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Production of effective antivenin to treat cobra snake (Naja naja oxiana) envenoming

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ABSTRACT

Conventional treatment of Naja naja oxiana (NNO) envenoming requires large volumes of equine antivenin raised against NNO crude venom. The poor efficiency of this antivenin is assumed to be due to the high molecular weight non-toxic proteins, a strong immunogen, present in the crude venom. These proteins cause depression of antibody formation against the low molecular weight toxic components of venom. In the present study the low molecular weight lethal components were isolated from crude venom of NNO venom by sephadex G_{50} get filtration chromatography. A sera was prepared by immunizing horses with toxic fraction. One milliliter of this serum neutralized 1.8mg of NNO crude venom. This high titer antivenin is thus 2.2 times more potent than the sera obtained against NNO crude venom.

Keywords: Naja naja Oxiana, venom, gel chromatography, Antivenin

INTRODUCTION

The cobra shakes comprise a great group of species found in Africa and Asia. The Asian cobra (Naja naja) venoms cause death by the action of their neurotoxic and cardiotoxic components (7, 8, 1, 14). Naja naja Oxiana (NNO) is an abundant snake in northeast of Iran and responsible for a large number of snakebite mortality (6). Two main toxins as well as a number of minor components and three basic polypeptides similar to cardiotoxins and cytotoxins were isolated from NNO crude venom (3). The treatment of cobra bite patient constitutes an medical emergency. To date, there is no specific treatment, except antivenin therapy (11). A polyvalent antivenin and also a monovalent cobra antivenin are prepared against NNO crude venom in Razi Institute (Iran). These antivenins showed poor efficiency to counter the venom (5). In present study, a monovalent cobra antivenin is prepared against a low molecular weight toxic components of NNO venom.

MATERIALS AND METHODS

Central Asian cobra (NNO) venom was collected from a large group of individual snakes caught in the northeast of Iran. Following milking, venom was immediately lyophilized and stored for using. Freund's complete adjuvant was obtained from

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tuberculosis department, Oil adjuvant was supplied in venomous animal and antivenin D. using olive oil and Arabic Gum. Albino mice (18-20 gr) were supplied by the central Animal house. Sephadex G50, and other chemicals and reagents were purchased from Pharmacia, Merck and Fluka Company.

Sephadex G50 gel filtration. Sephadex G50 was swollen in 0.1 M ammonium acetate and packed in two series column (3×100cm). 2000 mg lyophilized NNO venom was dissolved in 10 ml 0.1 M ammonium acetate and loaded to the columns. Elution was carried out with the same solution as above. A constant flow rate was adjusted to 60 ml/hr and a volume of 10 ml of effluent was collected per tube. The effluent was analyzed for protein by absorbance measurement at 280 nm. Individual fractions were pooled, desalted against distilled water and lyophilized.

Protein determination. Determination of the protein of fractions was carried out by the method of microkejeldal (15).

Toxicity assay. Toxicity of the crude venom and toxic fractions was assayed by i.v. injection in groups of mice (19±1 gr). Four mice were used at each dose level. The mortality rate was recorded 48hr after the injection. The lethality values were evaluated by the method of Sperman and Karber (2).

Immunization. The increasing amount of low molecular toxic component with Freund's complete adjuvant (in 3 first injections) and oil adjuvant (in later injections) were occulted into horses weighting 350-400 kg. The antigen was subcutaneously injected at weekly intervals during a period of two months. The animals were bled by jugular puncture 7 days after the last injection. Blood-clots were kept for 24hr at 4ºc. Crude antivenin was aspirated into containers, stored at 4ºc prior to purification.

Purification of the crude antivenin (plasma). The crude antivenins taken from immunized horses was precipitated with ammonium sulfate then cleaved with pepsin (13). The antibody molecule was split by pepsin to yield 2 identical fragments ($F(ab)_2$ and Fc). The Fc fragment was discarded.

Antivenin neutralizing capacity. Neutralization capacity of the antivenin was determined as follows: one ml of dilutions of NNO crude venom was mixed with the same amount of serum. The each mixture was incubated at 37ºc for 30 minutes and was injected intravenously into3 mice. Survival or death of the mice was recorded 24hr after the injection.

Ouchterlonys immuno-diffusion test. The test was performed in plates of agar. The crude venom of NNO and their fractions were used against the serum made of crude venom and serum made of toxic fractions (12). The plates were incubated at 37ºc and precipitate bands were observed after 48hr.

SDS-PAGE electrophoresis. Electrophoresis on 15% polyacrylamide gel were performed according to the method of Laemmli (4). Samples of the crude venom and their fractions as well as two low molecular and high molecular markers were lauded into wells. The plate (130×160×1 mm) was released in laboratory during at night. Proteins were stained with comasi-brilliant blue.

RESULTS

The lethal activity (i.v.) of the NNO crude venom is 7.8 µg / 18-20 gr mouse. Figure 1 shows the sephadex G 50 gel filtration chromatography of 2000 mg NNO lyophilized crude venom. In this test four main fractions were obtained. Fraction I and II that consist of high molecular weight protein are nontoxic in mice. Fraction III and IV, that possess low molecular weight protein (6-14 K Dalton), have toxic activity. The lethal toxicity fractions III and IV are 64.2 and 1.65 µg / mouse respectively. These toxic fractions were pooled and designated as the antigen for Hyperimmunization of animals. The amount of proteins of fractions are 200, 168, 1073, and 418 mg respectively.

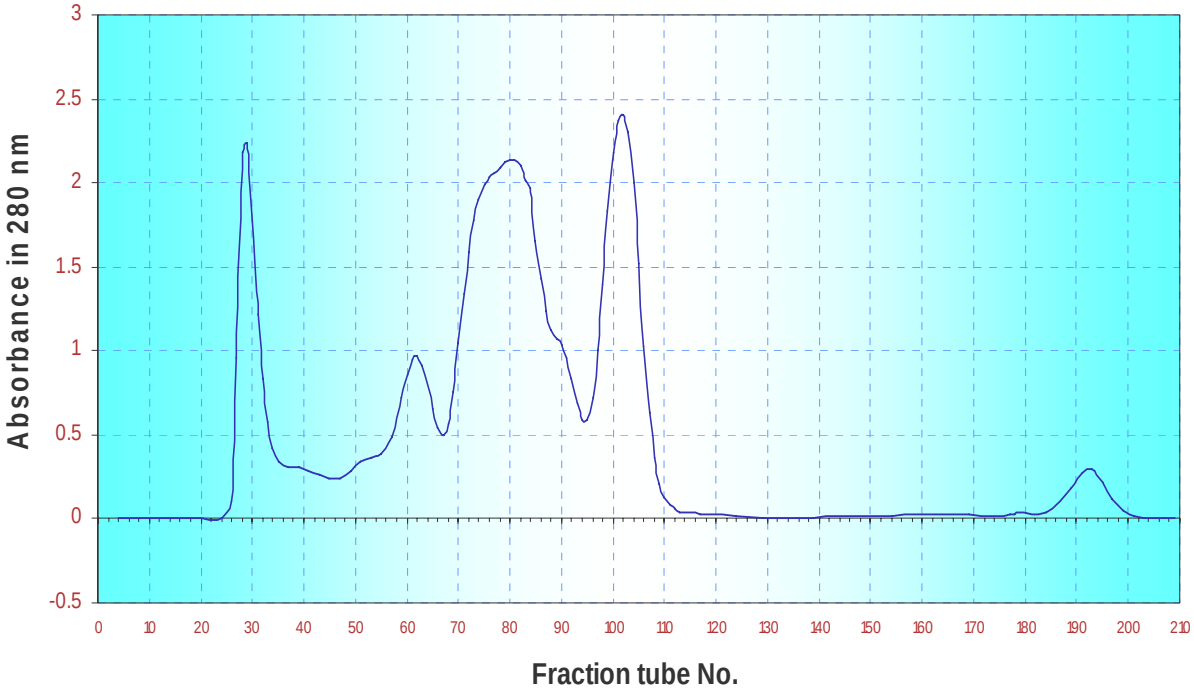

Figure 1. Sephadex G50 gel filtration chromatography of the NNO venom. 2000 mg of N.n.oxiana venom was applied on tow series columns (100×3 cm). Elution was carried out by 0.1 M ammonium acetate at a flow rate of 60 ml/hr. Fraction of 10 ml of effluent were collected per tube and monitored at 280 nm.

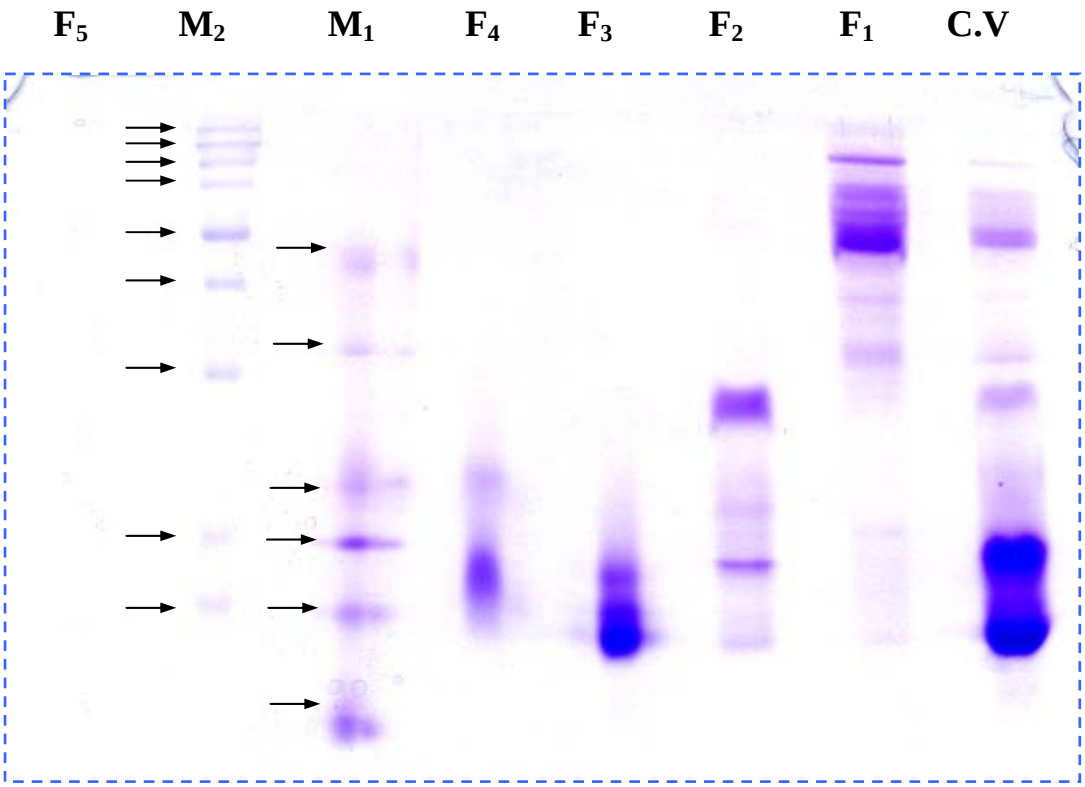

Figure 2. 15 % SDS Page gel electrophoresis of NNO crude venom and their fractions.
C.V: NNO Crude venom. **F_1- F_5:** Fractions of NNO venom. **M_1:** Low molecular marker (2.3, 3.4, 6.2, 14.3, 18.4, 29, 43 k Dalton). **M_2:** Wide molecular marker (10, 15, 25, 37, 50, 75, 100, 150, 250 k Dalton).

This shows that the toxic fractions constitute about 78 % by weight of the venom.

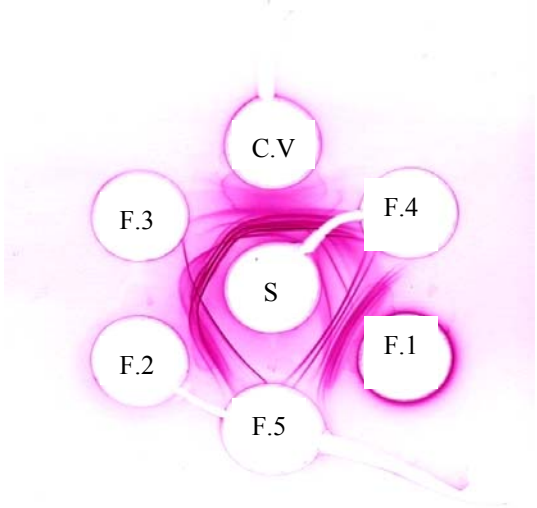

Figure 3. Immunodiffusion patterns of NNO crude venom and fractions against the serum obtained against NNO crude venom. **S:** Serum obtained against NNO crude venom. **C.V:** NNO crude venom. **F_1-F_5:** Fractions of NNO venom.

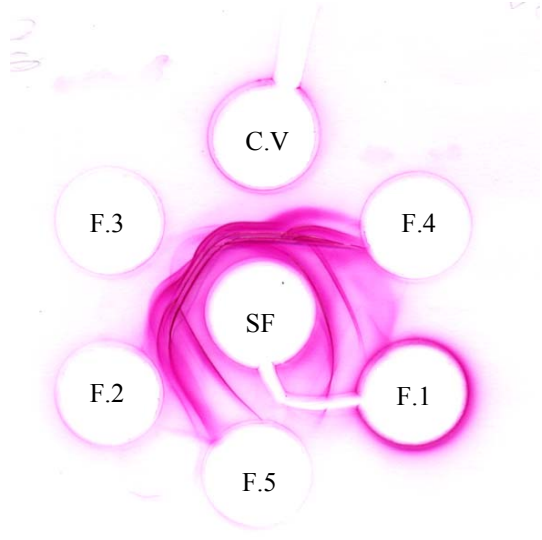

Figure 4. Immunodiffusion patterns of NNO crude venom and their fractions against the serum obtained against Low molecular toxic fractions.
SF: Serum obtained against low molecular toxic fractions. **C.V:** NNO crude venom. **F_1-F_5:** Fractions of NNO venom.

As shown in figure 2 fractions I and II consists of some high molecular weight proteins (25-250 k Dalton). Fraction III and IV consists of some low molecular weight proteins (6-14 k Dalton). In present study a antivenin was prepared against the low molecular weight toxic fractions of the NNO venom on horses. One milliliter of antivenin neutralized 1.8 mg of NNO crude venom. This high titer antivenin is thus 2.2 times more potent than the serum obtained against NNO crude venom. In ouchtorlony double diffusion, the serum obtained against the NNO crude venom, showed precipitation lines against the all of fractions (Figure 3). Whereas the antivenin raised against the low molecular weight toxic fractions, indicated precipitation lines only against these toxic fractions (Figure 4). It is well established, as shown in figure 4, the precipitation lines against nontoxic fraction II indicated that fraction III was slightly contaminated by fraction II.

DISCUSSION

The general method of isolation of low molecular weight toxic fraction (9) was applied with success using 2000 mg of NNO crude venom. The LD50 of toxic fraction III and IV are 64.2 and 1.65 µg /18-20gr mouse respectively. This result showed the fraction IV is very toxic and main toxins of venom were eluted with this fraction. But the toxicity value of fraction III is relatively weak. This means that this fraction was slightly contaminated by the nontoxic Fraction II. A monovalent antivenin is generally prepared by immunizing horses against NNO crude venom. This antivenin neutralizes 0.8 mg of the NNO crude venom. The low titer of this serum is presumably due to the low amount of antibody made against low molecular toxic components of venom. The poor efficiency of this antivenin may have some causes, including low antigenicity low concentration of the toxic components and depression of toxic antigen by other high Molecular nontoxic components. Sephadex G_{50} gel filtration chromatography of NNO

venom yielded 4 major fractions. Fractions I, a nontoxic peak, consists of some high molecular weight proteins. Fraction II also consists of a few high molecular weight nontoxic proteins. Finally fractions III and IV, two toxic peaks, consist of various low molecular proteins. The low molecular toxic fractions constitute 78% by weight of the venom. These toxic fractions responsible to snake bite envenoming. Therefore antibodies need to be prepared against these toxic components. But existing of high molecular proteins in antigen causes depression of antibody formation against the toxic components. Therefore these nontoxic proteins in the venom could be discarded. Although toxic fraction has a molecular weight of approximately 7-14 KD and is thus a poor antigen (10). However discarding of high molecular nontoxic proteins from antigen and injection high concentration of low molecular toxic fraction with Freund's adjuvant and also oil adjuvant raised a potent against low molecular toxic components of venom, is 2.2 times more potent than the serum raised against the crude venom. To summarize, this work shows that it is possible to improve the method in our laboratory to isolate animal toxins. Also when low molecular toxic components are used as antigen, the sera obtained against these components show a much higher neutralizing capacity.

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