Original Article

Effects of Adjuvant and Immunization Route on Antibody Responses against *Naja Naja oxiana* Venom

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How to cite this article: **Kashkooli S, Khamehchian S, Dabaghian M, Namvarpour M, Tebianian M. Effects of Adjuvant and Immunization Route on Antibody Responses against Naja Naja oxiana Venom. Archives of Razi Institute. 2023;78(4):1177-83.**

DOI: 10.32592/ARI.2023.78.4.1177

Article Info:
Received: 21 January 2023
Accepted: 2 March 2023
Published: 31 August 2023

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ABSTRACT

Naja naja oxiana (NNO) is one of the important venomous species in Iran. The current snakebite treatment is antivenom therapy that deals with hyper immunization of horses with crude or fractionated snake venom plus traditional adjuvants, like Freund's adjuvant. For improvement of antivenom production, it has been suggested to use different adjuvant systems or immunization procedures. In this study, humoral immune responses against immunogenic fractions of NNO venom (NNO3 and NNO4) and crude venom have been compared by usage of different adjuvant and immunization routes. Additionally, a new indirect enzyme-linked immunosorbent assay (ELISA) was set up for the detection of specific antivenom antibodies. This study was conducted on six different groups of female Dutch rabbits that were hyperimmunized using crude and fractionated NNO venom, along with Freund's and MF59 adjuvants through subcutaneous or intramuscular route. The immunization was performed four times with 10-day intervals and the levels of specific antibodiees were detected by indirect ELISA. The statistical analysis reveals a negligible variation in the antivenom titers among the venom-inoculated groups, regardless of the adjuvant type or the immunization route. Finally, it was concluded that the fractions are efficient for antivenom production, and it is possible to use MF59 adjuvant via subcutaneous routes as an alternative to Freund's adjuvants considering its fair immunopotentiation capacity and safety in animals.

Keywords: Adjuvant, Antivenom, Immunization, Nanja naja oxiana

1. Introduction

Snake bite is considered to be one of the most important non-infectious deadly agents all over the world. The World Health Organization (WHO) has estimated that about 2.7 million venomous snake bites occur worldwide each year, resulting in 80,000 to 138,000 deaths. The majority of these bites occur in Africa, South-East Asia, and South Asia (1). Accordingly, three quarters of the lands in Iran are potentially suitable for life of diverse species of venomous snakes (2). The most medically important venomous snake family in Iran is Elapidae, especially Asian cobra (Naja naja oxiana), and Viperidae (2, 3).

A unique and reliable treatment against snake venom activity is antivenom administration which is prepared in a second immunized host (4, 5). It is important to increase the antivenom efficiency to enhance the survival rate of victims. Some trends have been taken to produce potent and safe antivenom in horses and other hosts, including use of proper adjuvant, use of specific fractions of venom, and toxin modification to increase immunogenic properties and diminish the toxic effects of venom (5, 6).

Another significant issue is related to the care and health of the host animal. Although horse is the most preferred host animal for commercial antivenom production, but they may suffer from serious complications during venom/adjuvant mixture inoculation, such as tissue damages at injection site, fibrosis, fistula, inflammation, and granuloma. These could affect the quality of antivenom and reduce the production yield (7, 8).

Regarding animal welfare and to save time and production costs, use of proper adjuvant with the least side effects and the most immune reinforcement has a great deal of importance for antivenom production. Traditionally, Freund's complete and incomplete adjuvants (FCA/FIA) have been used for antivenom production, which often cause local reactions (9). However, MF59 as an oil in water emulsion adjuvant, has been confirmed and licensed for some common vaccines, such as influenza and hepatitis (10).

Most previous studies have recommended the use of crude venom in antivenom preparation. However, some previous studies have emphasized the use of toxic fractions and their efficiency in the hyper-immunization process (6, 11, 12).

This study aimed to investigate some parameters for antivenom production, including the impact of adjuvant type, antigen use, and route of injection. For this purpose, an alternative procedure was developed for hyperimmunization of rabbits with Naja naja oxiana (NNO) venom by a MF59 adjuvant. Toxic fractions were used instead of whole venom; moreover, MF59 adjuvant was tried for antivenom production in a rabbit model, to obtain more immune stimulation with less side effects. Moreover, these plans were tried in two routes, namely intramuscular (IM) and subcutaneous (SC) routes.

2. Materials and Methods

2.1. Venom Preparation

The NNO venom was obtained from Venomous Animal Department of Razi Vaccine and Serum Research Institute (RVSRI), Iran where it was prepared by milking some northeast Iranian NNO snakes. After collection, the venom was lyophilized and stored in -20 °C. Some of the crude venom was used for fractionation by chromatography with gel filtration method.

2.2. Venom Fractionation

Sephadex G50 was swollen in 0.1 M ammonium acetate and packed in two series column (3×100 cm). Afterward, 2,000 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 mentioned above. A constant flow rate was adjusted to 60 ml/h and a volume of 10 ml of effluent per tube was collected. The effluent was analyzed for protein by absorbance measurement at 280 nm. Individual fractions were pooled, desalted against distilled water, and lyophilized. After measuring the LD_{50} of the obtained fractions, the toxic fractions

(NNO3 and NNO4) were used for injection and comparison with the crude venom.

2.3. Protein Determination and Quality Control of Venoms

Protein content of the crude venom and the fractions (NNO3 and NNO4) were determined by Lowry method, using bovine serum albumin as a standard protein. The electrophoresis pattern and protein composition of venoms was established by sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE) electrophoresis on 20% polyacrylamide gel. Crude and fractionated venoms of NNO were loaded into wells in two sample forms, including samples with and without mercaptoethanol. The plate was stained with comasi-brilliant blue and leaved overnight in laboratory. The median lethal doses (LD_{50}) of the crude venom and fractions were estimated using the Sperman and Karber method (13).

2.4. Animal Immunization

In total, 12 new female Dutch rabbits that were about 1 month old and weighted 1.5±0.2 Kg were obtained from Razi Institute and maintained under identical, standard conditions. Each animal was housed in an individual cage and kept in suitable conditions in terms of climate, physiochemical parameters, cages, nutritions, microorganisms and parasites, and transportation. The rabbits were examined for signs of any disease, injury, and physical deformity.

2.5. Antigen Preparation and Injection Schedule

According to lethality assay (LD_{50}) assessment, the non-lethal doses of antigen (5-10% of LD_{50}) were formulated (1:1 v/v) with Freund's (RVSRI) or MF59 (4.3% squalene, 0.5% Tween-80, 0.5% Span-85) adjuvants and injected subcutaneously or intramuscularly four times with 10-day intervals into separated groups. The FCA was used for initial injection and the FIA was used for the later three ones.

According to table 1, injection schedule contained four injections with 10-day intervals into six separated groups of rabbits (each group contained three animals). The same dose of antigen (20 µg/kg) was determined for each group for the first and second inoculations and 30 and 40 µg /Kg for two additional ones. The animal sera were collected every 10 days and kept in +4 ℃ for later assays.

Table 1. Antigen formulation and rout of immunization of test groups

	Group A	**Group B**	**Group C**	**Group D**	**Group E**	**Group F**
Antigen	Crude venom	Fraction 3&4	Crude venom	Fraction 3&4	Crude venom	Fraction 3&4
Adjuvant	Freund's Adjuvant	Freund's Adjuvant	MF59	MF59	MF59	MF59
Injection Rout	Subcutaneous	Subcutaneous	Subcutaneous	Subcutaneous	Intramuscular	Intramuscular

2.6. Evaluation of Antibody Responses

Specific antibody responses of individual rabbit serums were evaluated by indirect ELISA method. Probability of coating fractions and parameters, including antigen proper coating dilution for both venom forms, blocker type, and percentage and incubation time for each ELISA stage, were investigated in the checker board test.

According to checker board analysis, the ELISA plates were coated with 1 µg/well of either crude or fractionated NNO antigen (venom) diluted in carbonate/bicarbonate buffer (0.05 M, pH 9.6). The plates were stored overnight at 4 ˚C and blocked with 3% bovine albumin serum for 1 h at 37 ˚C. After the wells were washed with the washing buffer (phosphate buffered saline, including 0.05% Tween 80), they were loaded with 100 µl of sample serum and incubated for 1 h at 37 ˚C. Following additional washing steps, 1:20000 diluted anti-rabbit IgG peroxidase conjugate were added to each well and kept in dark at 37 ˚C for 1 h. Finally, the wells were washed for 5 times and TMB substrate were added to each well. After 20 min of incubation, the reaction was stopped with sulfuric acid and optical absorbance was read in an ELISA plate

reader (biotek Company) at 450 nm.

2.7. Statistical Analysis

A two-sided Student's *t*-test or one-way analysis of variance followed by Tukey's or Dunnet multiple comparison tests were performed using GraphPad Prism (version 8.0) for Windows (GraphPad Software, San Diego, CA). A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. Venom Standardization

The estimated Median Lethal Dose (LD_{50}) of NNO venom was calculated as 7.8 µg µg per mouse. After fractionation of crude venom on Sephadex G50, four distinct peaks (Fractions 1-4) were isolated (Figure 1). Among them, fraction three had the highest and fraction two had the lowest values, compared to the other fractions. The protein concentration in crude as well as the third and fourth fractions was determined at 6.53, 3.34, and 1.76 mg, respectively.

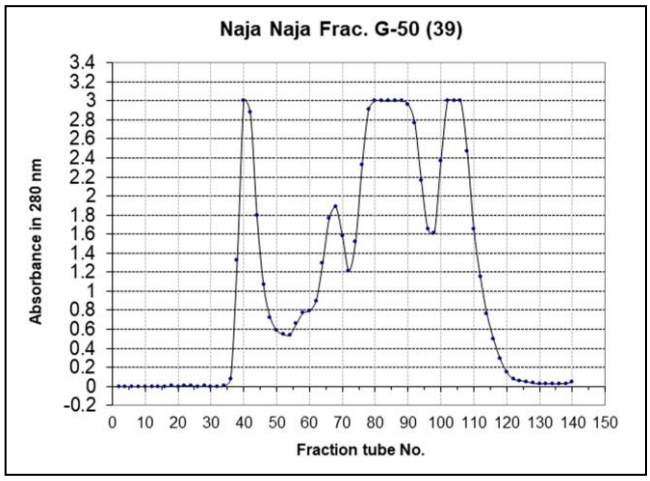

Figure 1. Chromatogram of *Naja naja oxiana* (*NNO*) venom by gel filtration chromatography (Sephadex G-50)
2000 mg of *NNO* venom was applied on columns (150×5 cm). Elution was carried out by 0.1 M ammonium acetate at a flow rate of 60 ml/hr. Fraction of 10 ml of effluent were collected per tube and monitored at 280 nm

The protein composition of the venom and fractions were carried out using SDS-PAGE (20%) under reduced (with mercaptoethanol) and non-reduced (without mercaptoethanol) conditions. As shown on figure 2, crude venom was divided according to molecular weight in a wide range that started from 4 KDa to 250KDa. However, the proteins with a molecular weight of lower than 25 kDa were separated in the third and fourth fractions.

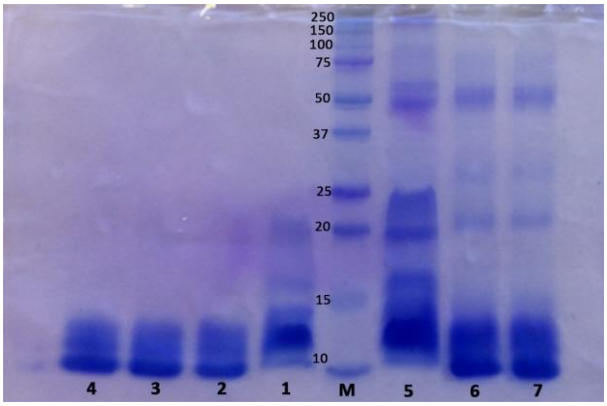

Figure 2. SDS-PAGE analysis of crude and toxic fractions (Fraction 3 and 4) of *Naja naja oxiana* (*NNO*) venom
Lane 1, Fraction 3 without mercaptoethanol; Lane 2, Fraction 4 without mercaptoethanol ; Lane 3 and 4, fraction 3 and 4 with mercaptoethanol; Lane 5, crude venom without mercaptoethanol; lane 6 and 7, crude venom with mercaptoethanol, Lane M, protein marker

3.2. Determination and Kinetics of Antibody Responses with ELISA

After immunization of animals, they were repeatedly monitored for antibody response against NNO crude venom or fraction 3-4. The mean of specific antibody levels was measured by optimized indirect ELISA method.

As shown in figure 3, the formulation of crude and toxic fractions with MF59 adjuvant represented that the level of antibodies in all groups increased with an upward trend and reached the highest level on day 40. This pattern was consistent with the results of groups that received venom and fractions with Freund's adjuvant. Moreover, similar results were observed in antibody levels after immunization of animals in two separate routes ($P>0.05$). Both IM and SC immunization could significantly increase the antibody response towards whole venom and fractions ($P<0.01$).

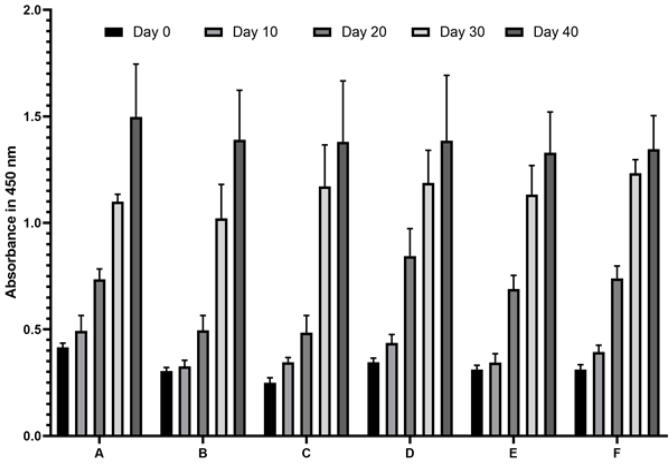

Figure 3: Evaluation of antibody levels against *Naja naja oxiana* (*NNO*) crude venom in different groups during immunization period.

The groups are indicated as: A, immunized with crude venom and Freund's adjuvant; B, immunized with fractions and Freund's adjuvant; C, immunized with crude venom and MF59 (Subcutaneously); D: immunized with toxic fractions and MF59 (Subcutaneously); E, immunized with crude venom and MF59 (Intramuscular); D: immunized with toxic fractions and MF59 (Intramuscular).

4. Discussion

Administration of antivenom is considered the sole effective and accepted treatment for snake envenomation (14, 15). Despite the long precedence of antivenom production, there are various problems in different manufacturing processes and some efforts are being made in manufacturing protocol to improvement the antivenom production (16, 17).

According to WHO Guidelines, animal immunization schedule is one of the critical stages in antivenin production process which could be optimized with different protocols, such as selection of appropriate adjuvants and immunization routes (15, 18). According to these strategies, Immunization protocol should be simple and economic with minimal usage of venom.

Adjuvants have a great deal of importance in antivenom production and should be chosen according to their impacts, side effects, and coasts. The FCA, as a most widely utilized adjuvant could result in severe *adverse effects and many producers fight for additional alternative adjuvants* and utilize less toxic ones with more effectiveness (15, 19). For this reason, it is recommended that Freund's adjuvant be used only at the beginning of the immunization process, not in the case of booster injections. This reduces the risk of developing granuloma formation.

This study focused on the use of MF59 adjuvant for antivenom production with with the aim of immunization of rabbits with NNO fractionated venom administered via two separate routes. According to Zolfagharian, Mohammadpour (12) the presence of high molecular weight fractions can have a suppressive effect on the induction of immune responses against small and immunogenic fractions (F3 and F4). They proposed that administration of fractions 3 and 4 with oil adjuvant, have greater results, compared to crude NNO venom (12).

Based on the comparison of MF59 and Freund's adjuvants, the formulation of venom with MF59 is less time-consuming and results in a less viscosity sample with easy inoculation. Regardless of the injection route, evaluation of antibody levels during bleeding phases represented the effectiveness of MF59 for antivenom production, compared to FCA. Altogether, the results demonstrated that formulation of NNO venom with MF59 adjuvant administered via both SC and IM routes are in conformity with Freund's adjuvant.

In a similar study, following the formulation of the *Androctonus australis hector* detoxified venom with adjuvants MF59 and Alum adjuvants, the amount of antibodies produced by the MF59-formulated venom was higher than the Alum-formulated venom and caused less inflammation in the immunized animal (20).

In their study, Waghmare, Salvi (8) used montanide adjuvants (IMS 3012, ISA 206, and ISA 35) and

concluded that these adjuvants had acceptable safety and efficacy for antivenom production. In addition, the effects of these adjuvants on the health of horses immunized with venom have been significant in comparison with those of Freund adjuvants (7).

In addition to the nature of adjuvant, the method and volume of injection has a great impact on the health of the host animal, which can be effective in controlling pain and emotional behaviors and even complications after the injection (21, 22). There are three commonly used SC, intradermal (ID), and IM routes for the administeration of venom to a host animal. However, injection of snake venom for animal hyper-immunization can be given through single or multiple sites (23).

Regarding the method of administration, it should be noted that the injection site should be in several areas and close to the main lymph nodes. This operation causes the injected toxin to spread to a wider area and a large number of APC cells are used and enter the reaction and subsequently induce an appropriate antibody response. However, in most cases, the subcutaneous method is used, which can stimulate DC and MQ and lymphocytes located in the retropharyngeal, submandibular, and subscapular lymph nodes (23).

Subcutaneous inoculation causes less pain but allows movement of possible fistulous track (24, 25). Moreover, ID injection provides easy monitoring, but leads to ulceration. The main advantage of IM injection is the easy inoculation of large volumes at a unique site, but it is hard to monitor the inoculation site and also causes constant potential pain (24, 26).

This study proposed that MF59 adjuvant administered through subcutaneous and IM routes can be a proper and safe choice for rabbit model in comparison with Freund's adjuvant. However, further investigation is suggested about long-lasting effects of MF59 on larger animals to see if it can be used for antivenom production.

Authors' Contribution

Study concept and design: M. T. and S. K.
Acquisition of data: S. K., S. Kh and M. T.
Analysis and interpretation of data: S. K. and M. T.
Drafting of the manuscript: S. K. and M. N.
Critical revision of the manuscript: M. T and M. D.
Statistical analysis: M. T.

Ethics

It is declared that all ethical considerations were taken into account in the preparation of the submitted manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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