs, all Wildlife Divisions of Assam,
2. The DFOs, all Territorial Divisions of Assam.
3. Dr. Robin Doley, Asstt. Prof., Dept. of Molecular Biology and Biotechnology, Tezpur University, Naapam, Tezpur.

PCCF (WL) & Chief Wildlife Warden, Assam.

GOVERNMENT OF ASSAM
OFFICE OF THE PRINCIPAL CHIEF CONSERVATOR OF FORESTS: :WILDLIFE::
BASISTHA:: GUWAHATI-29.

No. WL/FG.27/Tissue collection/09, Dt. 19/08/11.

To, Dr. Robin Doley, Asstt. Prof., Deptt. of Molecular Biology & Biotechnology, Tezpur University, Tezpur.

Sub: Grant of special purpose permit.

Sir,

The permission to collect snake venom samples from Assam can be accorded under Sec. 12 of Wildlife(Protection) Act, 1972 under the following terms & conditions during 2011-12.

1. All the provisions, relating to the National Parks, Sanctuaries and NTCA under the Wildlife (Protection) Act, 1972 shall be strictly adhered to.
2. No boundary mark of the Protected Area will be damaged, altered, destroyed, moved or defaced.
3. No other wild animal will be teased, molested or disturbed.
4. No damage to any flora or fauna and snake venom samples will be allowed to collect inside and outside the PAs.
5. The ground of the Park/Sanctuary will not be littered.
6. A Project Monitoring Officer authorized by the PA authority and the Research Officer, O/o PCCF(WL), Assam will monitor the activities to ensure the adherence of all the conditions stipulated herein.
7. The Park Authority will not take responsibility for arrangement of the food, lodging and conveyance.
8. The Park Authority will reserve the right to cancel/ terminate this permission at any time, whenever it is considered that the activities resulting from this permission is affecting the flora and fauna adversely or the permit holder is not abiding by the stipulations contained herein.
9. A copy of annual progress report with a soft copy may be submitted for the extension of the project and three copies of final report shall be furnished to the Research Officer, O/o the PCCF (WL), Assam for office record.
10. An amount of Rs.1,000/- as special purpose permit fees and Rs. 10,000/- will have to be deposited in the form of a "Fixed Deposit" pledged in favour of the Chief Wildlife Warden, Assam, Basistha, Ghy-29, as a security deposit which will be released immediately after fulfilling the Clause 9 and also on receipt of the NOC about satisfactory compliance of all the above stipulations.
11. Entry to the Protected Area would be as per the convenience of the local forest Authority and a register will have to be maintained by the researcher for entering in to the PA and equipment used with authentication of the local forest authority.

If agreed to all the above stipulations and on furnishing the documents and security deposit an undertaking as below will have to be signed by you before obtaining the permission for entering into the Protected Area for implementing the abovestated research.

Please take the necessary steps accordingly.

Yours faithfully,

PCCF (WL) & Chief Wildlife Warden, Assam.

Undertaking

I do hereby undertake that I shall abide by all the stipulations contained in this permission and I shall enter in to the PA at my own risk and in case of any violation of any of the stipulations, I shall be liable to be prosecuted under the relevant provisions of law.

Signature of the applicant. 26/9/11

PROCEEDINGS OF THE INSTITUTIONAL ANIMAL ETHICS COMMITTEE MEETING HELD ON 29th OCTOBER 2011 AT 11.30 AM IN THE CHAMBERS OF THE CHAIRMAN, DEPARTMENT OF STUDIES IN ZOOLOGY, MANASAGANGOTRI, MYSORE – 06

MEMBERS PRESENT

1. Prof. Mewa Singh — Chairman, IAEC
2. Dr. H. Krishnappa — Member & CPCSEA Nominee
3. Mr. D. R. Prahallada — Member
4. Prof. B. S. Vishwanath — Member
5. Prof. Shivabasavaiah — Member
6. Prof. H. N. Yajurvedi — Member & Convener IAEC

MEMBERS ABSENT :

1. Dr. Hari Krishna
2. Dr. Suresh Kumar

The Chairman welcomed the members. The committee noted that though some of the suggestions made in the earlier IAEC meeting regarding the preparation of the proposals were complied with, the Investigators have to prepare the proposals in still better manner, especially the protocols of experiments, requirement of animals in each experiment and restricting the number of animals per group only to requirement of the investigation. In addition names of all he individuals of the research group involved in proposed work have to be mentioned.

The committee unanimously agreed with the above suggestions and resolved to implement them.

The committee discussed at length about the proposals submitted by different Investigators and sought clarifications from Principal Investigators / Ph.D., guides / Ph.D., scholars about their animal requirements. In some of the proposals the animal number was reduced by suggesting modified protocols / alternatives. Following approvals were given.

a. The proposals submitted for class work by Chairpersons of different science Departments were accepted and animal requirement as shown in the table was approved (Nos. UOM/IAEC/11-16/2011).

Sl. No.	Name	Department	No. of animals indented	No. of auimals approved
1	Prof. Bharathi P. Salimath	Biotechnology, MGM	Mice – 180 Nos. Balb/c mice – 120Nos. Rabbit – 04 Nos.	Mice – 180 Nos. Balb/c mice – 120Nos. Rabbit – 04 Nos.
2	The Chairman	Biochemistry, MGM	Mice – 250 Nos. Rat – 150 Nos.	Mice – 250 Nos. Rat – 150 Nos.
3	The Chairman	Psychology, MGM	Rat – 20 Nos.	Rat – 20 Nos.
4	Dr. N. S. Devaki	Yuvaraja's College	Rat – 51 Nos.	Rat – 51 Nos.
5	The Chairman	Zoology, MGM, for Genetcs	Rat – 118 Nos.	Rat – 118 Nos.
6	The Chairman	Zoology, MGM	Rat – 493 Nos. Mice – 60 Nos.	Rat – 493 Nos. Mice – 60 Nos.

b. Dr. Shubha Gopal and Satisha K R (UOM/IAEC/17/2011) to utilize 128 mice for entire Ph.D. progrramme.

27/3/12

c. Prof. K. S. Rangappa and Rakesh K S (UOM/IAEC/18/2011) to reduce number of rats per group from 8 to 6 and total number from 440 to 330.

...2

d. Prof. V. A. Vijayan and Raghavendra B S (UOM/IAEC/19/2011): Requirement of 12 mice approved.
e. Prof. H. S. Prakash and Chandra Nayaka (UOM/IAEC/20/2011): Requirement of 4 rabbits approved and rabbits have to be procured from CPCSEA recognized breeders.
f. Dr. Kemparaju and Prathima R (UOM/IAEC/21/2011) : Requirement of 285 rats for entire Ph.D. programme approved.
g. Dr. Shailasree Sekhar and Ruma Karmakar (UOM/IAEC/22/2011) : Requirement of 96 rats approved.
h. Prof. H. S. Prakash and Ghffari (UOM/IAEC/23/2011) to reduce number of rats from 96 to 72 and to submit detailed plan of the work.
i. Prof. H. S. Prakash and Chethan J (UOM/IAEC/23-24/2011): To provide detailed plan of work showing experiment groups, treatments and animals in each group and requirement of 250 rats approved.
j. Prof. B. S. Vishwanath and Nanjaraj Urs A N (UOM/IAEC/25/2011): The proposal for 480 accepted in principle and individual experimental designs to be given in D-form.
k. Prof. B. S. Vishwanath and Yariswamy M (UOM/IAEC/26/2011) : Requirements of 200 mice approved.
l. Dr. M. Bhagya and Samson S (UOM/IAEC/27/2011) : Requirement of 22 lizards approved.
m. Dr. Asna Urooj and P. Vanitha Reddy (UOM/IAEC/28/2011) : Requirement of 124 rats approved.
n. Dr. Asna Urooj and Sudh Sairam (UOM/IAEC/29/2011) : Requirement of 150 rats approved.
o. Dr. M. Bhagya (UOM/IAEC/30/2011) :Utilization of unused different tissues of lizards approved earlier in other proposals permitted.
p. Prof. V. A. Vijayan and Prathibha K P (UOM/IAEC/31/2011) : Requirement of 12 mice approved.
q. Prof. Cletus J. M. D'Souza and Mamatha A M and Shubha M C (UOM/IAEC/32/2011) : Requirement of 02 rabbits approved. Rabbits to be procured from CPCSEA recognized breeders.
r. Dr. T. Shivanandappa and Mahsa Zarei (UOM/IAEC/33/2011) : Requirement of 270 mice approved.
s. Dr. T. Shivanandappa and Dileepkumar H V (UOM/IAEC/34/2011) : Requirement of 160 rats approved.
t. Dr. T. Shivanandappa and Niveditha (UOM/IAEC/35/2001) : Requirement of 120 rats approved.
u. Prof. B. S. Vishwanath and Zahra A (UOM/IAEC/36/2011) : Requirement of 46 rats approved.
v. Prof. B. S. Vishwanath and Vilas Hiremath (UOM/IAEC/37/2011) : Requirement of 75 rats approved
w. Prof. B. S. Vishwanath and Vikarm Joshi (UOM/IAEC/38/2011): Requirement of 456 Mice and 228 rats approved.
x. Prof. Cletus D. Souza (UOM/IAEC/39/2011) : Requirement of 02 hens approved.
y. Prof. Cletus D. Souza (UOM/IAEC/40/2011) : Requirement of 02 hens approved.
z. Prof. Cletus D. Souza (UOM/IAEC/41/2011) : Requirement of 02 Rabbits approved
aa. Prof. Cletus D. Souza (UOM/IAEC/42/2011) : Requirement of 48 rats approved.

Accepted

(H. N. YAJURVEDI)
CONVENER, IAEC

TEZPUR UNIVERSITY
TEZPUR UNIVERSITY ANIMAL ETHICAL COMMITTEE
TEZPUR- 784 028, ASSAM, INDIA
(CPCSEA Regd. No 754/CPCSEA)

Approval No: DoRD-Pro/TUAEC/10-56/15/Res-02 Dated: 06/06/2015

Certificate

This is to certify that the project title "*Isolation, purification and characterization of novel three finger toxin from cobra venom*" has been approved by the IAEC.

Name of Chairman: *Prof. C. L Mahanta*
Dean Research and Development, TU

Name of CPCSEA link nominee: *Dr. P. Chakravarty*
Associate Professor
Silchar Medical College, Silchar, Assam.

Chara Lata Mahanta
(C. L Mahanta) Dean
Dean, R&D
&
Chairperson
IAEC
Research & Development
Tezpur University
Chairperson
IAEC, Tezpur University
Tezpur-784 028, Assam, India

Pinaki
(Dr. P. Chakravarty)
CPCSEA Link nominee
IAEC

Dr. P. Chakravarty
M.B.B.S., D.M.C.H., M.D.
Associate Professor
Department of Pharmacology
SILCHAR MEDICAL COLLEGE & HOSPITAL

CPCSEA Nominee

UNIVERSITY OF MYSORE

Communication of decision of the Institutional Human Ethical Committee (IHEC)

IHEC -UOM No. 62 /Ph.D/2011-12

Protocol title: Search for novel treatments for snake venom poisoning
Name of the Student: Mr Nanjaraj Urs A.N Research Guide: **Dr. B.S.Vishwanath**
Department: DOS in Biochemistry, Manasagangotri, Mysore, India
[√] New review [ ] revised review
Date of review (D/M/Y): 15/12/2011
Decision of the IHEC: [√] Recommended [ ] Recommended with suggestions [ ] Revision/Resubmission
Suggestions: To give a declaration stating that the subject's blood sample will be used only for the specified purpose Revise and submit the consent form
Recommended for a period of: Effective from the date of PhD enrolment / registration

Please note*

- Inform IEC in case of any change of study procedure and investigator.
- This permission is only for period mentioned above.
- Brief report to be submitted to IHEC.

Dr Asna Urooj
Member Secretary
Human Ethical Committee
University of Mysore
MYSORE-570 006

Dr N.M.Srinivas
Chairman
Human Ethical Committee
University of Mysore
MYSORE-570 006