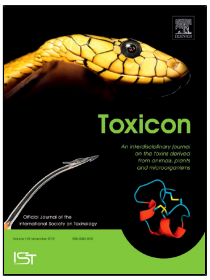
Journal Pre-proof



Development of a human scFv antibody targeting the lethal Iranian Cobra (*Naja oxiana*) snake venom

Fatemeh Kazemi-Lomedasht, Montarop Yamabhai, Jean-Marc Sabatier, Mahdi Behdani, Mohammad Reza Zareinejad, Delavar Shahbazzadeh

PII:	S0041-0101(19)30486-6
DOI:	https://doi.org/10.1016/j.toxicon.2019.10.006
Reference:	TOXCON 6225
To appear in:	*Toxicon*
Received Date:	19 August 2019
Accepted Date:	10 October 2019

Please cite this article as: Fatemeh Kazemi-Lomedasht, Montarop Yamabhai, Jean-Marc Sabatier, Mahdi Behdani, Mohammad Reza Zareinejad, Delavar Shahbazzadeh, Development of a human scFv antibody targeting the lethal Iranian Cobra (*Naja oxiana*) snake venom, *Toxicon* (2019), https://doi.org/10.1016/j.toxicon.2019.10.006

Development of a human scFv antibody targeting the lethal Iranian Cobra (*Naja oxiana*) snake venom

Fatemeh Kazemi-Lomedasht[1*], Montarop Yamabhai[2], Jean-Marc Sabatier[3], Mahdi Behdani[1], Mohammad Reza Zareinejad[1], Delavar Shahbazzadeh[1]

[1]Biotechnology Research Center, Venom and Biotherapeutics Molecules Laboratory, Pasteur Institute of Iran, Tehran, Iran

[2]Molecular Biotechnology Laboratory, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand.

[3]Institute of NeuroPhysiopathology, UMR 7051, Faculté de Médecine Secteur Nord, 51, Boulevard Pierre Dramard - CS80011, 13344 - Marseille Cedex 15, France

*Corresponding author: Fatemeh Kazemi-Lomedasht, Biotechnology Research Center, Venom and Biotherapeutics Molecules Laboratory, Pasteur Institute of Iran, Tehran, Iran. (T) 982166480780 (E) fa_kazemi@pasteur.ac.ir

Co-corresponding author: Delavar Shahbazzadeh, Biotechnology Research Center, Venom and Biotherapeutics Molecules Laboratory, Pasteur Institute of Iran, Tehran, Iran. (T) 982166480780 (E) shahbazzadeh@pasteur.ac.ir

Abstract

Snakebite is one of the health concerns worldwide. *Naja oxiana* is one of the venomous snakes with a high mortality rate. Anti-serum therapy is the only treatment of the victims. However, in some cases, antiserum injection leads to some side effects in host like serum sickness and anaphylactic shock. It is crucial to develop a neutralizing agent with low side effects. The human antibody library (non-immunized library) was used to isolate specific antibodies against *N.oxiana* venom components. Four rounds of biopanning were performed to enrich scFv-displaying phages against the venom of *N. oxiana*. Enrichment of scFv-displaying phages against *N. oxiana* venom was analyzed by polyclonal Enzyme-Linked Immunosorbent Assay (ELISA). Specific antibody fragments against *N. oxiana* venom were selected through monoclonal ELISA, and were expressed in *E. coli* bacterial cells. Purification of the selected clones was performed by using nickel affinity chromatography. Neutralization and protective capacity of specific antibody fragments were analyzed in C57BL/6 mice (i.v. injection). Results of biopanning and polyclonal ELISA demonstrate a successful enrichment process. Five specific antibody fragments with the highest signal in monoclonal ELISA were selected, expressed, and purified. The purity of expressed antibody fragments was monitored by SDS-PAGE and western blot. The selected antibody fragments were able to neutralize two LD_{50} of *N. oxiana* venom and protected all mice when injected 15 min post-envenomation. The data indicate that such selected antibodies are promising tools for further studies and in the development of novel protective agents against *N. oxiana* venom.

Keywords: *Naja oxiana*, snake venom, human antibody, phage display.

1. Introduction

Snakebite is one of the most prevalent causes of toxin-dependent death in many countries, including Iran. Based on the results of a ten years study, 58,704 snakebites resulted in 73 deaths from 2002 to 2012 (Dehghani et al., 2014). Viperidae and Elapidae families are responsible for most of the deaths in Iran. *Naja oxiana* (Caspian cobra) is belonging to the Elapidae family and is mostly found in the northeast of Iran (Dehghani et al., 2014; Firouz, 2005; Vazirianzadeh et al., 2008). Snake venom is a mixture of various proteins, enzymes, and peptides (Oghalaie et al., 2017; Pourhashem et al., 2018). Some of the major unique proteins of snake venom are matrix metalloproteinases (MMPs), C-type lectins, disintegrins, and phospholipases (Marcinkiewicz, 2013; Ogawa et al., 2005). About 95% of the toxic molecules are proteins and 5% are non-protein components (Otten and Blomkalns, 1998). For example, in the *N. oxiana* venom, neurotoxins I and II (Grishin et al., 1973; Grishin et al., 1974) are capable to block neuromuscular junctions, especially in eyes, tongue, throat and chest (Shashidharamurthy et al., 2002). Three-finger toxins (3FTxs) and phospholipases A2 (PLA_2) are some other important toxic components of *N. oxiana* venom (Wong et al., 2016). The fatal issue of *N. oxiana* venom depends on the age and size of the victim. Lethality also depends on the amount of venom injected, the site of injection, and the composition of venom (which for some species can vary with ontogeny and geographical distribution). Serum therapy is the oldest and mostly used treatment against snakebite. In this method, the anti-snake venom (ASV) antibodies are obtained from the blood of animals (mammals like horse) that were hyper-immunized by using snake venom (Taherian et al., 2018). Potential disadvantages include: anaphylactic shock, pyrogen reaction and serum sickness decreases ASVs usage in humans (Bawaskar, 2004; Laustsen et al., 2018a; Makhija and Khamar, 2010). The Crotalidae Polyvalent (ACP) antivenom is composed of IgGs (150 kDa) and is obtained from horse, whereas the Crotalidae Polyvalent Immune Fab (ACPFab) antivenom is composed by Fab fragments (50 kDa) and is obtained from sheep. In the clinical trials, FabAV was five-times more potent than ACP, with less cases of serum sickness reactions (Gold et al., 2002).

The single-chain variable fragment (scFv) has consisted of one variable region of heavy chain (VH) of immunoglobulin connected to another variable region of the light chain (VL) of immunoglobulin. Simple production in bacterial host is the greater advantage of scFvs (Huston et al., 1988). Phage display is potent technique for selection of specific antibody fragments against various targets. Using phage display technology, a unique clone is isolated among the wide set of clones (Rami et al., 2017). McCafferty et al. in 1990 showed that scFv fragments could be displayed on the surface of phages as the active proteins (Ahmad et al., 2012; McCafferty et al., 1990). There are three types of antibody libraries, including immune, non-immune, and synthetic libraries (Zhao et al., 2016). In the immune library, the different host species (mouse, camel, sheep, horse, etc.) are immunized by using the antigen of interest and the genes encoding the antibody are used for constructing the library. Immune library represents the strongest binding

affinity and specificity. However, a new library should be constructed for each antigen in immune libraries. In addition, obtaining a human antibody from immune libraries is unreachable. However, non-immunized libraries are constructed easily and various antibodies against a wide range of antigens could be isolated (Ahmad et al., 2012; Pansri et al., 2009; Pucca et al., 2014; Silva et al., 2018). Given the importance of snakebite in Iran, we used human non-immunized scFv library (Yamo I) (Pansri et al., 2009) to isolate specific antibodies against *N. oxiana* venom. Five specific antibody fragments were selected and their abilities to neutralize *N. oxiana* venom were evaluated.

2. Materials and Methods

2.1. Biologicals

The *N. oxiana* crude venom (lyophilized powder) was obtained from the Pasteur Institute of Iran. Human scFv library (named Yamo I phage display scFv library) was kindly received from Montarop Yamabha (Pansri et al., 2009). Anti-M13-HRP and anti-His-HRP antibodies were purchased from Roche (Basel, Switzerland). VCSM13 helper phage was purchased from Amersham-Pharmacia (Buckinghamshire, United Kingdom) and the Ni-NTA resin was purchased from Qiagen (Bremen, Germany). Horse polyclonal antiserum (10 ml with ability of neutralization of 500 LD50 of N.*oxiana*)(obtained from hyper-immunized horse with Iranian snakes including *N. oxiana*, *M. lebetina*, *E. carinatus*, *and P. persicus*) was obtained was obtained from RaziVaccine and SerumResearch Institute.

2.2. Enrichment of scFv-displaying phages against *N. oxiana* venom

Four rounds of biopanning were performed to select the high affinity antibodies against *N. oxiana* venom. Briefly, a 96-well plate (Maxsorp Nunc, Roskilde, Denmark) was coated overnight at 4 ° C with 100µl of *N. oxiana* venom (100 µg/ml) in sodium bicarbonate buffer (pH 9.2). As control, wells coated only with 100 µl of sodium bicarbonate buffer were used. The wells were blocked with skim milk 4 % at RT for 1 h. The blocking buffer was removed from the wells and 100 µl of 10^{12} cfu (colony forming units) of phage library were added to each well, and plate was incubated at 37 ^{o}C for 1h. The wells were washed five times with PBST (Phosphate-Buffered Saline with Tween 20) (0.05% (v/v) Tween 20 in PBS). For the elution of bounded phages, 100 µl of TEA (Triethylamine, 100 mM, pH 10.0) were added to the wells and incubated for 10 min at RT. Then, TEA was neutralized with 100 µl of 1M Tris-HCl (pH 8.0). About 180 µl of eluted phages (output phages) were inoculated into a 15 ml tube containing 5 ml of *E. coli* TG1 cells (in 2xTY medium and at log phase, OD_{600nm} 0.4-0.6). The *E. coli* TG1 cells were incubated at 37 ^{o}C without shaking for 1 h. Eight ml of 2xTY with ampicillin (100 µg/ ml) and glucose (20 % (v/v) were added to the 15 ml tube and incubated in the same condition while shaking at 250 rpm for 1 h. About 10^{7} cfu of helper phage (VCSM13) were added to the tube and incubated for 30 min at RT without shaking. The tube was centrifuged at 5,000 x*g* for 15 min, the supernatant was removed and the pellet was resuspended into 330 ml of 2xTY containing kanamycin (70 µg/ml) and ampicillin (100 µg/ml) and incubated overnight at 37 ^{o}C while

shaking at 250 rpm. The pellet of bacterial cells was removed by centrifugation at 5,000 x*g* for 15 min. The phage particles in the supernatant were precipitated by incubating on ice for 1 h in PEG/NaCl (20% polyethylene glycol 6000 and 2.5 M NaCl) solution. The recombinant phage particles were collected at 10,000 x*g* for 30 min and resuspended in PBS buffer (input phages). The input phages were used for the next round of biopanning. The stringency of each round of biopanning was increased through increasing of tween-20 concentration in PBST buffer (0.05, 0.1, 0.2 and 0.4 % (v/v)). After each round of biopanning, phage titrations in positive (well coated with venom) and control (well coated with sodium bicarbonate buffer) wells were performed to estimate the enrichment progress (Homayouni et al., 2016; Kazemi-Lomedasht et al., 2016; Kazemi-Lomedasht et al., 2015b; Kazemi-Lomedasht et al., 2017).

2.3. Polyclonal ELISA

Polyclonal phage ELISA was performed to analyze the progress of the biopanning process (Kazemi-Lomedasht et al., 2015a). In this method, the input phages after each round of biopanning were examined for antigen binding. Briefly, a 96-well plate was coated with 100 µg/ml of *N. oxiana* venom at 4 ^{o}C overnight. Control wells were also coated with sodium bicarbonate buffer and incubated in the same condition. After blocking and washing of wells (five times with PBST (0.05 % (v/v)), 10^{12} cfu of input phages from each round of biopanning were added to the wells and incubated for 1 h at RT (not shaking). The wells were washed and incubated with anti-M13 HRP conjugated (1:7,000 in PBS) for 1 h at RT (not shaking). The TMB solution (3, 3', 5, 5'-Tetramethylbenzidine) was added to the wells and after 15 min the reaction was stopped using 100 µl of 2N H_2SO_4. The intensity of absorbance was measured at a wavelength of 450 nm using an ELISA plate reader.

2.4. Monoclonal ELISA

Monoclonal ELISA was performed as described in Section 2.2, with the difference that monoclonal scFv-displaying phages were added to each well. In total, 70 colonies were randomly picked from the third and fourth rounds of biopanning, which were cultured in 96-well plate containing 200 µl 2xTY and ampicillin (100 µg/ml). The plate was incubated at 37 ^{o}C while shaking until the bacterial cells reach to the log phase (OD_{600nm} 0.4-0.6). Then, 10^8 cfu of VCSM13 helper phage were added to the wells and the plate was incubated at 37 ^{o}C without shaking for 30 min. After addition of kanamycin (50 µg/ml), incubation was continued in a shaker incubator for 16 h at 37 ^{o}C. The plate was centrifuged at 3,200 x*g* for 10 min and ELISA was performed on the collected supernatant, as described in Section 2.2. The colonies that showed signal intensities at least three times greater than those of control wells were considered to be positive and were further subjected to sequencing.

2.5. Expression and purification of soluble scFv antibodies

The positive clones in monoclonal phage ELISA (clones that are carrying phage encoding scFv antibodies) sub-cloned into pET-26b vector between *Nco* I and *Not* I restriction sites. The recombinant vector transformed into *E. coli* BL-21 (DE3) cells to produce soluble scFv antibodies, as previously described (Kazemi-Lomedasht et al., 2015a; Kazemi-Lomedasht et al.,

2016; Kazemi-Lomedasht et al., 2015b). The colonies were cultured in 300 µl of LB medium containing 100 µg/ ml ampicillin until reach to log phase (OD_{600nm} 0.4-0.6). Expression of scFv antibodies was induced at 30 °C by 1 mM IPTG (Isopropyl-β-D-thiogalactoside) and 16 h incubation. Pellet of the cells was collected at 8,000 x*g* for 10 min. The pellet was resuspended in 15 ml of lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 8.0). Cells were sonicated for 5 min (30s pulse and 30s break) and incubated for 1 h at 4 °C while shaking. The cells were then centrifuged for 30 min at 10,000 x*g* (4 °C). The supernatant was loaded on Ni-NTA column that was pre-equilibrated with the wash buffer (50 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 8.0). The eluted fraction of soluble scFvs (50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 8.0) was dialyzed against PBS buffer. Production of soluble scFv antibodies was analyzed by 15% coomassie brilliant blue sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition. To confirm the existence of His-tag on the recombinant scFv antibodies and to estimate the molecular weight of scFvs, western blot was performed using nitrocellulose membrane, anti-His HRP conjugated antibody (1:500 in PBS) and 4-chloro-1-naphthol (4cn) substrate.

2.6. Specificity of expressed scFv antibodies

Specificity and cross reactivity of selected scFv antibodies with various Iranian snake venoms (*N. oxiana* , *M. lebetina*, *E. carinatus,* and *P. persicus*) were tested by ELISA. Briefly, 100 µg/ml of mentioned crude venoms, as well as *N. oxiana* crude venom, were coated overnight at 4 °C. The wells were blocked with 4 % skim milk and then, 100 µl of purified scFv antibodies (100 µg/ml) were added to the wells and incubated for 1 h at 37 °C. Polyclonal horse antiserum was used as positive control. Binding of scFv antibodies to immobilized crude venoms was detected by anti-His HRP conjugated antibody (1:500 in PBS).

2.7. Affinity assay

The affinity of selected scFv antibodies was measured by Beatty et al. method using the below equation (Beatty, 1987).

$$K\ aff = n - 1/\ 2(n\ [Ab] - [Ab']),\ [Ag]/\ [Ag'] = n$$

First, 100 µl of two different concentrations of *N. oxiana* crude venom (100 µg/ ml and 10 µg/ ml) were coated overnight at 4 °C. Various amounts of scFv antibodies (0.1-100 nM) were added to the wells and incubated for 1 h at 37 °C. After washing, 100 µl of anti-His HRP conjugated antibody (1:500 in PBS) were added to the wells and ELISA was developed by TMB and 2N H_2SO_4. The optical density of the wells was measured at a wavelength of 450 nm. Affinity was calculated according to the above formula where [Ag], [Ag′] referred to the concentrations of 100 and 10 µg/ ml of crude venom, whereas [Ab] and [Ab′] were the concentrations of scFv antibodies that represent 50 % binding in the [Ag] and [Ag′] curves.

2.8. Competitive ELISA

In order to determine whether the selected scFv antibodies can detect the solution form of venom and neutralize it, the competitive ELISA method was designed. If selected scFv antibodies are

able to detect the solution form of venom, the intensity of observed signal in the antibodies will decrease (binding of scFv antibodies to the immobilized venom will decrease) by increasing the concentration of antigen (solution form of venom). Briefly, a 96-well plate was coated overnight with 100 µg/ml of venom at 4 °C. After blocking, 10 µg/ml of scFv antibodies pre-incubated (at 37 °C for 1 h) with various concentrations of crude venom (0-100 µg/ ml) were added to the wells. The wells were washed with PBST and 100 µl of anti-His HRP conjugated antibody (1:500 in PBS) were added, and incubation was continued for 1 h at 37 °C. The highest OD is 0 % inhibition whereas the lowest OD (OD background) represents 100 % inhibition.

2.9. *In vivo* assays

2.9.1. Median lethal dose (LD_{50}) determination

All animal challenges were approved by the ethical committee of Pasteur institute of Iran (IR.PII.REC.1396.5) and all experiments were performed in accordance with relevant guidelines and regulations. Median lethal dose (LD50) of *N. oxiana* venom was identified according to the Spearman Karber method (Hamilton et al, 1977). About 200 µl of various concentrations of *N. oxiana* venom (0-30 µg in PBS) were injected intravenously (i.v.) to six-week C57BL/6 mice (18g). Mice were divided in two groups of 6 animals (test and control groups). After 24 h, the mice were examined for their survival rate. The control group received 200 µl of PBS buffer.

2.9.2. Neutralization assay

In order to check the neutralization capacity of horse polyclonal antiserum, various concentrations of *N. oxiana* venom (1-20 LD_{50}) were mixed with a fixed amount of antiserum (200 µl), incubated at 37 °C for 30 min and injected through i.v. route to the mice. Control group only received 2 LD50 of *N. oxiana* venom. After 24 h, the surviving mice were counted and the effective dose was obtained as an amount of neutralized venom per ml of antiserum. To check the neutralization capacity of scFv antibodies, 2 LD_{50} of *N. oxiana* venom with different molar ratios of scFv antibodies (pool of antibodies (C13, C24, C39, C43, and C45) were used) (ratios of 1:3, 1:5, 1:10, 1:20, 1:30, and 1:40) in 200 µl PBS were incubated at 37 °C for 30 min and injected (i.v.) to the mice. The lowest amount of antibodies that led to the survival of 100% of the mice was expressed as a protective dose. To monitor the protective effect of antibodies, 2 LD_{50} of venom were injected (i.v.). After various times (0-30 minutes), the pool of scFv antibodies (300 µg) was injected (i.v.) into mice. The number of surviving mice and the neutralization capacity were determined.

2.10. Statistical analysis

Statistical analysis was performed by using the GraphPad Prism 5.0 Software (GraphPad, San Diego, CA). Analysis between two groups was performed by students t-test and $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Enrichment of scFv-displaying phages against the venom of *N. oxiana*

Enrichment of scFv antibody library against the venom of *N. oxiana* was qualitatively monitored after each round of biopanning. The *E. coli* TG1 cells (log phase) were infected by dilution series

(10^{-1}-10^{-7}) of output phages (bounded phages to immobilized venom) and incubated for 30 min at 37 ^{o}C. The number of grown colonies in each dilution was counted. Table 1 shows results of enrichment. The data highlight the successful enrichment of scFv-displaying phages against *N. oxiana* venom.

3.2. Phage-ELISA

The phages obtained after each enrichment process (input phages) were used to determine the signal strength in the ELISA. The signal intensity in the phage-ELISA experiment was greater with increasing the biopanning rounds. An uptrend in figure 1 indicates the successful progress of enrichment of the scFv antibody library after each round of biopanning. For phages obtained from the first round of biopanning, a slight increase in the signal was observed. With the increase of biopanning rounds, the signal strength increased.

3.3. Screening

Nine monoclonals had a positive signal in monoclonal phage ELISA (the observed signal in the positive well was at least 3 times greater than the signal observed in the control well (well without venom)). Five clones (C13, C24, C39, C43, and C45) with the highest ODs in ELISA were selected to obtain scFv antibodies with the highest affinity toward their target (Fig. 2). Sequencing was performed on five selected antibodies and the sequences thus obtained were subjected to IMGT numbering tools (http://www.imgt.org/) and IgBLAST tools of NCBI (https://www.ncbi.nlm.nih.gov/igblast/) to identify the framework and CDR regions of antibodies (Table 2).

3.4. Expression and purification of selected scFv antibodies

Expression of the antibodies was induced by 1 mM IPTG and 16 h of incubation. The soluble scFvs were purified by nickel affinity chromatography (Ni-NTA resin). The purity of antibodies was checked by 15% SDS-PAGE analysis (Coomassie brilliant blue staining) (Fig. 3A). Western blot was performed using anti-His-HRP conjugated antibody to detect the expression of selected antibodies (Fig. 3B). SDS-PAGE and western blotting highlighted a protein band of about 28 kDa. The concentration of selected antibodies was identified by BCA kit according to the manufacturer's protocol.

3.5. Binding specificity of selected scFv antibodies

Specificity and reactivity of selected antibodies to other snake venoms (*N. oxiana*, *M. lebetina*, *E. carinatus,* and *P. persicus*) were checked using the ELISA method. Horse polyclonal antiserum was used as a positive control. Results indicate the specificity of the selected scFv antibodies to *N. oxiana* venom. No cross-reactivity between selected scFv antibodies and other snake venoms (*M. lebetina*, *E. carinatus,* and *P. persicus*) was observed. However, the horse polyclonal antiserum detected all the mentioned snake venoms in ELISA specificity assay (Fig. 4).

3.6. Binding affinity

The binding affinity of purified scFv antibodies was measured by the ELISA method of Beatty et al (Beatty, 1987). Determined k_{aff} for C13, C24, 39, C43, and C45 are shown in Table 3.

3.7. Competitive ELISA assay

Results showed that, by increasing the concentration of the soluble form of *N. oxiana* venom, the binding of scFv antibodies to the immobilized *N. oxiana* venom decreased. These data suggest that scFv antibodies detected the solution form of *N. oxiana* venom better than the immobilized form (Fig. 5).

3.8. *In vivo* results

3.8.1. LD50 calculation

Various doses of *N. oxiana* crude venom were injected through i.v. route to groups of six mice and the median lethal dose (LD_{50}) was calculated according to the procedure of Spearman Karber (Hamilton et al, 1977). The calculated LD50 of *N. oxiana* crude venom was 10 µg/mouse after 24 h (Table 4).

3.8.2. *In vivo* neutralization assay

The effective dose 50% (ED_{50}) to address the neutralization capacity of antiserum was assessed by pre-incubation of antiserum with various concentrations of the *N. oxiana* venom. The data showed that 200 µl of antiserum were able to neutralize 10 LD_{50} of the *N. oxiana* venom (Table 5). This indicates that each ml of antiserum neutralizes 50 LD_{50} of venom (equivalent to 500 micrograms of venom). The neutralization capacity assay of the pool of scFv antibodies was carried out (i.v. injection) by pre-incubating a fixed amount of venom (2 LD_{50}) with increasing doses of scFv antibodies. The protective dose of scFv antibodies was established according to the neutralization capacity data. Results showed that all of the mice survived the lethal dose of *N. oxiana* venom in the lowest protective dose of scFv antibodies (300 µg). The achieved protective dose for scFv antibodies corresponds to a molar ratio of 1:30 (venom:antibody) (Table 6). To study the protective efficacy of scFv antibodies and time effects on envenoming, 2 LD_{50} of venom were injected (i.v. route) to mice. After various times, 300 µg of scFv antibodies were injected (i.v. route) to mice, and the protection potential was examined. Results showed that administering 300 µg of scFv antibodies protected all mice after 15 min of envenoming whereas, after 30 min, only 16.7 % of all mice survived. In the control group (receiving PBS buffer), all mice died (Table 7).

4. Discussion

Here, we used a human scFv non-immunized library to isolate specific antibodies targeting *N. oxiana* snake venom. The scFv antibodies have small size and low molecular weight (28 kDa), as well as 'high' tissue penetration capability (Yokota et al., 1992). The small size of scFvs makes them appropriate tools for a large-scale and cost-effective production in microbial host (Bates and Power, 2019). In addition, because of the small size of scFvs, they can be quickly cleared from the blood stream (Oriuchi et al., 2005; Winthrop et al., 2003). In case of an antivenom development, fast clearing could be a serious advantage of the scFvs. However, lack of Fc domain in scFvs results in low thermostability, high aggregation rate as well as increased

immunogenicity (Bates and Power, 2019). Thus, high and frequent dose administration is needed (Bates and Power, 2019). It has been reported that scFv could be used for treatment of Crohn's disease, rheumatoid arthritis, breast cancer, medical diagnosis, etc. (Ahmad et al., 2012). However, the most widespread formats of antibodies currently used in the clinic are IgGs (Kaplon and Reichert, 2019; Walsh, 2018).

In many studies, human scFv phage libraries were constructed and evaluated (Bai et al., 2015; Li et al., 2015; Okamoto et al., 2004). The scFv antibodies were found to neutralize different snake venom components: Crotoxin of *Crotalus durissus terrificus,* long alfa neurotoxin of *Naja kaouthia*, BthTXI, BthTX-II, and PLA_2 of *Bothrops jararacussu, BaP1*of *Bothrops asper,* and Vipoxin of *Vipera ammodytes meridionalis* (Laustsen et al., 2018a). The scFv antibodies also neutralized venoms of different animal species such as bee, scorpion, and spider (Laustsen et al., 2018a; Pucca et al., 2019). Another type of antivenom (IgG) for *N. oxiana* was produced by immunization of camel(Khamehchian et al., 2014). Some studies reported on scFvs to neutralize the venoms of other snakes from the *Naja* family (Danpaiboon et al., 2014; Kulkeaw et al., 2009). In a study, the neurotoxicity of black mamba venom was neutralized using an oligoclonal mixture of human IgGs (Laustsen et al., 2018b). In Laustsen study, specific antibody fragments scFv against dendrotoxin of black mamba snake were isolated using the phage display technique. The scFv genes were sub-cloned into a mammalian expression vector to achieve whole antibodies (IgGs). The results showed that an oligoclonal mixture of human IgGs neutralized the neurotoxicity of dendrotoxin in the whole venom (Laustsen et al., 2018b).

It has been reported that the distribution of large size IgGs was limited to the intravascular compartment. It is 'ideal' for the neutralization of toxins that act systemically (Laustsen et al., 2018a). However, antibody fragments like scFvs are able to neutralize toxins in circulation, toxins in bitted site and toxins in systemic target, i.e. neuromuscular junction(Laustsen et al., 2018a). Elapid snakebites and scorpion stings contain toxins that act systemically. However viper snakebites express toxins that act both locally and systemically (Laustsen et al., 2018a). The crude venom virtually contains all toxins of the venom and is an appropriate target for biopanning and antibody selection goals(Amaral et al., 1997). A previous study in our laboratory showed that all fractions of *N. oxiana* are lethal to mice (unpublished work). The venom of *N.oxiana* was fractionated by fast protein liquid chromatography and superdex 200 column (Kazemi-Lomedasht et al., 2019). All the fractions (8 fractions) were administered through i.v. route in six-week C57BL/6 mice. Results showed that all groups of mice died after 12 h. Therefore, we used the crude venom for the biopanning procedure to get antibodies against the whole components of the *N. oxiana* venom (Kazemi-Lomedasht et al., 2018). However, most of the studies focused on the development of specific antibodies against toxins for antivenom application (Laustsen et al., 2015). Biopanning was performed to enrich the scFv library against crude venom of *N. oxiana*. Four rounds of biopanning were achieved to select antibodies with both high affinity and specificity. In each round of biopanning, the percentage of tween-20 in the washing solution was increased to stringent the washing steps and decrease elution of non-

specific antibodies. Polyclonal ELISA confirmed a successful biopanning process. Specific antibody fragments with the ability of binding to the crude venom were selected using monoclonal phage ELISA. Selected clones (C13, C24, C39, C43, and C45) were expressed in *E. coli* BL-21 (DE3) cells. Expression was confirmed by SDS-PAGE and western blot analysis. All selected antibodies detected the solution form of *N. oxiana* venom in competitive ELISA assay. Lethality of *N. oxiana* venom was abolished by a molar ratio of 1:30 (venom: scFv antibodies), and all mice survived. However, with a molar ratio of 1:20, about 67% of the injected mice survived. Therefore, the molar ratio of venom: antibody is also crucial for an efficient neutralization of *N. oxiana* venom. Thus, in lower molar ratios (1: 3, 1: 5, 1: 10, and 1: 20), the total venom of *N. oxiana* was not completely neutralized by the selected antibodies. This may be due to the non-immunized origin and low affinity of the antibody fragments. In addition, the small size of scFvs may lead to high dose administration (high molar ratio of antibody: venom) (Bates and Power, 2019). Selected antibodies protected all mice after 15 min of envenomation. By increasing the time between envenomation and scFvs administration, the protective efficacy of the selected antibodies was significantly decreased. After 30 min of envenomation, only 16% of mice survived. These data strongly suggest that the delay between envenomation and antivenom administration is of the most importance to achieve an effective protective effect. To the best of our knowledge, the current study is the first report on the neutralization of the lethal Iranian *N. oxiana* venom by human scFv antibody using the phage display approach. Overall, production of antivenom using the recombinant technology (recombinant production of specific antibodies targeting toxin) is economically cost effective (H Laustsen et al., 2016). Taken together, the data are promising because a non-immunized library was used. This suggests one may further work on the affinity of such antibodies to 'improve' their protective effects *in vivo*. In addition, testing of other formats of human libraries would be of potential interest, especially for the sake of comparison with our data. Furthermore, application of panning on the purified toxins instead of the crude venom to achieve specific antibody fragments against each toxin would be promising.

In conclusion, using the human scFv library displayed on the phage surface, five specific antibodies targeting the Iranian *N. oxiana* snake venom were selected. The selected antibodies were able to detect the native format of *N. oxiana* venom in competition ELISA assay. The selected antibodies neutralized 2 LD_{50} of *N. oxiana* venom and protected all mice after 15 min of envenomation. Altogether, the results indicate that using non-immunized scFv antibodies is a promising approach to develop novel efficient antivenoms against lethal snakes (and potentially other venomous animals).

Acknowledgments

This work was financially supported by Pasteur Institute of Iran, Tehran, Iran (to Fatemeh Kazemi-Lomedasht; grant number 974).

Conflict of interest

There is no any conflict of interest to declare.

Author contribution

FKL designed the study, did the research and analyzed the data. She also prepared draft of manuscript. MY Prepared the library and read the draft of manuscript. JMS improved the manuscript. MB interpreted the data. MRZ did the animal study. DSH designed the study.

References

Ahmad, Z.A., Yeap, S.K., Ali, A.M., Ho, W.Y., Alitheen, N.B.M., Hamid, M., 2012. scFv antibody: principles and clinical application. Clinical and developmental immunology 2012.

Amaral, C.F., Campolina, D., Dias, M.B., Bueno, C.M., Chávez-Olortegui, C., Penaforte, C.L., Diniz, C.R., Rezende, N.A., 1997. Time factor in the detection of circulating whole venom and crotoxin and efficacy of antivenom therapy in patients envenomed by Crotalus durissus. Toxicon 35, 699-704.

Bai, X., Kim, J., Kang, S., Kim, W., Shim, H., 2015. A novel human scFv library with non-combinatorial synthetic CDR diversity. PloS one 10, e0141045.

Bates, A., Power, C.A., 2019. David vs. Goliath: the structure, function, and clinical prospects of antibody fragments. Antibodies 8, 28.

Bawaskar, H., 2004. Snake venoms and antivenoms: critical supply issues. JOURNAL-ASSOCIATION OF PHYSICIANS OF INDIA 52, 11-13.

Beatty, J.D., Beatty,B.G., Vlahos,W.G, 1987. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. J. Immunol. Methods 100, 173-179.

Danpaiboon, W., Reamtong, O., Sookrung, N., Seesuay, W., Sakolvaree, Y., Thanongsaksrikul, J., Dong-din-on, F., Srimanote, P., Thueng-in, K., Chaicumpa, W., 2014. Ophiophagus hannah venom: proteome, components bound by Naja kaouthia antivenin and neutralization by N. kaouthia neurotoxin-specific human ScFv. Toxins 6, 1526-1558.

Dehghani, R., Fathi, B., Shahi, M.P., Jazayeri, M., 2014. Ten years of snakebites in Iran. Toxicon 90, 291-298.

Firouz, E., 2005. The complete fauna of Iran. IB Tauris.

Gold, B.S., Dart, R.C., Barish, R.A., 2002. Bites of venomous snakes. New England Journal of Medicine 347, 347-356.

Grishin, E., Sukhikh, A., Lukyanchuk, N., Slobodyan, L., Lipkin, V., Ovchinnikov, Y.A., 1973. Amino acid sequence of neurotoxin II from Naja naja oxiana venom. FEBS letters 36, 77-78.

Grishin, E., Sukhikh, A., Slobodyan, L., Ovchinnikov, Y.A., Sorokin, V., 1974. Amino acid sequence of neurotoxin I from Naja naja oxiana venom. FEBS letters 45, 118-121.

H Laustsen, A., Engmark, M., Milbo, C., Johannesen, J., Lomonte, B., Maria Gutierrez, J., Lohse, B., 2016. From fangs to pharmacology: the future of snakebite envenoming therapy. Current Pharmaceutical Design 22, 5270-5293.

Homayouni, V., Ganjalikhani-Hakemi, M., Rezaei, A., Khanahmad, H., Behdani, M., Lomedasht, F.K., 2016. Preparation and characterization of a novel nanobody against T-cell immunoglobulin and mucin-3 (TIM-3). Iranian journal of basic medical sciences 19, 1201.

Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotný, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R., 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. Proceedings of the National Academy of Sciences 85, 5879-5883.

Kaplon, H., Reichert, J.M., 2019. Antibodies to watch in 2019, MAbs. Taylor & Francis, pp. 219-238.
Kazemi-Lomedasht, F., Behdani, M., Bagheri, K.P., Habibi-Anbouhi, M., Abolhassani, M., Arezumand, R., Shahbazzadeh, D., Mirzahoseini, H., 2015a. Inhibition of angiogenesis in human endothelial cell using VEGF specific nanobody. Molecular immunology 65, 58-67.
Kazemi-Lomedasht, F., Behdani, M., Habibi-Anbouhi, M., Shahbazzadeh, D., 2016. Production and characterization of novel camel single domain antibody targeting mouse vascular endothelial growth factor. Monoclonal antibodies in immunodiagnosis and immunotherapy 35, 167-171.
Kazemi-Lomedasht, F., Behdani, M., Rahimpour, A., Habibi-Anbouhi, M., Poshang-Bagheri, K., Shahbazzadeh, D., 2015b. Selection and characterization of specific Nanobody against human immunoglobulin G. Monoclonal antibodies in immunodiagnosis and immunotherapy 34, 201-205.
Kazemi-Lomedasht, F., Oghalaie, A., Behdani, M., Shahbazzadeh, D., 2019. Anti-tumor activity of Iranian cobra snake (Naja oxiana) venom on lung cancer cell line.
Kazemi-Lomedasht, F., Pooshang-Bagheri, K., Habibi-Anbouhi, M., Hajizadeh-Safar, E., Shahbazzadeh, D., Mirzahosseini, H., Behdani, M., 2017. In vivo immunotherapy of lung cancer using cross-species reactive vascular endothelial growth factor nanobodies. Iranian journal of basic medical sciences 20, 489.
Kazemi-Lomedasht, F., Rahimi Jamnani, F., Behdani, M., Shahbazzadeh, D., 2018. Linear mimotope analysis of Iranian cobra (Naja oxiana) snake venom using peptide displayed phage library. Toxin Reviews, 1-9.
Khamehchian, S., Zolfagharian, H., Dounighi, N.M., Tebianian, M., Madani, R., 2014. Study on camel IgG purification: A new approach to prepare Naja Naja Oxiana antivenom as passive immunization for therapy. Human vaccines & immunotherapeutics 10, 1633-1638.
Kulkeaw, K., Sakolvaree, Y., Srimanote, P., Tongtawe, P., Maneewatch, S., Sookrung, N., Tungtrongchitr, A., Tapchaisri, P., Kurazono, H., Chaicumpa, W., 2009. Human monoclonal ScFv neutralize lethal Thai cobra, Naja kaouthia, neurotoxin. Journal of Proteomics 72, 270-282.
Laustsen, A.H., Gutiérrez, J.M., Knudsen, C., Johansen, K.H., Bermúdez-Méndez, E., Cerni, F.A., Jürgensen, J.A., Ledsgaard, L., Martos-Esteban, A., Øhlenschlæger, M., 2018a. Pros and cons of different therapeutic antibody formats for recombinant antivenom development. Toxicon 146, 151-175.
Laustsen, A.H., Karatt-Vellatt, A., Masters, E.W., Arias, A.S., Pus, U., Knudsen, C., Oscoz, S., Slavny, P., Griffiths, D.T., Luther, A.M., 2018b. In vivo neutralization of dendrotoxin-mediated neurotoxicity of black mamba venom by oligoclonal human IgG antibodies. Nature communications 9, 3928.
Laustsen, A.H., Lohse, B., Lomonte, B., Engmark, M., Gutiérrez, J.M., 2015. Selecting key toxins for focused development of elapid snake antivenoms and inhibitors guided by a Toxicity Score. Toxicon 104, 43-45.
Li, K., Zettlitz, K.A., Lipianskaya, J., Zhou, Y., Marks, J.D., Mallick, P., Reiter, R.E., Wu, A.M., 2015. A fully human scFv phage display library for rapid antibody fragment reformatting. Protein Engineering, Design and Selection 28, 307-316.
Makhija, I.K., Khamar, D., 2010. Anti-snake venom properties of medicinal plants. Der Pharmacia Lettre 2, 399-411.
Marcinkiewicz, C., 2013. Applications of snake venom components to modulate integrin activities in cell–matrix interactions. The international journal of biochemistry & cell biology 45, 1974-1986.
McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J., 1990. Phage antibodies: filamentous phage displaying antibody variable domains. nature 348, 552.
Ogawa, T., Chijiwa, T., Oda-Ueda, N., Ohno, M., 2005. Molecular diversity and accelerated evolution of C-type lectin-like proteins from snake venom. Toxicon 45, 1-14.
Oghalaie, A., Behdani, M., Yardehnavi, N., Shahbazzadeh, D., Kazemi-Lomedasht, F., 2017. Cytotoxicity, anti-adhesive and anti-angiogenic effects of Caspian Cobra snake (Naja oxiana) venom on human endothelial cells.

Okamoto, T., Mukai, Y., Yoshioka, Y., Shibata, H., Kawamura, M., Yamamoto, Y., Nakagawa, S., Kamada, H., Hayakawa, T., Mayumi, T., 2004. Optimal construction of non-immune scFv phage display libraries from mouse bone marrow and spleen established to select specific scFvs efficiently binding to antigen. Biochemical and biophysical research communications 323, 583-591.

Oriuchi, N., Higuchi, T., Hanaoka, H., Iida, Y., Endo, K., 2005. Current status of cancer therapy with radiolabeled monoclonal antibody. Annals of Nuclear Medicine 19, 355-365.

Otten, E.J., Blomkalns, A., 1998. Venomous animal injuries. Emergency medicine concepts and clinical practices. New York: Mosby, 924-940.

Pansri, P., Jaruseranee, N., Rangnoi, K., Kristensen, P., Yamabhai, M., 2009. A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. BMC biotechnology 9, 6.

Pourhashem, Z., Yardehnavi, N., Behdani, M., Kazemi-Lomedasht, F., 2018. An in silico study to find angiogenesis inhibitory role for Naja oxiana snake venom cytotoxins.

Pucca, M.B., Cerni, F.A., Oliveira, I.S., Jenkins, T., Timothy, P., Argemí, L.M., Sørensen, C.V., Ahmadi, S., Barbosa, J.E., Laustsen, A.H., 2019. Bee updated: Current knowledge on bee venom and bee envenoming therapy. Frontiers in Immunology 10, 2090.

Pucca, M.B., Cerni, F.A., Peigneur, S., Arantes, E.C., Tytgat, J., Barbosa, J.E., 2014. Serrumab: A novel human single chain-fragment antibody with multiple scorpion toxin-neutralizing capacities. Journal of immunotoxicology 11, 133-140.

Rami, A., Behdani, M., Yardehnavi, N., Habibi-Anbouhi, M., Kazemi-Lomedasht, F., 2017. An overview on application of phage display technique in immunological studies. Asian Pacific Journal of Tropical Biomedicine 7, 599-602.

Shashidharamurthy, R., Jagadeesha, D., Girish, K., Kemparaju, K., 2002. Variation in biochemical and pharmacological properties of Indian cobra (Naja naja naja) venom due to geographical distribution. Molecular and cellular biochemistry 229, 93-101.

Silva, L.C., Pucca, M.B., Pessenda, G., Campos, L.B., Martinez, E.Z., Cerni, F.A., Barbosa, J.E., 2018. Discovery of human scFvs that cross-neutralize the toxic effects of B. jararacussu and C. d. terrificus venoms. Acta tropica 177, 66-73.

Taherian, A., Fazilati, M., Moghadam, A.T., Tebyanian, H., 2018. Optimization of purification procedure for horse F (ab) 2 antivenom against Androctonus crassicauda (Scorpion) venom. Tropical Journal of Pharmaceutical Research 17, 409-414.

Vazirianzadeh, B., Chitins, P., Vahabe, A., Mozafari, A., 2008. Epidemiological study of patients with snake biting in the hospitals of Ahvaz, Iran. Journal of Experimental Zoology, India 11, 497-500.

Walsh, G., 2018. Biopharmaceutical benchmarks 2018. Nat. Biotechnol 36, 1136-1145.

Winthrop, M.D., DeNardo, S.J., Albrecht, H., Mirick, G.R., Kroger, L.A., Lamborn, K.R., Venclovas, Č., Colvin, M.E., Burke, P.A., DeNardo, G.L., 2003. Selection and characterization of anti-MUC-1 scFvs intended for targeted therapy. Clinical cancer research 9, 3845s-3853s.

Wong, K.Y., Tan, C.H., Tan, N.H., 2016. Venom and purified toxins of the spectacled cobra (Naja naja) from Pakistan: insights into toxicity and antivenom neutralization. The American journal of tropical medicine and hygiene 94, 1392-1399.

Yokota, T., Milenic, D.E., Whitlow, M., Schlom, J., 1992. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer research 52, 3402-3408.

Zhao, A., Tohidkia, M.R., Siegel, D.L., Coukos, G., Omidi, Y., 2016. Phage antibody display libraries: a powerful antibody discovery platform for immunotherapy. Critical reviews in biotechnology 36, 276-289.

483

484 Tables

485 Table 1. Enrichment evaluation by phage titration. The titer of output phages increased with
486 increasing biopanning rounds.

Biopanning round	Input phages (cfu)	Output phages (cfu)
1	10^{12}	3×10^{4}
2	10^{12}	5×10^{5}
3	10^{12}	4×10^{7}
4	10^{12}	9×10^{7}

487 cfu: colony forming units.

488 Table 2. Sequencing results. The framework and CDR regions were identified by IMGT and
489 IgBLAST tools of NCBI.

Clone	CDR1	CDR2	CDR3
VH			
C13	WWLHQQWWLV	HLLQWEH	CERRGILYQLLFCFY
C24	GGSISSGGYY	IYYSGST	ARDGGYCSSTSCYSDAFDI
C39	GGTFSSYA	IIPIFGTA	ATPEPFGYN
C43	WVLTQHWNV	HMLGMMM	CTDNRTLWFGDHIRYGR
C45	GGTFSSYA	IIPIFGTA	ATPEPFGYN
VL			
C13	KLGDKY	QDS	QAWDSSSVV
C24	AVTLVVITM	MSV	AHIQAAALVGC
C39	SGINVGTYR	YKSDSDK	MIWHSSARG
C43	AVTLVVITM	MSV	AHIQAAALVGC
C45	KAVDKY	SRM	RSGTAVTWY

490

491 Table 3. Affinity of specific antibody fragments.

Clone	Affinity constant (k_{aff}) (M^{-1})
C13	3.5×10^{7}

C24	2.1×10^7
C39	5.6×10^8
C43	2×10^7
C45	3.2×10^7

492

493 Table 4. Median lethal dose (LD50) of *N. oxiana* crude venom.

Concentration (µg/ mouse)	Mice (died/ total)	Death (%)
1	0/6	0
2.5	1/6	16.66
5	2/6	33.33
10	3/6	50
15	4/6	66.66
20	6/6	100
30	6/6	100

494

495

496 Table 5. Neutralization potency of horse polyclonal antiserum against lethality of *N. oxiana*
497 venom

LD50	Antiserum (µl)	ED_{50} (µg)	Mice (survived/total)	Survived (%)
3	200	250	6/6	100
5	200	250	6/6	100
10	200	250	6/6	100
15	200	110	4/6	66.7
20	200	60	2/6	33.3

498

499 Table 6. Neutralization capacity of the pool of scFv antibodies against lethality of *N. oxiana*
500 venom

Ratio (venom: scFv antibody) (mol/mol)	Mice (survived/total)	Survived (%)
1:3	0/6	0
1:5	0/6	0
1:10	2/6	33.3
1:20	4/6	66.7
1:30	6/6	100
1:40	6/6	100

501

502 Table 7. Protective effect of scFv antibodies on envenoming of mice by *N. oxiana*

Administration time (after envenoming) (min)	Mice (survived/total)	Survived (%)
5	6/6	100
10	6/6	100
15	6/6	100
20	5/6	83.3
25	3/6	50
30	1/6	16.7

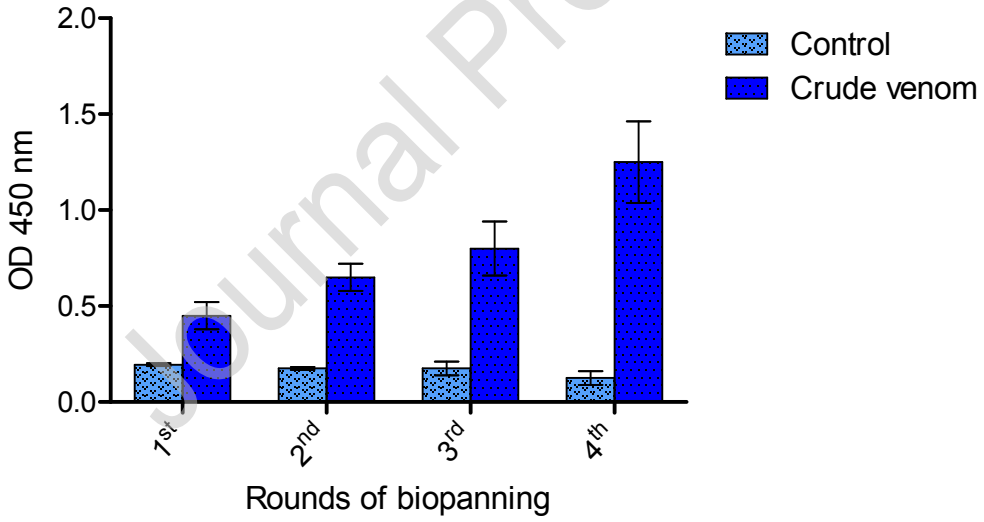
503

504

505

506

507 Figures



508

509 Figure 1. Polyclonal ELISA. The binding of scFv-displaying phages rescued during the selection
510 rounds was evaluated by ELISA. The ELISA test was performed on Input phages. Results are
511 expressed as means ± SD (n = 3).

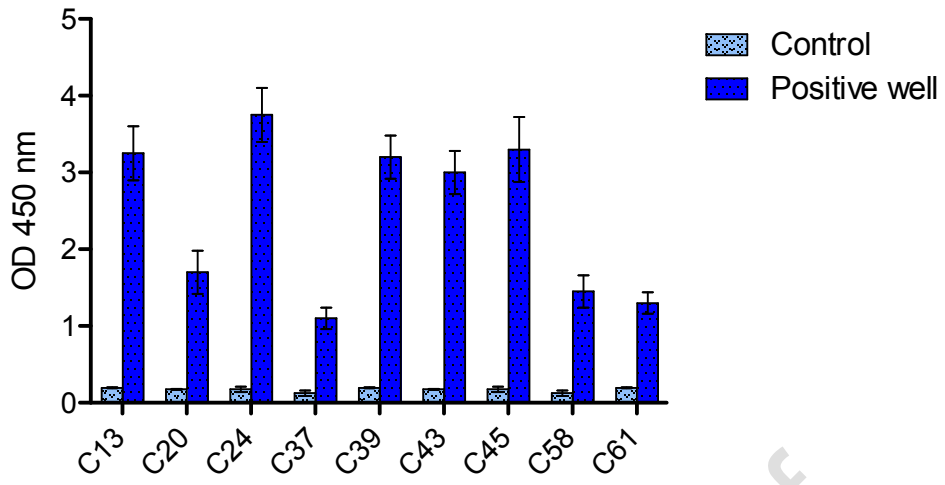


Figure 2. Monoclonal ELISA. Monoclonal scFv-displaying phages presenting the highest binding signals against *N. oxitana* venom. Seventy colonies were randomly picked from the third and fourth rounds of biopanning. Colonies with highest ODs (C13, C24, C39, C43, and C45) were selected for further assays. Results are expressed as means ± SD (n = 3).

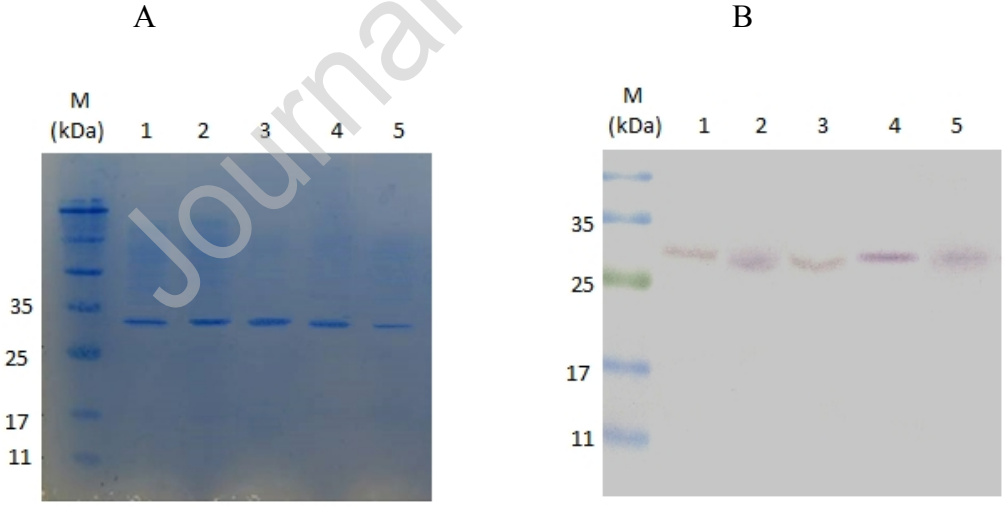


Figure 3. Purification of scFvs. A. Coomassie brilliant blue stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15%). SDS-PAGE was performed under reducing condition. B. Western blots analysis. Detection of selected antibodies was performed by

anti-His-HRP conjugated antibody. M (protein markers), 1 (C13), 2 (C24), 3 (C39), 4 (C43), and 5 (C45).

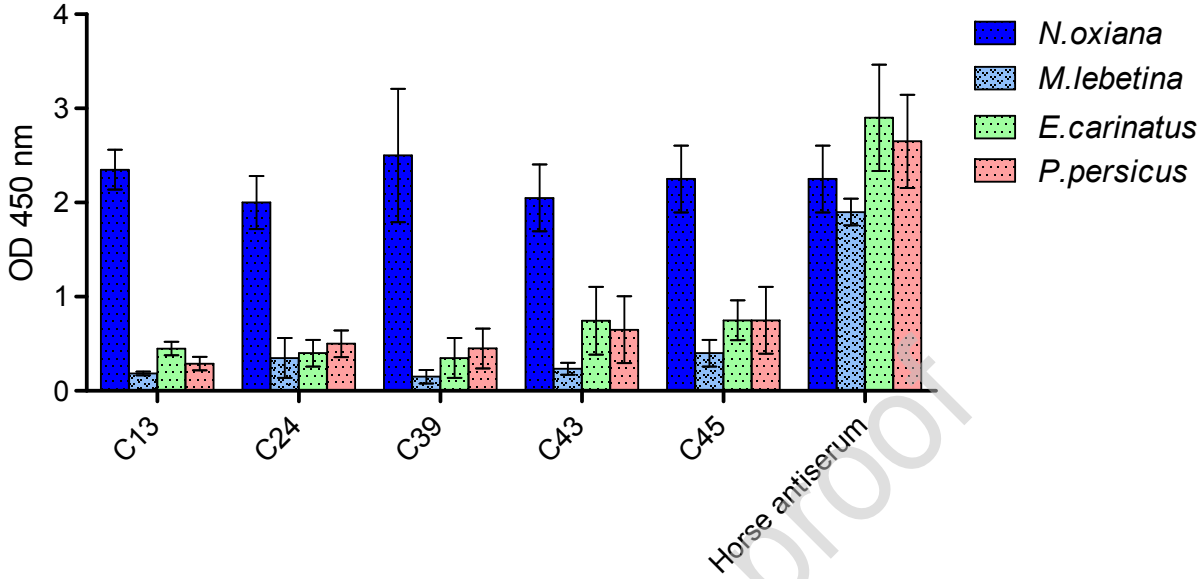
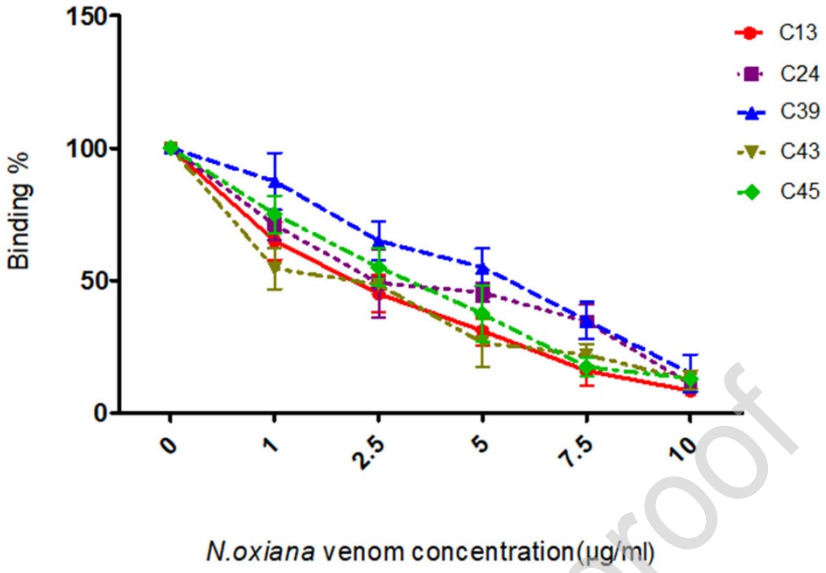


Figure 4. Binding specificity. All of the selected scFv antibodies detected *N. oxiana* venom in ELISA experiments whereas none of them was able to detect other snake venoms (*M. lebetina*, *E.carinatus,* and *P. persicus)*. Horse polyclonal antiserum detected all the tested snake venoms. Results are expressed as means ± SD (n = 3).



534

535 Figure 5. Competitive ELISA assay. The selected scFv antibodies were pre-incubated (at 37 °C
536 for 1 h) with various concentration of *N. oxiana* venom (0-100 µg/ml) and then added to the
537 wells containing immobilized *N. oxiana* venom. Data showed that scFv antibodies detected the
538 solution form of *N. oxiana* venom better than the immobilized form. Results are expressed as
539 means ± SD (n = 3).

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

All animal challenges were approved by the ethical committee of Pasteur institute of Iran (IR.PII.REC.1396.5) and all experiments were performed in accordance with relevant guidelines and regulations (the ARRIVE guidelines, EU Directive 2010/63/EU for animal experiments).

- Non-immunized human antibody library was used to isolate specific antibody fragments (scFvs) against *N. oxiana* venom using phage display technology
- Enrichment of scFv-displaying phages against *N. oxiana* venom was monitored by polyclonal ELISA.
- Specific antibody fragments against *N.oxiana* venom were selected through monoclonal ELISA.
- Neutralization and protective capacity of selected antibodies was analyzed in C57BL/6 mice.
- The selected antibodies protected all mice after 15 min envenomation.
- The results indicate use of selected antibodies as promising tool for further studies and in the development of novel protective agents against *N.oxiana* venom .