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A NEUROTOXIN ISOLATED AND PURIFIED FROM THE VENOM OF *NAJA NAJA* (INDIAN COBRA) ENVISAGED AS A POTENT THERAPEUTIC TOOL TO TREAT MOVEMENT DISORDERS

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ABSTRACT

Naja Naja (Indian cobra) venom essentially contains a neurotoxin and group of enzymes. In the present study, a neurotoxin from the venom of elapid snake *Naja naja* (Indian cobra) was isolated and purified to homogeneity by fast protein liquid chromatography (FPLC) method on Mono-Q HR-5/5 column. The molecular weight of the toxin was 7 kDa as confirmed by SDS-PAGE and LD_{50} was 0.25 mg/kg body weight in mice. Further, the mode of action of purified toxin was investigated, the toxin inhibited acetylcholine contractile responses of frog rectus abdominis muscle acting on nicotinic receptors. At higher concentration, the toxin abolished acetylcholine sensitivity and produced an irreversible neuromuscular blockade. These results demonstrate that a purified neurotoxin isolated from *Naja naja* (Indian cobra) venom blocks the neuromuscular transmission at postsynaptic level. Thus, *Naja naja* neurotoxin is envisaged as a potent therapeutic tool alternative to botulinum neurotoxin in the treatment and management of painful neuromuscular movement disorders.

KEYWORDS: *Naja naja* (Indian cobra), Snake venom, Neurotoxin, Neuromuscular blocking activity, Movement disorders, Fast protein liquid chromatography (FPLC).

INTRODUCTION

Snake venoms are known to contain a low molecular weight peptide toxins and number of enzymes that have evolved to assist in the capture and digestion of prey as well as for use in

defense against predators. Elapidae family snake venoms contain toxins that exert a potent neurotoxic action by inhibiting the release of acetylcholine from nerve terminals of neuromuscular junctions.[1] *Naja naja* (Indian cobra) is one of the most venomous elapid snakes found throughout India responsible for the majority of fatal snake bite cases. *Naja naja* (Indian cobra) neurotoxin causes postsynaptic neuromuscular blockade as well as paralysis of respiratory muscles; the latter often resulting in death of victims.[2]

It was established that the respiratory failure caused by cobra venom in dogs and rabbits is due to its peripheral curare like action.[3-5] The neuromuscular blocking effect of Formosan cobra (*Naja naja atra*) is mainly due to non-depolarizing curare like action.[6] On the other hand, a component isolated from *Naja naja* (Indian cobra), which possessed most of the toxicity of the original venom depolarized frog sortorius muscles.[7]

The purified neurotoxin isolated electrophoretically, blocks neuromuscular transmission by competing with acetylcholine without affecting the release of acetylcholine on nerve stimulation in rat phrenic diaphragm preparation or causing contracture of the chick biventer cervicis or frog rectus abdominis muscle. These findings concluded that cobra neurotoxin behaves just like d-tubocararine.[8, 9] A frequency independent neuromuscular block observed in these nerve-muscle preparations was suggestive of absence of possible presynaptic effect. These results demonstrate that the cobra neurotoxin in some cases can imitate d-tubocararine, but its neuromuscular blocking activity is different from that of curare in many respects.[10]

In the present study, the isolation, purification and characterization of a low molecular weight and homogeneous neurotoxin from the venom of *Naja naja* (Indian cobra) was undertaken. Further, the neuromuscular blocking activity of purified neurotoxin on a frog rectus abdominis muscle preparation was carried out. The results obtained are in full agreement with the studies reported.

MATERIALS AND METHODS

Venom and chemicals

Lyophilized whole venom of *Naja naja* (Indian cobra) was a product of Haffkine Institute, Mumbai (India). All other chemicals were obtained from the commercial sources and were of analytical grade.

Venom fractionation and purification

Naja naja (Indian cobra) venom was dissolved in 20mM triethanolamine hydrochloride buffer pH 7.5 and 200µl was loaded and fractionated by fast protein liquid chromatography (LKB-Pharmacia Biotechnology) on Mono Q-HR 5/5 column, which was equilibrated with a same buffer. The column was eluted with the buffer followed by a linear salt gradient 0.5M NaCl in buffer pH 7.5 with a flow rate of 1.5 ml/min at 1MPa pressure **[11]**. Fraction 1 was refractionated under the same conditions for further purification. Refractionated fraction 1 containing purified neurotoxin was used throughout this study.

Protein Assay

Protein concentration of the FPLC fraction 1 was determined by Bradford's method[12] using Bovine serum albumin as standard.

Molecular weight determination

The molecular weight of fraction 1 (neurotoxin) was confirmed by SDS-PAGE with tris buffer pH 8.8 using pre-stained molecular weight standards (New England Biolabs).[13, 14]

Lethality of fraction 1 (LD_{50} Value)

Six groups of mice, each comprising of overnight fasting albino (Mus-musculus) mice weighing 25-40 gm were used. Group -1 (control) was administered 0.4 ml saline. Group-2-6 were injected with varying concentrations of fraction-1 (neurotoxin). Deaths were recorded 48 hours after the injection and LD_{50} was calculated by Spearmann-Karber method.[15]

Neurotoxicity

0.3 ml of fraction-1 was injected intraperitoneally in mice (18-20 gms).

Neuromuscular blocking activity

An isolated frog's rectus abdominis muscle preparation was mounted in the Dale's organ bath (20 ml capacity) containing aerated frog's Ringer solution (0.6% NaCl + 0.14% KCl + 0.012%$CaCl_2$ + 0.02% $NaHCO_3$) and the tissue was stabilized for 20 minutes. Acetylcholine (80µg/ml) contractile response of muscle was recorded on a kymograph as a control. Then, muscle preparation was washed with Ringer solution and allowed to relax for 2 minutes. Subsequently, acetylcholine contractile responses of muscle treated with d-tubocurarine (10 µg/ ml) and increasing doses of fraction-1 (neurotoxin) were recorded. Neostigmine (100 µg/ ml) was used to see the reversal of the neuromuscular blockade.[16]

RESULTS

Isolation of neurotoxin

Fig. I shows the chromatographic profile of the fractionation of *Naja naja* (Indian cobra) venom on Mono Q HR-5/5 column. It was observed that whole venom was fractionated into six components. A major fraction-1 had typical neurotoxic activity in mice. Subsequently, fraction-1 was refractionated into 8 fractions under the same conditions (Fig. II).

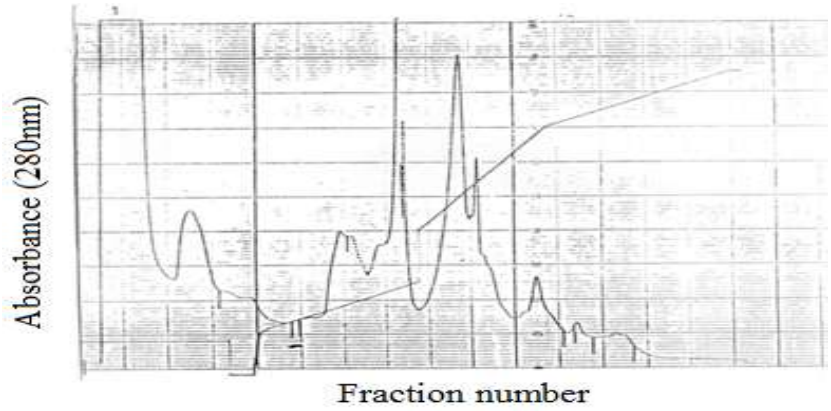

FIGURE- I: Fractionation of *Naja naja* (Indian cobra) venom by fast protein liquid chromatography on a Mono Q-HR 5/5 column. [The elution was done with 20 mM triethanolamine hydrochloride buffer pH 7.5 followed by a linear salt gradient 0.5M NaCl in buffer at pH 7.5. Fraction-1 had neurotoxic activity was refractionated under the same conditions for further purity].

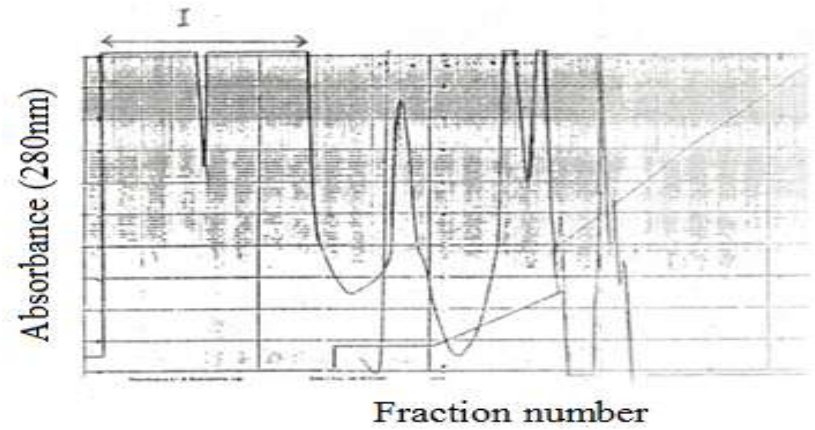

FIGURE-II: Refractionation of *Naja naja* (Indian cobra) venom fraction-1 by fast protein liquid chromatography on a Mono Q-HR 5/5 column

Protein concentration

The protein concentration of fraction -1 confirmed from the calibration curve was 0.2 mg/ml.

Molecular weight

The fraction -1 showed a single band with a molecular weight of 7 kDa. Electrophoresis was carried out in a 15% polyacrylamide slab gel by tris buffer pH 7.5 according to the method of Reisfield et al (1962). Gels were stained with Coomassie blue R-250 dye (Fig. III).

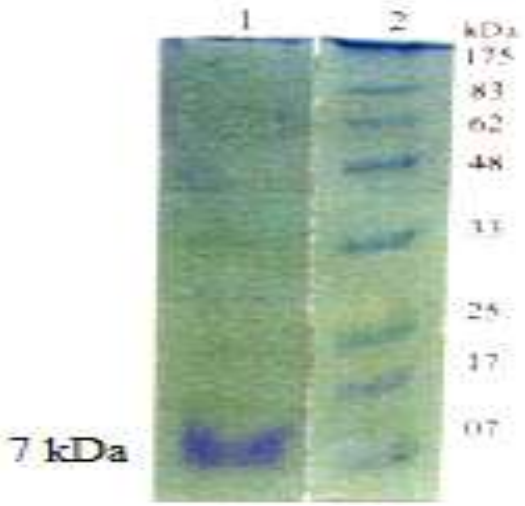

FIGURE III: SDS-PAGE pattern of FPLC-isolated and

purified neurotoxin (fraction-1) from *Naja naja* venom (Lane-1) with standard molecular weight markers (Lane-2).

Neurotoxicity

Fraction-1 demonstrated typical neurotoxicity symptoms when injected intraperitoneally in mice (18-20 gms).

Lethality: The LD_{50} value of fraction -1 was 0.25 mg/kg body weight in mice.

Neuromuscular Blocking Activity of Fraction-1 (Neurotoxin)

Fig. IV shows acetylcholine contractile responses on a frog's rectus abdominis muscle in presence of increasing doses of purified neurotoxin (fraction-1) from the venom of *Naja naja* (Indian cobra). An irreversibleblock was produced at the concentration of 0.4 µg neurotoxin (Table I&II). A progressive reduction in the height of acetylcholine contractile responses was indicative of neuromuscular blockade by neurotoxin (Fig.V).

Table I. Acetylcholine contractile response of frog's rectus abdominis muscle treated with d-tubocurarine.

Drug/sample	Dose	Contractile Response	Height of contraction	Percentage Reduction
a. Acetylcholine	80 µg/ml	Normal	50 mm	100%
b. Acetylcholine + d-tubocurarine	80 µg/ml + 10 µg/ml	Decreased	22 mm	44%

Table II. Acetylcholine contractile responses of frog's rectus abdominis muscle treated with increasing doses of purified *Naja naja* neurotoxin (fraction 1)

Drug/sample	Dose	Contractile Response	Height of contraction	Percentage Reduction
a. Acetylcholine	80 µg/ml	Normal	50 mm	--
b. Acetylcholine + Fraction 1	80 µg/ml + 0.05mg	Decreased	15 mm	30%
c. Acetylcholine + Fraction 1	80 µg/ml + 0.1 mg	Decreased	22 mm	44
d. Acetylcholine + Fraction 1	80 µg/ml + 0.2 mg	Decreased	30 mm	60
e. Acetylcholine + Fraction 1	80 µg/ml + 0.3 mg	Decreased	36 mm	72
f. Acetylcholine + Fraction 1 + Neostigmine	80 µg/ml + 0.4 mg + 100 µg/ml	Irreversible Block	Base line	100

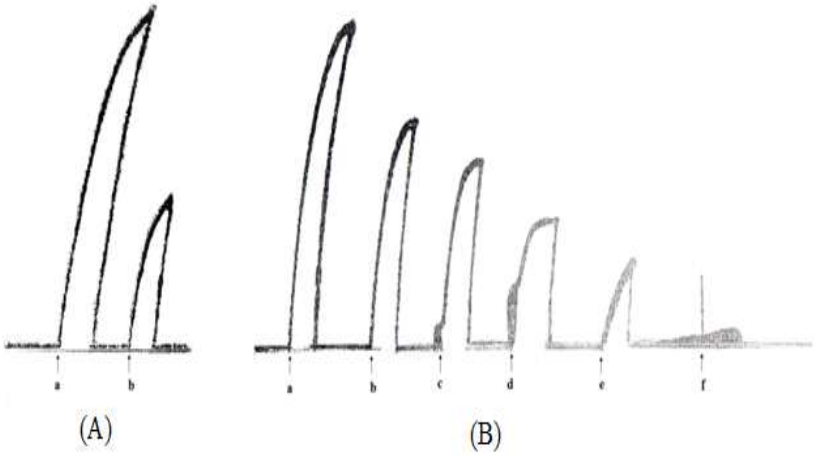

Figure IV: (A) Acetylcholine contractile response of frog's rectus abdominis muscle treated with d-tubocurarine. (B) With increasing doses of purified *Naja naja* neurotoxin (fraction 1)

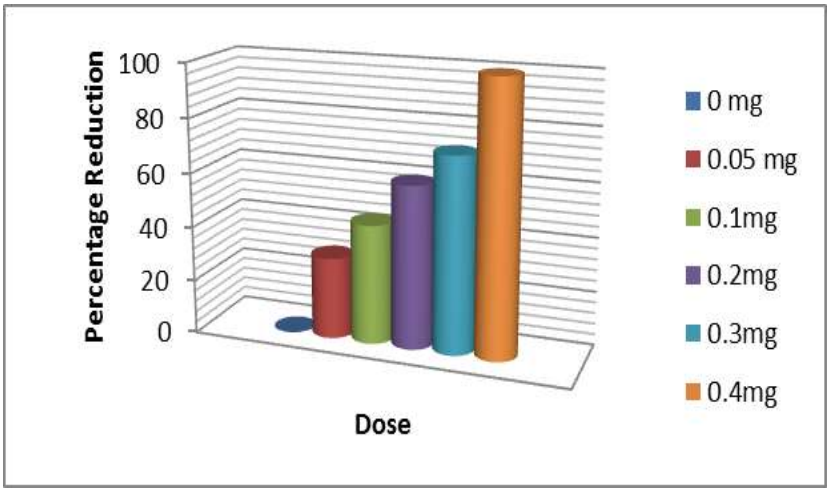

Figure V: Percentage reduction of acetylcholine contractile responses of frog's rectus abdominis muscle treated with increasing doses of *Naja naja* (Indian cobra) venom neurotoxin (fraction-1).

DISCUSSION

Elapidae and Hydrophidae snake venoms contain pharmacologically active postsynaptic or α-toxins that interfere with the function of the neuromuscular junction.[17] Postsynaptic toxins bind to the acetylcholine receptors at the postsynaptic membrane of the neuromuscular junction and prevent the binding of acetylcholine. The effect is to produce a non-depolarizing type of neuromuscular blockade. To date, a large number of postsynaptic neurotoxins have been isolated from the venoms of snakes such as cobras, kraits and sea snakes.

In contrast to a low molecular weight (7 kDa) postsynaptic purified Naja naja (indian cobra) neurotoxin, botulinum toxin is a high molecular weight (150,000 - 167,000 kDa) presynaptic neurotoxin and one of the most poisonous (LD_{50} 1.3 -2.1 ng/kg IV or IM) biological substance produced by an anaerobic bacterium clostridium botulinum. All the serotypes of Botulinum neurotoxin interfere with neural transmission by blocking the release of acetylcholine at neuromuscular junction from presynaptic motor neurons causing muscle paralysis. Botulinum toxin currently used in the treatment and management of wide variety of medical conditions, especially strabismus, focal dystonias, cervical dystonia, hemifacial spasm, blepharospasm, cerebral palsy, achalasia, hyperhidrosis, chronic migraine and spastic movement disorders. It elicits the production of high antibody titers in patients compelling to go in for different isoforms of the toxin. Botulinum neurotoxin causes a permanent neuromuscular blockade at the motor end plate and known to have many adverse effects.[17-19]

Our findings on the mode of action of the *Naja naja* neurotoxin suggested the postsynaptic neuromuscular blocking activity on frog rectus abdominis muscle. Thus, a further study of a purified postsynaptic *Naja naja* neurotoxin isolated from the crude cobra venom may be suggested as an alternative possible potent therapeutic tool to a presynaptic botulinum neurotoxin for the treatment and management of painful movement disorders.

CONCLUSION

In vitro neuromuscular preparations have been proven to be invaluable tools in the examination of snake venoms and isolated neurotoxins. They will continue to play a role in further elucidating the mechanism of action of these highly potent toxins and may provide highly specific research tools and also lead compounds for pharmacological agents.

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