Research article

Comparison of venom from wild and long-term captive *Gloydius caucasicus* and the neutralization capacity of antivenom produced in rabbits immunized with captive venom

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ARTICLE INFO

ABSTRACT

Gloydius caucasicus (NIKOLSKY, 1916) is a member of the Viperidae family in Iran.

Comprehensive understanding of the toxigenic characteristics of snake venom is important for clinical monitoring of snakebite patients and effective therapy. We compared the toxic activities of venoms and the neutralization capacity of antivenoms produced with venoms from wild adult (WA) with long-term captive adult (LCA) of *G. caucasicus* in order to obtain more effective antivenom from LCA in therapy, and subsequently protect *G. caucasicus* from overharvesting for its venom, which poses a real threat of extinction for the species. Our results showed that LD_{50} of WA and LCA were 16.8 µg/dose and 17.7 µg/dose, respectively. Lower hemorrhagic and necrotic ($p \geq 0.05$), and higher coagulative and edematogenic activities ($p \leq 0.05$) were observed in WA compared with LCA venom. Also, captive-born neonates exhibited weaker toxic activities compared with captive adult snakes, which could be an age-related difference. Study data illustrated that effective capacity of LCA antivenom to neutralize the toxic activities of WA viper venom. According to the results, about 0.4–4 µl of LCA antivenom is required to neutralize the toxic activities of 1 µg of WA venom, indicating its efficacy in treatment of snakebites in humans. On this basis, it is recommended that capture of wild snakes for their venom be discontinued to reduce their future extinction risk.

1. Introduction

Venomous snakes, i.e. species in the suborder Serpentes which inject venom through bites, cause death in humans. Venom is a mixture of biologically active substances consisting of enzymatic and nonenzymatic polypeptides and other substances which are mostly toxic [1]. These compounds can affect the biological functions of the human body, leading to edema, hemorrhage, inflammation, and severe poisoning. Snakes inject venom through unique fangs into the body of the prey in order to paralyze or digest the prey or to defend against threats [2, 3, 4]. In June of 2017, snakebite envenoming was added to the priority list of the neglected tropical diseases (NTD) by the World Health Organization (WHO) (https://www.who.int/neglected_diseases/en/). Therefore,

disease control, prevention and development of therapeutic methods are high on the agenda [5].

Vipers are among the world's most venomous snakes whose venoms often have hemotoxic activity. The Caucasian pit viper is a venomous and viviparous snake that is found across southeastern Azerbaijan, northwest to northeast of Iran, Kopet Dagh Mountains (Southern Turkmenistan), and northwestern Afghanistan, and is commonly found throughout the Alborz Mountains. It belongs to the *Gloydius halys/Gloydius intermedius* species complex, which consists of nine taxa of the Crotalinae subfamily (Viperidae family), including: *G. halys, G. cognatus, G. h. caucasicus, G. caraganus, G. rickmersi, G. stejnegeri, G. changdaoensis, G. shedaoensis*, and *G. intermedius* [6]. These pit vipers occur in a diverse array of habitats across a wide range in the Palearctic region from Azerbaijan, Iran and

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Central Asia to Mongolia, Eastern Siberia and China [7, 8]. Although several phylogenetic, morphological, ecological, and captive-breeding studies have attempted to investigate this group, it yet remains an ambiguous species complex [8, 9, 10, 11]. Recently, the Caucasian pit viper (*Gloydius caucasicus*) has been elevated to species rank based on phylogenetic and phylogeographic analyses [6], suggesting the need for further future research across the western range of the complex in the Palearctic.

Several studies have described analysis of snake venom using proteomic methods which help us gain a better understanding of venom compositions. Various proteomic tools (gel-based and free proteomics) have been developed over the past decades, such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), tandem mass spectrometer (MS/MS) and high-performance liquid chromatography (HPLC) Each approach has its advantages and disadvantages. Gel-based proteomics rely on one-dimensional (SDS-PAGE) and or two dimensional (2-DE) separation techniques. SDS-PAGE is an analytical technique widely used for gel electrophoretic separation of proteins based on their molecular weight (MW). The protein mixture is denatured by adding SDS and beta-mercaptoethanol to break disulfide bonds of proteins. In 2-DE, protein mixtures are separated by using two different properties of the proteins in two dimension steps: First, proteins are separated by their isoelectric point (PI) and, second based on their relative molecular weights [12]. MS has been used for protein identification whereas chromatography has been preferably used for separation of proteins. MS/MS is used to measure molecular weight and amino acid sequences of proteins. MS is based on the positive ion generation and is a technique with high speed. LC-MS is a hybrid technique which is more specific and sensitive than HPLC, but more expensive and complex. The HPLC is used to separate or purify the components (proteins) from a mixture based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase. Reverse-phase HPLC (RP HPLC) separates the amino acids based on their hydrophobicity. In RP HPLC, hydrophobic molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase, and hydrophilic molecules in the mobile phase will pass through the column and are eluted first. Overall, HPLC is a fast and high-accuracy method to identify sample components, but it is costly [13, 14].

Immunotherapy using antivenins has been used for over a century; however, it still remains one of the most effective treatments for snake-bite envenoming according to the WHO recommendations [15, 16]. Assessment of neutralization potential of antivenoms based on the median effective dose is essential to reduce possible adverse after-treatment effects such as severe allergic reactions [17, 18, 19]. The only antivenom available for viperid bites in Iran is a polyvalent antivenom against five viperid venoms (*Macrovipera lebetina, Montivipera raddei, Echis carinatus, Pseudocerastes persicus*, and *G. caucasicus*). However, the Padraserum Institute has recently developed a bivalent antivenom against two venomous species including *M. raddei* and *G. caucasicus*, two species which occur in north and northwest of Iran. This antivenom is under final examination and we hope it can be used more effectively than the polyvalent one.

Captive breeding of snakes in controlled environments have fundamental importance for therapeutic antibody production, in addition to preventing their extinction due to overharvesting, natural causes and habitat destruction [20, 21, 22, 23]. Furthermore, comparison of venom proteins and biological properties between venoms from captive-bred and wild snakes are essential in order to obtain antivenom for effective therapeutic interventions [24], manage snake bites in humans, and prevent the destructive effects of venom on host tissues. In Iran, no studies have been performed on venom of captive *G. caucasicus*, production of antivenom from its venom, and evaluation of the antivenom's neutralization efficacy.

In this study, we used proteomics methods including SDS-PAGE, 2-DE, and RP HPLC to compare venom protein profiles. The present study aimed to compare crude venom protein and peptide profiles in wild adult (WA), long-term captive adult (LCA), and captive-born neonates (CN) of the Caucasian pit viper. We investigated the toxic effects of venoms from WA, LCA and CN *G. caucasicus* vipers. In addition, one of the main targets of this research was to produce antivenom from LCA *G. caucasicus* and evaluate the antivenoms' neutralizing effect against the toxic activities of WA *G. caucasicus* venom.

2. Material and methods

2.1. Collection of snakes from the wild, captive breeding, and venom milking

Three sample groups of *G. caucasicus* including wild adult, long-term captive adult, and captive-born neonates were studied. This research was licensed by the Iranian Department of Environment under permit No. 97/9061. In 2018, 20 Caucasian pit vipers (10 adult males and 10 adult females; WA) were collected from Lar Valley (Lar National Park), central Alborz Mountains. All maintenance and handling of snakes were conducted following the regulations and relevant guidelines [25]. WA venom was milked immediately after snakes were captured. The snakes were then transferred to the University of Tehran. The incoming vipers were quarantined for a period of four weeks. To eradicate ectoparasites, the vipers were treated with a dilute ivermectin spray. Then, they were housed individually in tubes. The tubes were stacked in racks measuring 120 cm long × 45 cm wide × 50 cm high, consisting of 6 floors with 4 tubes per floor. The tube size was 120 cm long × 45 cm wide × 50 cm high. A hiding place, a bowl of water, a piece of stone, and clean sawdust (as substrate) were provided in each tube. The heating system consisted of six heating strips, 100 cm long × 25 cm wide, 32 Watt. The thermal gradient ranged from 30–32 °C to 20–24 °C (along the one-third of the posterior and anterior ends of the tubes, respectively). Adequate moisture was provided by spraying water into the tubes as needed. The humidity level prior to spraying was 30%, which increased to 55% after water spraying. The relative humidity averaged 30–45%. Once a week, newborn and adult mice (*Mus musculus albinus*) were offered as food. From 9 to 12 September, 17 vipers were born and placed into separate tubes. Each tube was 30 cm long × 10 cm wide × 10 cm high and comprised a hiding place, a paper substratum, and a water bowl. After the first skin shedding, none of the neonates accepted food readily, so they had to be force-fed or stimulated to eat. After a year, the snakes accepted newborn mice without human intervention. After offering food for several months, the adult vipers were put into hibernation and kept at temperatures ranging from 6–8 °C. In the third week of March 2019, they emerged from hibernation and were placed together to be able to exhibit courtship behavior and copulate. The same procedure was repeated for 2020.

LCA venom was milked before the second hibernation (n = 19) of snakes that had been kept in captivity for more than a year under favorable and controlled conditions. Also, CN venom was milked from six-month-old captive-born neonates (n = 17). After extraction, crude pooled venom of each group was freeze-dried and stored at -20 °C. A stock solution (10 mg/ml) was prepared for each group by dissolving 10 mg of lyophilized venom powder in 1 ml of double distilled water (DDW). The solution was then centrifuged for 10 min at 10000 rpm at 4 °C to remove cellular debris.

2.2. Animals

Healthy male Balb/c mice (20 ± 2 g weight) and male albino New Zealand rabbits (2.5–3.5 kg) were obtained from the Pasteur Institute of Iran (Karaj, Iran) and kept in stainless steel cages under controlled conditions at a regulated temperature of 18–22 °C, humidity of 50 ± 5%, and a 12/12 h light/dark cycle. During this period, the snakes were fed a standard commercial feed. Animal use procedures were conducted in accordance with the regulations of the ethics committee of university of Tehran and the animal welfare agreement of the Pasteur Institute of Iran (approval number: IR.PII.REC.1394.38).

2.3. Protein assay

Protein concentration of the crude venoms from the three groups was estimated using the Bradford Coomassie brilliant blue assay, in which bovine serum albumin (BSA) was used as standard. Subsequent measurements were carried out at 595 nm with a micro-plate reader [26].

2.4. Proteomics analysis

2.4.1. Mono-dimensional electrophoresis (SDS-PAGE)

Separation of protein and peptide components in each crude venom (WA, LCA and CN) was carried out according to the technique by Laemmli [27] by SDS-PAGE over 12.5% polyacrylamide gels (w/v), under reducing (with 5% (v/v) 2-mercaptoethanol, 2-ME; 95 °C, 10 min) and non-reducing (without 2-ME) conditions. Thirty micrograms of crude venom was separately mixed with sample buffer [50 mM Tris–HCl pH 6.8, 10% glycerol, 0.1 M DTT, 2% (w/v) SDS, and 0.1% bromophenol blue], and then incubated at 95 °C for 5 min and loaded onto the SDS-PAGE gel. Protein gels were visualized by staining with Coomassie brilliant blue R-250 (CBBR), Ultra-Pure Grade (Thermo Fisher Scientific; USA). Unstained protein molecular markers ranging from 14.4 kDa to 116 kDa (Bio Basic; Canada) were used for proteomic analysis.

2.4.2. Two-dimensional gel electrophoresis (2-DE)

2-DE for crude venom of WA, LCA and CN snakes was carried out separately using a modification of a previous study [28] and in accordance with laboratory guidelines. Three hundred micrograms of each venom sample were separately dissolved in 100 μl of rehydration solution containing 8 M urea, 2% (w/v) CHAPS (Co. Sigma), 20 mM DTT (dithiothreitol, Co, Merck, Germany), 0.5% (v/v) immobilized pH gradient (IPG) buffer (Merck), and 0.002% (w/v) bromophenol blue. Seven-centimeter IPG strips (Merck KGaA, Darmstadt, Germany) with a linear pH ranging from 3 to 10 were equilibrated using two equilibration buffers (reduction and alkylation), each for 15 min. Then, strips were loaded and run on 12.5% polyacrylamide Laemmli gels (m/v) (18 × 16 cm) using the SE600 Ruby system (with a programmable power control, 70v for 30 min, followed by 120V for 40). Thereafter, gels were stained with Coomassie brilliant blue and scanned on an Epson Imagescanner III. Finally, analysis of images was done using ImageMaster 2D 7.0 software (GE Healthcare, U.S.A.). Each venom sample was run three times.

2.4.3. Reversed phase-high performance liquid chromatography (RP HPLC)

Two milligrams of crude venom were dissolved in 200 μl of solution A and centrifuged for 10 min at 10,000 g at 4 °C. Then, 100 μl of the supernatant was injected into the HPLC system. The separation of proteins of crude venom samples (WA, LCA, and CN; each venom sample, separately) was performed using RP HPLC on reverse-phase C18 column at an analytical scale (5 μm particle size, pore size 300 Å. 250 mm × 4.6 mm; MicroTech). The flow-rate was adjusted to 1 mL/min and the column was developed with a linear gradient of solution A (water containing 0.1% trifluoroacetic acid; TFA) and solution B (70% acetonitrile, CNCH3 containing 0.1% TFA), and absorption was monitored at 214 nm (absorption wavelength of peptide bond). Column elution conditions were as follows: B = 10% at t = 20 min, 45% at t = 60 min, 45% at t = 85 min, 75% at t = 110 min, 90% at t = 120 min and 0% at t = 130 min. After elution, the fraction was lyophilized and stored at 20 °C. Chromatography data was obtained by the Agilent EZChrom Elite version 3.2.0 software (Agilent Technologies, California; USA).

2.5. Biological activity of crude venom samples of adult wild, adult captive and neonatal captive vipers

2.5.1. Median lethal dose assay

The median lethal dose (LD_{50}) is the amount of venom that kills 50% of injected mice. LD50 was determined using the Reed & Munch (1938) method [29], according to WHO guidelines. All animal subjects were obtained from the animal center at the Pasteur Institute of Iran. Various doses of crude venom from WA, LCA and CN vipers were injected intra-peritoneally (IP) to five groups of mice and an untreated control group. The control group received the phosphate-buffered saline buffer alone (PBS, 8 g of NaCl, 0.2 g of KCl, Na_2HPO, and 0.24 g of KH_2PO_4, distilled water was added to a total volume of 1 L) using the same protocol and conditions. The number of deaths and survival rates in each group was recorded after 48 h LD50 values were calculated using Spearman and Karber's analysis.

2.5.2. Coagulation activation

The prothrombin time (PT) and active partial thromboplastin time (aPTT) tests were used to evaluate coagulation time in the extrinsic and intrinsic pathways. In the PT test, different doses of venom (0.5, 1, 2, 4, 6, 8, 16, 32 and 64 μg) and PT reagent (thromboplastin with calcium) were pre-incubated for 10 min at 37 °C and mixed. Clotting time was recorded by addition of 100 μL of fresh citrate plasma. The normal range for the PT test is 11–13 s. For the aPTT test, different doses of venom (0.5, 1, 2, 4, 6, 8, 16, 32 and 64 μg) and aPTT reagent (cephalin plus kaolin) were incubated for 10 min at 37 °C. Then, 100 μL of fresh citrate plasma and subsequently, 100 μL of $CaCl_2$ were added and the clotting time was recorded. Human normal plasma without venom sample was used as control plasma for the tests. The normal range for aPTT is 30–35 s.

2.5.3. Edematogenic activity

Edematogenic activity was measured using Lomonte et al.'s method [30]. Different doses of venom were injected subcutaneously in the right footpad of mice and PBS was administered in the contralateral paw (left foot) as negative control. Control animals received PBS alone. Minimum Edematogenic Dose (MED) was considered as the venom dose that induced an increase of 30 % in footpad volume 1 h after injection [31].

2.5.4. Hemorrhagic activity

To assess the hemorrhagic activity of crude venom on albino rabbits, the method described by Kondo et al. (1960) [32] was used. Dilutions of several doses (0.5, 1, 2, 4, 8, 16, 32 and 64 μg) of each crude venom pool were injected intra-dermally (ID) to an area on the abdomen of albino rabbits weighing about 2.5–3.5 kg. Control animals received PBS without the venom sample using the same protocol. After 24 h, the rabbits were killed by anesthesia, their dorsal skin was removed and response intensity was estimated from the inside of skin. The Minimum Hemorrhagic Dose (MHD) of venom was defined as the smallest amount of venom (μg) causing hemorrhagic spots of approximately 9 mm in diameter within 24 h after ID injection. Mean cross-diameters of hemorrhagic spots is an indicator of response intensity.

2.5.5. Necrotic activity

Necrotic activity of venom was estimated using the method followed by Tan et al. [33] on albino rabbits. Different doses of venom (0.5, 1, 2, 4, 8, 16, 32 and 64 μg) were injected intra-dermally to the albino rabbits. PBS without venom sample was used as negative control. Minimum Necrotizing Dose (MND) was defined as the minimum amount of venom (μg) which results in a necrotic lesion of 5 mm in diameter within 72 h after ID injection.

2.6. Immunization of rabbits to obtain of antivenom

Rabbits were immunized with venom associated with Freund's adjuvant according to WHO guidelines in a two-month immunization protocol [19]. Rabbits were first injected intra-dermally with a sub-lethal dose of venom and Complete Freund's Adjuvant (CFA, Sigma Co. Roedermark, Germany) mixture. Every ten days, subsequent booster injections were also given intra-dermally with Incomplete Freund's Adjuvant (IFA, Sigma Co. Roedermark, Germany). Blood samples were taken from rabbits before each injection, which were then centrifuged at 3000 rpm for 20 min at 4 °C to produce serum, and finally stored at -20 °C

until use. As control, three rabbits received sterile PBS plus Freund's adjuvant. Each venom sample (WA and LCA) was injected into three rabbit groups to obtain antivenom.

2.6.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA, a plate-based assay, was conducted in order to analyze WA and LCA antivenoms. Serum obtained from rabbits immunized with LCA venom was assessed for detection of raised antibody based on the ELISA technique. To this end, 1 μg of lyophilized venom of a captive viper (dissolved in 0.1 M carbonate-bicarbonate buffer; pH 9.6) was coated in individual wells of a 96-well microtiter plate and incubated at 4 °C overnight. The coating buffer was removed and the plate was washed three times with PBS. All wells were filled with 100 μl of blocking buffer (%2 Bovine Serum Albumin (BSA) in PBS) and the plate was incubated at room temperature for 1 h under gentle shaking. After removing unbound BSA molecules by patting the plate on a sink, 100 μl of serum from immunized rabbits (as primary antibody) was diluted with PBS in a serial dilution from 1:500 to 1:128000, followed by adding each dilution to one individual well. Next, the plate was incubated at 37 °C for 1 h under gentle shaking. After that, each well was washed with PBST several times and 100 μl of goat anti-rabbit IgG-HRP conjugate (as secondary antibody) diluted with PBS at 1:5000 was loaded into each well, followed by incubating the plate at 37 °C for 1 h. Finally, 100 μl of TMB (Tetramethylbenzidine) solution was added to each well and stored for 15 min in the dark. To stop the reaction, the stopping solution (H_2SO_4, 2N) was added and the absorption of all wells was determined at 450 nm using a spectrometer-based ELISA Reader. All six series of injections were done in this test. The ELISA procedure was simultaneously performed for serum obtained from rabbits immunized with WA venom. Furthermore, cross-reactivity was also evaluated as follows: venom of WA vipers was coated into the wells, followed by adding serum from rabbits immunized with LCA viper venom as primary antibody; the remaining steps were performed as above.

2.6.2. Ouchterlony double immunodiffusion

For analysis of the interaction of raised antibody levels in rabbits against venom, 1 g of agarose powder was dissolved in 100 ml of PBS and heated up to 100 °C until a homogenous solution was obtained. Then, the solution was poured into a plate and set aside until a solidified gel was formed. Wells were created in the gel by a puncher. Three plates were prepared in this manner for the following tests: In the first plate, 20 μg of WA venom was loaded into the central well. Increasing amounts (25, 50, 100, 150, and 200 μl) of antivenom from rabbits immunized with WA viper venom were added to peripheral holes. In the second plate, 20 μg of LCA viper venom was loaded into the central well, and increasing amounts (25, 50, 100, 150, and 200 μl) of antivenom from rabbits immunized with LCA viper venom were added to peripheral holes. In the third plate, the cross-reactivity and quantitative Ouchterlony tests were performed to confirm the binding of WA viper venom to the antivenom obtained from rabbits immunized with LCA venom. To this end, 300 μl of antivenom from rabbits immunized with captive viper venom was loaded into the central hole followed by addition of increasing amounts of WA venom from 5 to 40 μg in peripheral wells. PBS was loaded into each well in the plates. The plates were kept in a humidified box at room temperature for 24 h.

2.6.3. Neutralization studies

For each neutralization experiment, a fixed concentration of crude venom as the challenge dose was separately mixed with variable doses of immunized rabbit antivenom. The mixtures were then adjusted to a constant volume with saline solution and pre-incubated at 37 °C for 30 min. Then, a certain volume of mixture was injected ID or IP into groups of 4 animals according to the method described above (Part 2.4. Biological activity of crude venom). In all the experiments, the control group was given a mixture of saline solution with the venom "challenge dose" alone.

A fixed amount of venom (5 LD_{50}) was prepared as the challenge dose for the lethality neutralization test in order to obtain the median effective dose (ED50) of antivenom. ED50 is expressed as μl antivenom/mouse or antivenom per challenge dose of venom that is required to protect 50% of mice injected with the venom "challenge dose" [34]. The deaths and survival rates were recorded 48 h after injection, and ED50 was calculated using the Spearman and Karber's formula. In neutralizing the edema-forming activity, antivenom ED50 was defined as the volume of antivenom which reduces the edema induced by the venom challenge dose by 50% [31]. Six MED of venom was used as the challenge dose. The effective dose of antivenom to neutralize the hemorrhagic activity was expressed as the venom/antivenom ratio or the volume of antivenom that reduced the size of the hemorrhagic spots (induced by the challenge dose) by 50% [34]. In neutralization of the necrotic activity, ED of antivenom was defined as the volume of antivenom which reduced the size of the necrotic lesion by 50% after injection of the challenge dose [35]. One MND was used as the challenge dose in the neutralization test of antivenom.

We evaluated the capacity of WA viper's antivenom and LCA viper's antivenom in neutralization of toxic activities induced by their venom. In doing so, we assessed the capacity of WA viper's antivenom in neutralization of WA venom, the capacity of LCA viper's antivenom in neutralization of LCA viper' venom, and also the capacity of LCA viper's antivenom in neutralization of WA viper's venom (cross-neutralization).

2.7. Statistical analysis

Statistical analyses were done using the STATISTICA software package (version 25, Stat- Soft Inc. Tulsa, OK, USA) and GraphPad Prism V.8.0 (GraphPad Software Inc, San Diego, CA, USA). Two-way ANOVA and Tukey's multiple comparisons post-hoc tests were used. Each experiment was repeated three times and the data were reported as means ± SD (N = 3) with 95% confidence interval (CI). A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Comparison of protein concentrations in venom

Protein concentration of crude venoms of WA, LCA, and CN vipers under the same conditions were 38.5, 36.5 and 42 μg/μl, respectively, as determined by the Bradford assay.

3.2. SDS- PAGE and 2-DE

SDS-PAGE analysis of venoms under similar conditions showed identical protein bands in the molecular weight (MW) range between 14.4 kDa and 116 kDa in all venom samples (Figure 1). Notable differences in protein bands were not detected in reduced/non-reduced conditions among the three groups of venoms. In reduced SDS-PAGE, LCA venom showed two visible bands below 14.4 kDa. Venom from the two groups in captivity (LCA and CN) showed one visible band below 25 kDa. One sharper band was observed near 116 kDa in WA venom on acrylamide gel.

2-DE gels of venom samples from WA, LCA and CN *G. caucasicus* are shown in Figure 2. All three venom samples showed similar molecular weights and pI values of isolated proteins. All gel images revealed greater number of spots at the pI range of 5–8, and in the molecular mass zones of approximately 40–80 kDa, 25kDa, and above 116 kDa. In addition, in all samples, spots were detected with molecular weights ranging from 8.5 to 25 kDa, and pI values of 4.5 or less. We detected approximately 300 protein spots on the 2-DE gel images for the three groups (Figure 2). Although, CN venom showed sharper protein spots in some areas, whereas WA and LCA venoms had sharper spots elsewhere. Spots in 18.5–25 kDa, and pI 4.5 and less were observed in all samples.
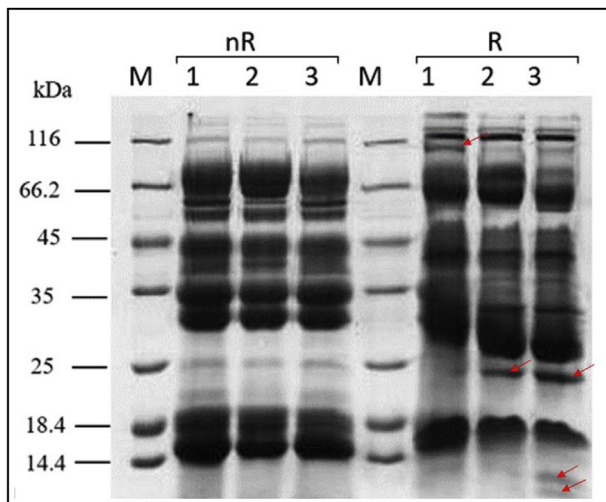
Figure 1. Comparison of SDS-PAGE profiles of WA and LCA *G. caucasicus* venoms. Electrophoretic separation of venom was performed under non-reducing (nR) and reducing (R) conditions using 12.5% acrylamide gels, and stained with Coomassie G-250. In each well, 30 μg of venom was applied. Lanes 1, 2 and 3 correspond to WA, LCA, and 3 CN venom, respectively. Line M: protein molecular marker (14.4–116 kDa). Arrows show the two visible bands below 14.4 kDa in lane 3/R, one visible band below 25 kDa in lines 2/R and 3/R, and one sharper band near 116 kDa in line 1/R. Arrows show sharper bands compared with other venom specimens. WA, wild adult; LCA, long-term captive adult; and CN, captive-born neonate (please refer to Fig. S1 for higher quality).

3.3. HPLC

For all groups, RP HPLC separation of venom proteins on C18 displayed similar profiles. Thirteen proteins and peptide fractions were separated by HPLC for each group. Figure 3 shows the chromatographic profiles of each venom.

3.4. Biological activities of crude venom samples

3.4.1. LD50

LD_{50} of crude venom of WA vipers was estimated to be 16.8 μg/mouse, which is equivalent to 0.84 μg/g body weight of each mouse after IP injection. LD50 for LCA vipers was 18.5 μg/mouse, which is equivalent to 0.92 μg/g body weight. Also, LD50 for CN vipers was determined to be 22 μg/mouse, equal to 0.77 μg/g body weight. This result indicates that mortality of mice after injection with venoms of WA, LCA and CN vipers was respectively 58, 56 and 45%, respectively.

3.4.2. Toxic activities of crude venom samples

The PT and aPTT tests were run at different concentrations of crude venom samples. In all venom samples, the clotting time of plasma in both intrinsic and extrinsic pathways decreased as venom concentrations increased from 2 to 60 μg. The WA and LCA venoms in concentration of more than 20 μg were reduced clotting times in PT and PTT tests (Table 1). The minimum amount of venom causing coagulation in the intrinsic pathway of blood coagulation (aPTT test) compared to the normal range (33.1 ± 1.6 s) was about 7.82 ± 1.18 μg for WA, 10.19 ± 1.38 μg for LCA, and 22.60 ± 2.12 μg for CN. The minimum amount of venom causing coagulation in the external pathway of blood coagulation (PT test) compared to the normal range (12 ± 1.1 s) was about 2 μg for WA, 3.5 μg for LCA, and 5 μg for CN. Crude venom of CN vipers significantly delayed coagulation time at concentrations of 2–20 μg in the PT test and 2 to 60 in the aPTT test (Table 1).

As shown in Table 2, 24 h after ID injection, the size of the hemorrhagic spots and necrotic lesions enlarged in all venom samples with the increase in venom concentrations (Figure 4A, B, and C). Minimum
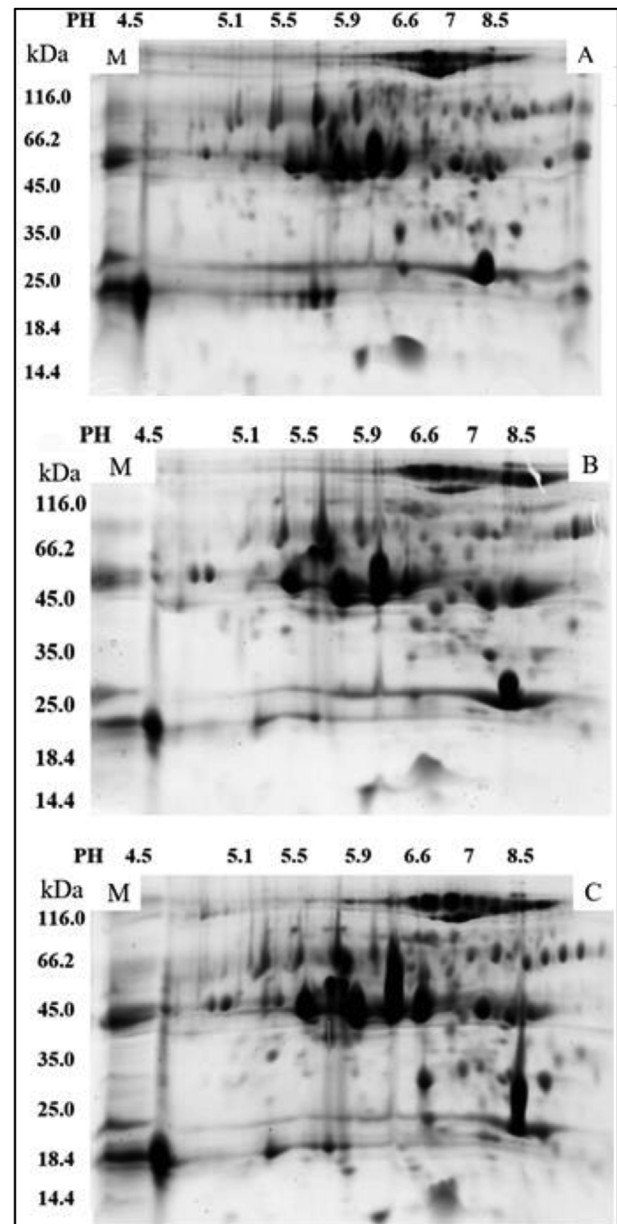

Figure 2. 2-DE profiles of WA, LCA and CN *G. caucasicus* venoms. A: WA, B: LCA and C: CN. Line M, protein molecular weight marker (14.4–116 kDa). Each venom sample equivalent to 300 μg was resolved in the first (IEF, isoelectric focusing) dimension in 7-cm linear IPG strips (PH 3–10), and in the second dimension in 21-cm 12.5% SDS-PAGE gel. 2-DE profiles were similar for the three groups. WA: wild adult; LCA: long-term captive adult; and CN: captive-born neonate (please refer to Figs. S2A, S2B, and S2C for higher quality).

Hemorrhagic Dose (MHD) resulting in a 10-mm spot was 3.93 ± 0.82 μg for WA, 3.36 ± 0.8 μg for LCA, and 6.44 ± 0.97 μg for CN. Minimum Necrotizing Dose (MND) resulting in a 5-mm lesion was 1.01 ± 0.15 μg for WA, 0.88 ± 0.15 μg for LCA, and 1.71 ± 0.53 μg for CN (Table 3). There was no statistically significant difference in the hemorrhagic spots and necrotic activities between the two groups of adult vipers (WA and LCA). CN had lower hemorrhagic and necrotic activities compared with LCA at venom concentrations of 4–64 μg ($p \leq 0.05$).

For this purpose, immunized rabbit sera with venom of WA and LCA Overall, the statistical analyses revealed a significant difference (P < 0.0001) between venom of LCA and CN vipers in terms of toxic activities (hemorrhagic, necrotic, edema, and coagulation). Furthermore, the analyses only showed a significant difference (P < 0.0001) in edema activity between venom of WA and LCA vipers.
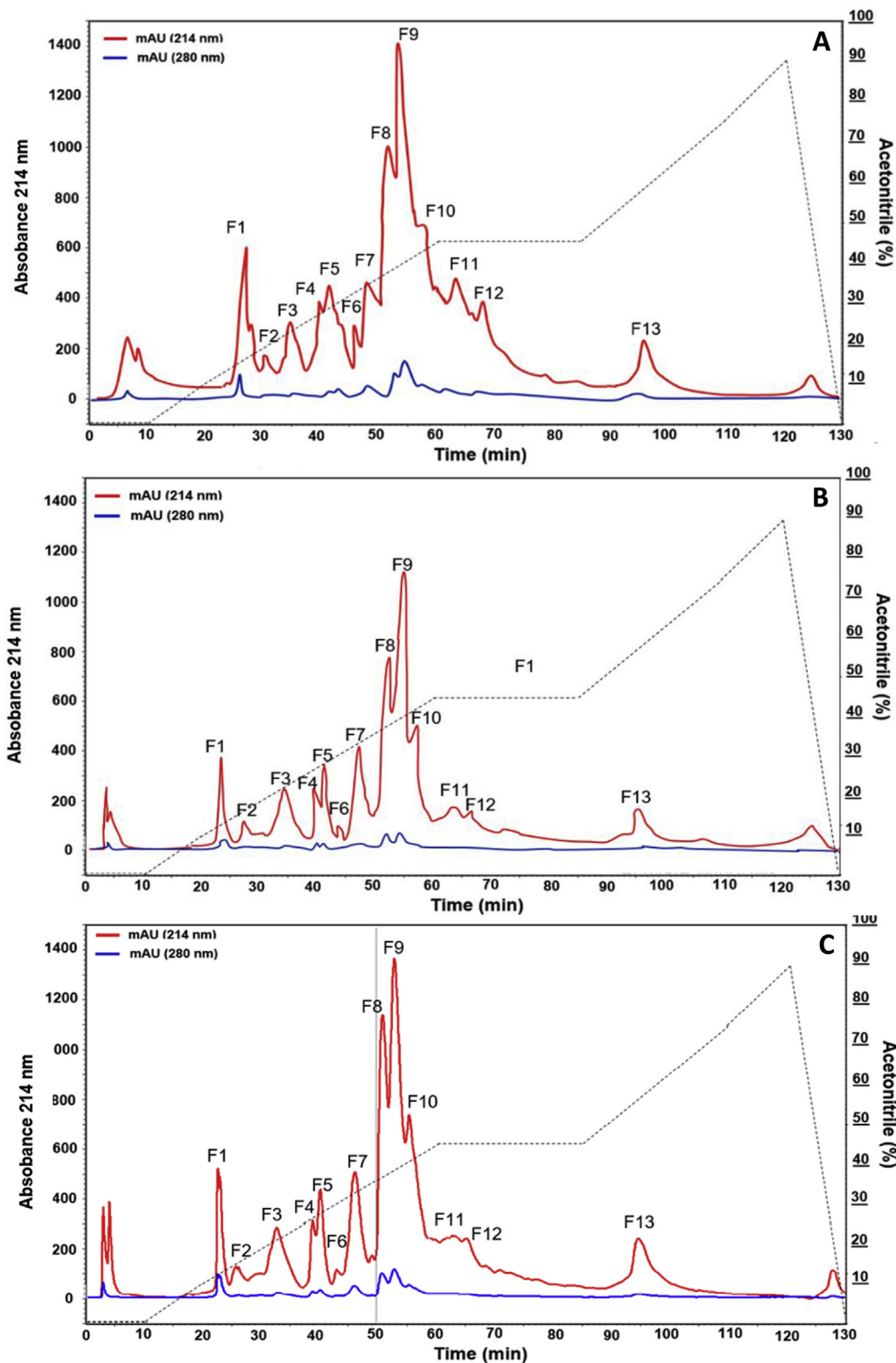
Figure 3. HPLC profiles of WA and LCA *G. caucasicus* venoms. A: WA, wild adult; B: LCA, long-term captive adult, and C: CN, captive-born neonate.

3.5. Results of neutralization studies

3.5.1. ELISA

The antivenom obtained from rabbits immunized with venom from LCA vipers effectively interacted with venom. The antibody titration profile revealed that antibody levels began to rise right after the first injection of venom at 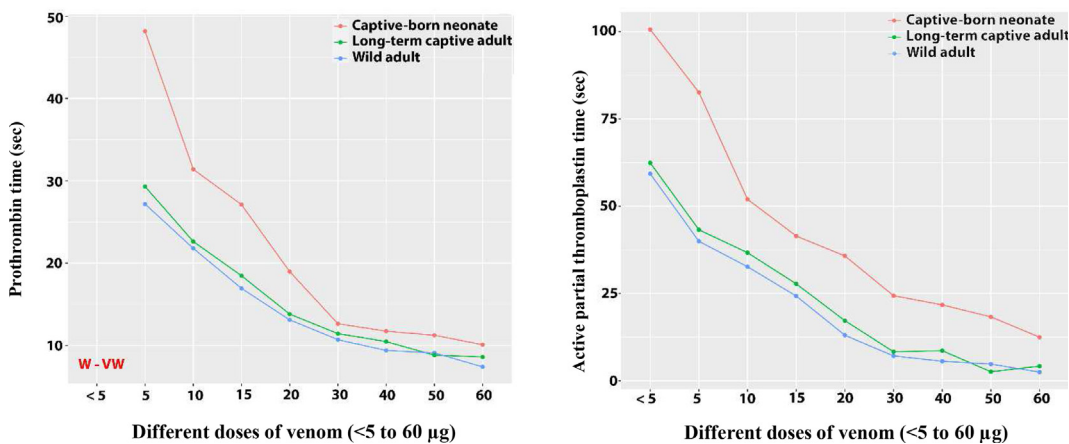1:500 serum dilution and remained high at 1:64000. At 1:64000 serum dilution, the antivenom reacted with venom at optical density (OD) equal to 1.086. The amount of antivenom serum reached its peak at this stage and from here to the 6th injection, the antibody titer did not change. These titrations imply that the corresponding antibodies can successfully induce an active immunization in rabbits (Figure 5A). With regard to rabbits immunized with venom from WA vipers, a similar titration profile was obtained; however, at 1:64000

Table 1. Results of PT and aPTT assay on crude venom obtained from WA, LCA, and CN *G. caucasicus* based on varying doses of venom. Graphs below Table 1 compare clotting times among WA, LCA, and CN: Prothrombin time (left) and active partial thromboplastin time (right) in different doses of venom (<5–60 μg), W: weak clot, VW: very weak clot.

Venom samples used in the tests	Test (time)	Different doses of venom (<5–60 μg)								
		<5	5	10	15	20	30	40	50	60
WA		W	27.17 ± 2.67	21.80 ± 2.31	16.93 ± 3.63	13.1 ± 1.14	10.70 ± 1.90	9.40 ± 1.44	9.10 ± 2.33	7.40 ± 1.40
LCA	PT (sec)	W	29.30 ± 3.40	22.63 ± 3.60	18.47 ± 2.80	13.80 ± 3.30	11.43 ± 2.26	10.47 ± 1.40	8.83 ± 1.21	8.60 ± 1.60
CN		VW	48.17 ± 2.97	31.40 ± 2.51	27.10 ± 2.72	18.97 ± 2.37	12.63 ± 1.07	11.73 ± 3.86	11.23 ± 1.23	10.10 ± 1.10
WA		59.30 ± 5.72	39.97 ± 4.23	32.67 ± 3.38	24.27 ± 4.86	13.03 ± 3.02	7.13 ± 2.12	5.60 ± 2.21	4.80 ± 4.20	2.50 ± 2.05
LCA	aPTT (sec)	62.43 ± 5.76	43.27 ± 5.73	36.70 ± 4.75	27.77 ± 4.16	17.20 ± 4.54	8.33 ± 4.15	8.63 ± 2.72	2.60 ± 2.25	4.20 ± 2.74
CN		100.63 ± 4.31	82.63 ± 6.85	51.97 ± 5.25	41.47 ± 4.86	35.77 ± 6.21	24.37 ± 3.52	21.73 ± 3.93	18.30 ± 4.03	12.47 ± 3.95
		***P*-value PT and PTT between different venom samples used**								
p-value of PT (WA vs. LCA)		0.567	0.567	0.9163	0.7448	0.9456	0.9346	0.8668	0.9911	0.8346
p-value of PT (LCA vs. CN)		<0.0001	<0.0001	0.0002	0.0003	0.042	0.8346	0.8176	0.4887	0.7543
p-value of PTT (WA vs. LCA)		0.6139	0.5824	0.4477	0.5448	0.4246	0.9304	0.6329	0.7853	0.8654
p-value of PTT (LCA vs. CN)		<0.0001	<0.0001	<0.0001	0.0003	<0.0001	<0.0001	0.0006	<0.0001	0.0397

WA: wild adult, LCA: long-term captive adult, CN: captive-born neonate, PT: prothrombin time, aPTT: active partial thromboplastin time, W: weak clot, VW: very weak clot. P-value < 0.05 was considered statistically significant.
Human normal plasma without venom sample was used as control plasma in tests. Control plasma has a PT about 12 ± 1.1 s, and a PTT about 33.1 ± 1.6 s, which corresponded to the normal range. Results are presented as mean ± S.D.

3.5.2. Ouchterlony

As seen in Figure 6A, the thickness of the formed precipitin lines is dose dependent. The multiple lines indicate that against any concentration of the whole venom, corresponding antibodies are produced in rabbits. Figure 6B shows the same assay for venom from LCA viper that yielded similar results. The cross-reactivity test for antivenom obtained from rabbits immunized with LCA viper venom against WA viper venom showed successful precipitin line formation, with higher doses of WA viper venom forming thicker lines, indicating the capability of this antivenom for binding and neutralizing venom of WA viper (Figure 6C).

3.5.3. Neutralization of toxic activities

The neutralizing efficacy of antivenoms obtained from venoms of WA and LCA vipers was assessed against the toxic activities of WA and LCA vipers' venom (Table 3). Our neutralization data showed that the ED50 of

serum dilution, the corresponding antivenom reacted with venom at OD equal to 1.21 (Figure 5B). The antivenom from rabbits immunized with LCA venom was completely reactive to venom of WA vipers. In this case, the 1:64000 serum dilution reacted with venom at OD equal to 0.97, suggesting that this antivenom can efficiently neutralize the lethality of venom from WA vipers (Figure 5C).

WA and LCA vipers' antivenoms was 206.312 ± 2.04 μl/5LD50 and 215 ± 4.12 μl/5LD50 against WA and LCA venoms, respectively. In cross-neutralization test, ED50 of LCA antivenom against lethality of WA venom was 224.83 ± 1.32μl/5LD50. Accordingly, both WA and LCA antivenoms had neutralizing potency against the lethal activity of WA viper venom; however, the effective dose of LCA antivenom used was higher than WA antivenom.

There was no significant difference in the doses of WA and LCA antivenoms used to neutralize hemorrhagic (P = 0.657), necrotic (P = 0.1422) and edema-inducing (P = 0.2257) activities. According to the results (Table 3), the effective dose of LCA antivenom needed to neutralize hemorrhagic and necrotic activities was slightly higher than the effective dose of WA antivenom. As shown in Figure 4D, LCA antivenom reduced the size of necrotic lesions caused by injection of various concentrations of WA venom. The minimum volume of WA and LCA antivenoms that completely prevented clotting induced by WA venom was 180 ± 0.32 μg and 178 ± 6.32 μg per challenge dose of venom, respectively. For the neutralization test of coagulant activity, a dose of 60 μg of WA and LCA venoms was used as the challenge dose. Table 3 shows ED of antivenoms obtained from venoms of WA and LCA vipers for neutralization of their toxic activities.

Table 2. Hemorrhagic spots (mm), necrotic spots (mm), and edema activities (%) induced by different doses of venoms (0.5–64 μg per animal) from WA, LCA and CN *G. caucasicus*. Graphs below Table 2 compare these tests among WA, LCA, and CN; ND: not detected.

Venom samples	Tests	Different doses of venom (0.5–64 μg per animal)								
		0.5	1	2	4	8	16	32	64	
										MHD (95% CI)
WA		ND	0.93 ± 0.31	6.07 ± 0.93	9.80 ± 1.76	15.47 ± 1.99	27.43 ± 1.99	33.90 ± 2.44	39.50 ± 5.86	3.93 ± 0.82
LCA	Hemorrhagic spots (mm)	ND	1.50 ± 0.98	7.80 ± 1.04	12.50 ± 2.46	16.37 ± 1.65	29.63 ± 2.80	35.20 ± 5.45	42.63 ± 5.90	3.36 ± 0.8
CN		ND	1.00 ± 0.30	4.43 ± 0.40	7.57 ± 0.95	11.67 ± 0.90	17.47 ± 3.37	26.13 ± 1.92	31.33 ± 2.80	6.44 ± 0.97
										MND (95% CI)
WA		ND	4.47 ± 0.91	7.87 ± 0.55	17.00 ± 0.98	25.00 ± 3.34	35.60 ± 2.60	44.47 ± 3.37	50.80 ± 2.63	1.01 ± 0.15
LCA	Necrotic lesion (mm)	ND	4.87 ± 1.27	8.20 ± 1.01	19.30 ± 2.05	28.70 ± 3.47	38.53 ± 3.01	46.37 ± 1.90	54.03 ± 4.13	0.88 ± 0.15
CN		ND	1.93 ± 0.85	4.27 ± 0.71	13.20 ± 2.23	15.83 ± 2.28	24.23 ± 2.18	32.37 ± 6.25	37.73 ± 6.00	1.71 ± 0.53
										MED (95% CI)
WA		4.67 ± 0.58	7.60 ± 0.50	10.10 ± 0.46	16.53 ± 2.08	28.97 ± 1.81	40.83 ± 3.27	52.40 ± 3.46	62.07 ± 5.96	8.69 ± 0.95
LCA	Edema activity (%)	3.67 ± 0.58	7.17 ± 0.25	9.17 ± 0.12	12.43 ± 0.85	25.53 ± 2.38	35.47 ± 1.29	45.13 ± 3.01	49.57 ± 4.08	11.40 ± 1.24
CN		ND	3.93 ± 0.35	7.30 ± 0.10	11.37 ± 2.27	19.80 ± 2.78	28.33 ± 3.96	35.6 ± 4.61	40.97 ± 4.71	19.1 ± 2.04
P-value of toxic activities in different doses of venom between different venom samples used										
Hemorrhagic spots (mm) (WA vs. LCA)		>0.999	0.6890	0.6981	0.4242	0.9071	0.5640	0.8172	0.3164	
Hemorrhagic spots (mm) (LCA vs. CN)		>0.9999	0.7089	0.2678	0.0651	0.189	<0.0001	0.0003	<0.0001	
Necrotic lesion (mm) (WA vs. LCA)		>0.9999	0.9823	0.9877	0.5594	0.2297	0.39191	0.6715	0.3222	
Necrotic lesion (mm) (LCA vs. CN)		>0.9999	0.3919	0.2478	0.0229	<0.0001	<0.0001	<0.0001	<0.0001	
Edema (WA vs. LCA)		0.8924	0.9788	0.9056	0.1598	0.2718	0.0472	0.005	<0.0001	
Edema (LCA vs. CA)		0.4512	0.3137	0.6745	0.8786	0.0318	0.006	0.0002	0.0008	

WA: wild adult, LCA: long-term captive adult, CN: captive-born neonate, ND: not detected, MHD: minimum hemorrhagic dose which is the lowest dose of venom that causes a hemorrhagic reaction with approximately 10 mm, MED: minimum edematogenic dose which is the smallest amount of venom that causes a 30% increase in footpad volume, MND: minimum necrotizing dose which is the minimum amount of venom resulting in a 5 mm necrotic lesion. Results are presented as mean ± S.D. P-value < 0.05 was considered statistically significant. As negative control, phosphate-buffered saline (PBS) buffer (8 g of NaCl, 0.2 g of KCl, Na2HPO, and 0.24 g of KH2PO4, distilled water was added to a total volume of 1 L) was ID injected. Mice for edema test and rabbits for necrotic and hemorrhagic tests were used.
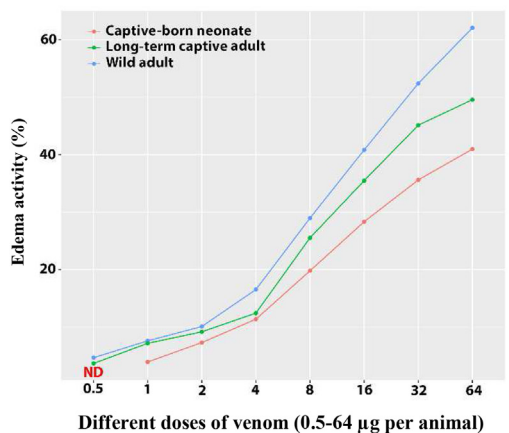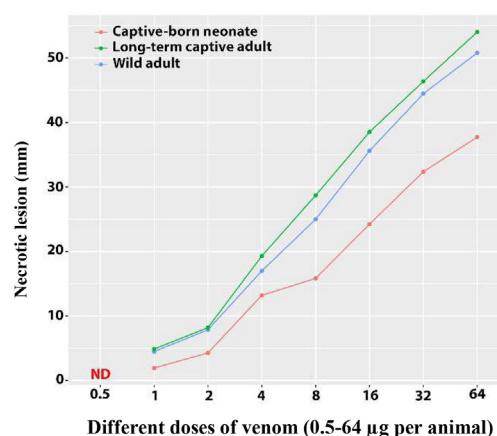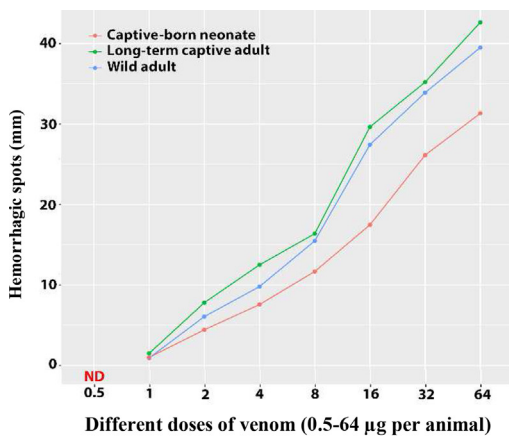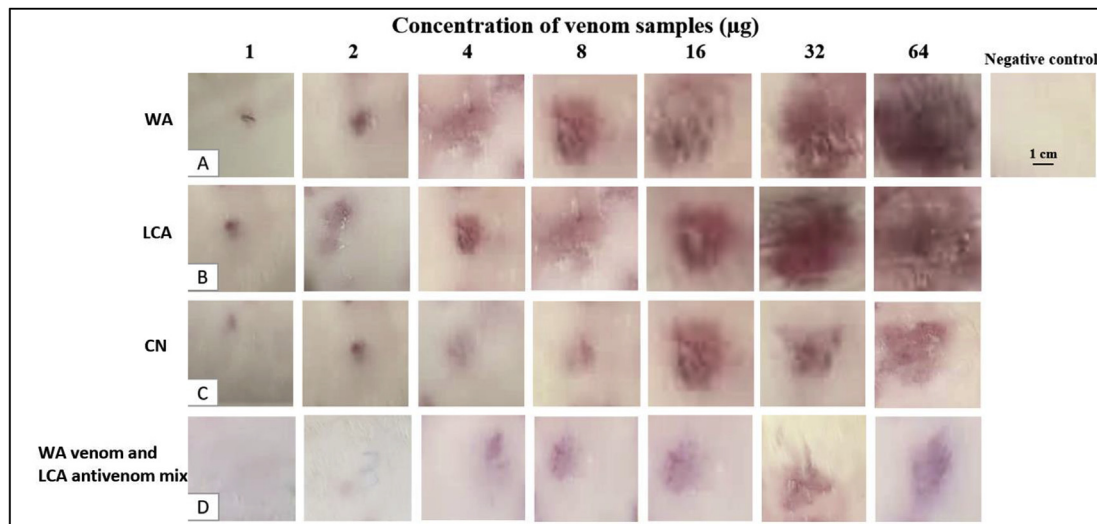
Figure 4. Necrotic activity of *G. caucasicus* venom and its neutralization by LCA antivenom. Necrotic lesions on the external aspect of rabbit skin 24 h after intradermal injection of varying doses of venom. The diameter of the necrotic lesions enlarged with increase in venom dosage. Rows A, B, and C show the necrotic activity of venoms of WA, LCA, and CN vipers; Row D represents neutralization of the necrotic activity of WA viper's venom (doses 1–64 μg) using effective dose 50% of antivenom of LCA vipers. Negative control: PBS without venom sample. WA: wild adult, LCA: long-term captive adult, and CN: captive-born neonate.

Table 3. Neutralization of toxic activities of WA and LCA *G. caucasicus* venoms by antivenoms obtained from rabbits immunized with WA and LCA venoms.

Venom of snake	Challenge dose (CD) of venom (μg/animal)	Effective dose (ED)* of antivenom (μL antivenom/CD venom)	
		WA antivenom	LCA antivenom
	Lethality (in mice)		
WA	5 LD50	206.312 ± 2.04	224.83 ± 1.32
LCA		ND	215 ± 4.12
	Hemorrhage (in rabbits)		
WA	5 MHD	38.25 ± 3.48	41.86 ± 4.58
LCA		ND	48.91 ± 4.56
	Necrosis (in rabbits)		
WA	1 MND	3.79 ± 0.49	4.18 ± 0.58
LCA		ND	4.78 ± 0.4
	Edema (in mice)		
WA	6 MED	89.88 ± 4.2	86.2 ± 3.2
LCA		ND	77.49 ± 3.82
	Coagulation		
WA	Dose causing larger clots*	181 ± 2.53	178 ± 6.32
LCA		ND	145 ± 8.05

WA: wild adult, LCA: long-term captive adult, CN: captive-born neonate, CD: challenge doses, MHD: Minimum Hemorrhagic Dose, MND: Minimum Necrotizing Dose, MED: Minimum Edematogenic Dose, ND: Not detected; the effective dose of antivenom obtained from wild vipers was not determined against captive vipers' venom, 5 MHD, edema activity, 6 MED; necrotic activity, 1 MND. WA and LCA antivenoms were produced from venoms of WA and LCA vipers used in the present study. Results are presented as mean ± S.D.

  * A dose of venom causing complete and larger clotting, known as the challenge dose, was 60 μg for WA and LCA vipers (in the PT and aPTT tests). Also, the minimum volume of antivenom which completely prevented clotting is expressed as the effective dose.

4. Discussion

    Snakes play a significant role in the environment as natural predators of pests, keeping their populations under control. Also, snakes are used in medicine for production of antisera. *G. caucasicus* is a unique species as it possesses both environmental and medicinal importance and its overharvesting has led to a substantial population decline of the species in Iran. Venom of *G. caucasicus* has specific pathophysiological functions and medical effects [36]. Comparison of the toxic activities of venom from *G. caucasicus* specimens kept in captivity (captive snakes) and those collected from the natural environment (wild snakes) is essential in order to produce effective antivenoms for treatment of snakebites in humans and preserve natural populations of the species [24].

    In this study, we compared the protein profiles of venom of WA, LCA and CN *G. caucasicus* vipers in Iran. Based on the results of SDS-PAGE, 2-DE, and fractionation by RP HPLC, the proteins of crude venom of the three groups showed almost similar profiles. This finding is in concordance with Galizio *et al.*'s study [37], which found no significant difference in protein composition between pooled venoms of recently wild-caught and long-term captive *Bothrops jararaca* (*B. jararaca*) using 1-DE and shotgun proteomic techniques. In our study, the SDS-PAGE analysis of venom from adult (WA and LCA) and neonatal (CN) vipers revealed two sharper bands in CN vipers compared to adults. In addition, the analysis showed one sharper band (near 116 kDa) for WA and one visible band (25 kDa) for LCA vipers. A full pattern of venom decomplexation was obtained for each sample using 2-DE, as illustrated by the
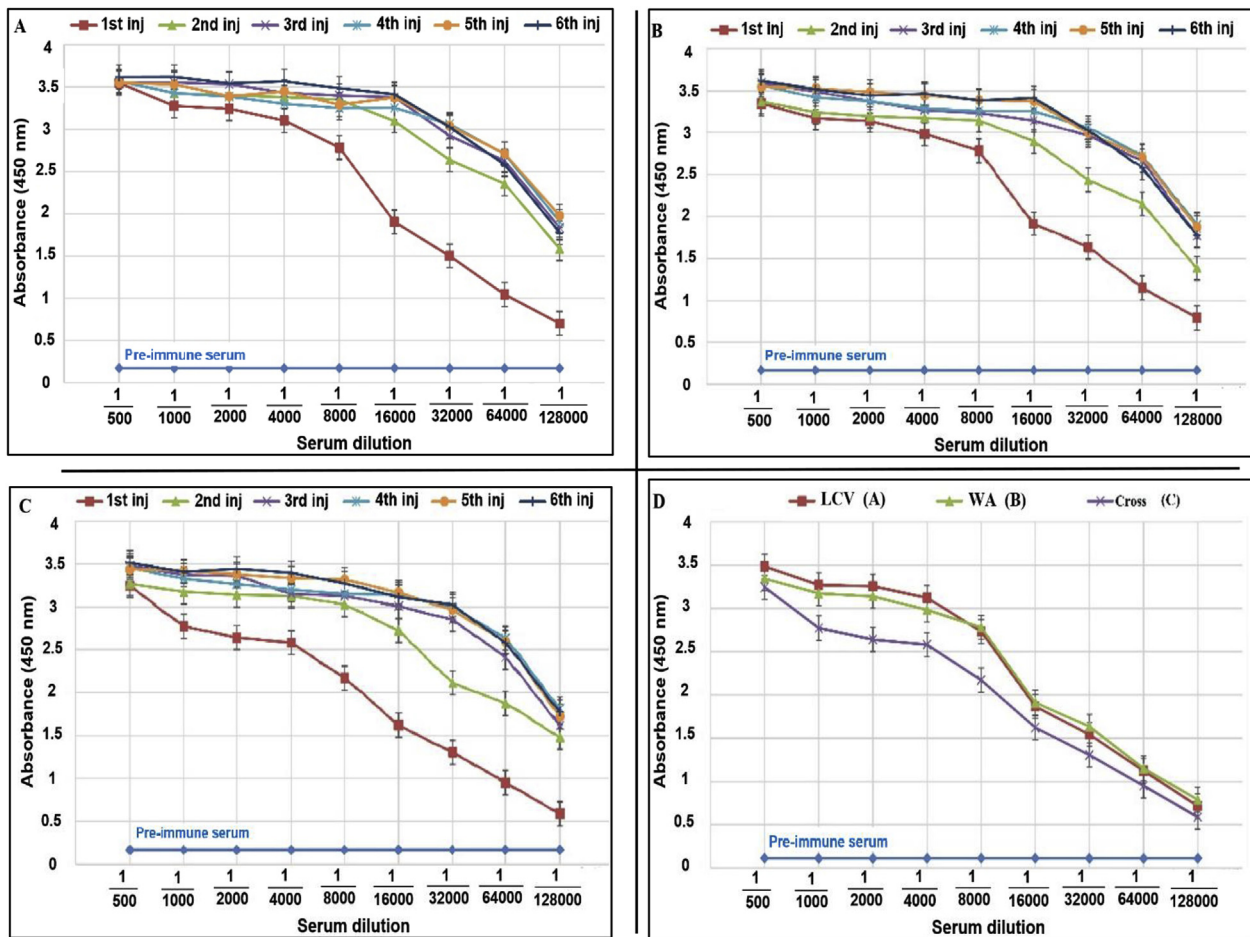
Figure 5. Results of ELISA tests. (A) Antivenom from rabbits immunized with LCA venom against the same venom; (B) Antivenom from rabbits immunized with WA venom against the same venom; (C) Antivenom from rabbits immunized with LCA venom against WA venom; (D) Compression of 3rd injection of (A), (B), and (C). WA, wild adult; LCA, long-term captive adult *G. caucasicus*. The titration profile represents the triplicate assay ±SD.
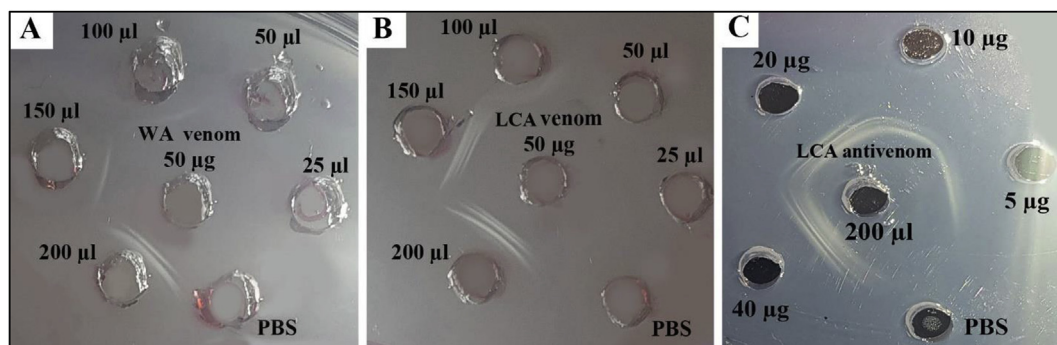

Figure 6. Antigen-antibody interactions of antivenoms of WA and LCA *G. caucasicus* with their venoms using the Ouchterlony double immunodiffusion method. (A) WA venom against antivenom from rabbits immunized with WA venom. Increasing amounts of antivenom from rabbits immunized with *G. caucasicus* venom were loaded into the peripheral wells; (B) The same assay was performed for LCA venom and similar results were obtained. Increasing amounts of antivenom from rabbits immunized with LCA venom were loaded into the peripheral wells; (C) Cross-reactivity Ouchterlony test. Antivenom from LCA venom-immunized rabbits was loaded into the central well. Increasing amounts of WA venom from 5 to 40 µg were placed into the peripheral wells. Sedimentary lines with increasing intensity indicate the capability of this antivenom at binding to WA venom. PBS was used as negative control. WA: wild adult, LCA: long-term captive adult (please refer to Figs. S6A, S6B, and S6C for higher quality).

protein spots (Figure 2). 2-DE demonstrated higher quantity of some proteins (sharper spots) in LCA vipers, which could be due to living and breeding conditions as well as regular feeding. Gao *et al.* [38] found an age-related variation in the proteomic profile of *Gloydius brevicaudus* venom using 1-DE and 2-DE, and reported that the abundance of compounds such as phospholipase A2 (PLA2) was higher in venom of adults than in neonates [36, 38]. Furthermore, in a study using proteomic analysis on venoms of wild and captive Eastern brown snakes (*Pseudonaja textilis*), McCleary *et al.* [39] found that venom variation may be a result of geographic distribution and has no correlation with time in captivity. In our study, HPLC allowed for fractionation of sufficient amounts of venom fractions. We recommend future studies for identification of the

collected fractions obtained from HPLC and protein spots obtained from 2-DE.

Our study on mice showed that the intraperitoneal-LD$_{50}$ of venom of WA and LCA vipers was nearly alike (16.8 vs. 17.7 µg/mouse). Our results are in congruence with Joseph's [40], which demonstrated that clotting time was shorter at higher venom concentrations (p < 0.05) and the clots formed were larger. Although Joseph et al. [40] showed that the intrinsic pathway is missing or weak in venomous snakes, we found that the intrinsic coagulation pathway (PTT) appears to be stronger in WA than in LCA vipers. Freitas-de-Sousa at el.'s study [24] on venoms from wild and captive *Bothrops atrox* snakes (as a venomous pit viper) showed higher coagulant activity caused by venom from captive snakes. We agree that further studies are needed to evaluate the coagulation factors involved in the coagulation cascade pathways as these components are highly useful for pharmaceutical applications. Our findings revealed that WA and LCA vipers produced venom with almost similar toxic functions. Moreover, the necrotic and hemorrhagic activities were almost similar in both groups at different doses. The size of hemorrhagic and necrotic spots was greater in captive vipers, but not significantly different. Therefore, it appears that there is a direct relationship between hemorrhagic and necrotic effects. Lee et al. [41] estimated the MHD of venom for samples of *Agkistrodon halys* from Japan and China to be 1.78 µg and 0.42 µg, respectively. Hemorrhage induced by venom may be due to a variety of hemorrhagic proteins such as snake venom metalloproteinases (SVMPs), which are abundant enzymes in venom of viperine snakes [42, 43]. One of the most obvious clinical signs following snakebite by *Agkistrodon* is edema and inflammation [44]. According to our findings, the edema-inducing activity of WA venom was higher than LCA, which was significant at concentrations of 16 µg and above (p < 0.05). MED-induced inflammation was observed 5 h after mouse paw injection with venom of WA and LCA vipers, representing an acute inflammatory response as described by Román-Domínguez et al. [45]. According to Galizio et al.' study [37], variability in the toxic activity (edematogenic) of venom from a wild venomous pit viper (*B. jararaca*) is more likely to be caused by genetic/populational distinctions, and not due to "captivity vs. wild" conditions.

Our results showed that hemorrhagic spots, necrotic lesions and edema caused by LCA venom were greater compared to venom of CN vipers, which is consistent with Saad et al.'s [46, 47]. Nevertheless, venom from captive-born neonates showed significantly (p < 0.05; Table 2) lower and weaker coagulation activity. This finding is in opposition to those of Kamiguti et al. [48] and Furtado et al. [46], who found stronger coagulant activity for venom of juvenile pit vipers (*B. jararaca*). This may be due to differences in species and variation in venom compositions [25]. Overall, changes (increase or decrease (in the intensity of toxic activities of venom from "captive vs. wild" and "adult vs. neonate" snakes may be related to the type of prey consumed and habitat shifts for adaptive venom evolution [49, 50, 51, 52]. A study conducted by Gibbs et al. [53], illustrated that adult snakes are likely influenced by different diets, e.g. changes in the abundance of PLA2.

On the other hand, we argue that differences in snakes' toxic effects are likely age-related and depend on dosage of venom injection. According to previous studies, the toxicity of venom is affected by age and size of the snakes, nutrition, stress, temperature, breeding conditions, climatic and geographic distributions [54, 55]. In addition, differences in toxicity of venom may be due to a variation of enzymes or an age-related change in venom compounds [54, 55].

Also, the antibody titer plateaued between the 2nd and 6th injections. A similar titration profile was obtained at 1:64000 serum dilution for both WA and LCA under similar concentrations and time intervals as shown in 3.5.1 section. These outcomes confirm that all rabbits were completely protected against the lethal effects of the venoms. On this basis, it could be supposed that injection of the venom/antivenom mixture by intradermal routs resulted in no necrotic lesions in rabbits' skin. These findings are greatly in accordance with the neutralizing effects of the venom/antivenom mixture against the hemorrhagic and procoagulant effects of venom when the mixture is injected

subcutaneously into rodents [56, 57, 58]. Theakston and colleagues [59] showed high titer of rabbit antisera, 6 months after booster dose of *Echis carinatus* venom. Thus, in the present study, the antivenom produced in rabbits immunized with LCA venom could effectively produce immunity against the toxic effects of WA venom. ELISA allows for rapid and differentiated diagnosis of snakebite patients with similar clinical features in the clinic. These findings imply effective and successful immunization against venom of LCA and WA vipers.

We investigated the neutralization ability of the antivenom obtained from venom of WA and LCA Caucasian pit vipers. Higher doses of LCA antivenom were needed for neutralization of WA venom's lethality (5 LD50) in this study. Lee at el [41]. reported that *Agkistrodon halys* antivenom had anti-lethal activity against 4 LD50 of *A. halys* venom. In their study, LD50 of *A. halys* venom was 44.89 µg for a 16 g mouse, when intra-peritoneally injected.

As shown in Table 2, the effective dose of LCA antivenom was a little higher than WA antivenom for neutralizing the hemorrhagic and necrotic activities of the "challenge dose" of LCA venom. However, compared to WA antivenom, a slightly higher dose of LCA antivenom was needed to neutralize coagulation and edema induced by the "challenge dose" of WA venom. According to the effective doses in Table 2, approximately 0.5–2.5 µl of WA antivenom and 0.4–2.8 µl of LCA antivenom are required to neutralize the hemorrhage, edema, and lethality caused by 1 µg of venom. It is noteworthy that for neutralization of the necrotic effect caused by 1 µg of WA venom, about 4 µl of WA and LCA antivenom was needed. Our results demonstrate that the venom obtained from LCA vipers could be an appropriate source for production of sufficient amounts of antivenom in Iran. The present research in accordance with the previous studies shown determination of effective dose of antivenom needed for each toxic activities [19, 60]. According to other studies, the efficiency of envenomation immunotherapy is crucial to the purity of the antivenom and its neutralizing potency in treatment of snakebite patients [19, 60]. Ainsworth et al. [61] suggested that an antivenom targeting snake venom toxins is capable of treating basic snakebite pathologies. For example, determining the effective dose of antivenom to neutralize coagulopathy can be very effective in treating blood clots (as the most important clinical syndrome) in the future [62]. Freitas-de-Sousa at el [24]. reported that only the less abundant components of venom from wild and long-term captive *Bothrops atrox* showed variability, suggesting that antivenoms obtained from captive snakes could be effectively used for production of antivenom. The most noteworthy achievement of our research was to argue that there is no longer a need to hunt wild vipers from the wild to produce antivenom, which in turn can help preserve wild snake populations and ensure their long-term survival.

Nevertheless, there were a number of limitations in our study which should be addressed in future research, such as evolution of venom in neonatal wild vipers. Moreover, identification of the different toxic proteins, particularly major toxic components of venom and their potential variation extracted from venom of WA and LCA vipers will be of considerable use in various fields of medical and pharmaceutical industries. By performing other experiments and with better quality control, the efficacy of the produced antivenom can be improved in the future. Our findings could be beneficial to pharmaceutical applications and production of LCA antivenom with higher efficacy [25]. Even though the present study focused on the toxic activities of venom from wild adult, long-term captive adult, and captive-born neonate *G. caucasicus* vipers, the restricted collection location of our samples (exclusively from populations of the Lar Valley, Lar National Park, central Alborz Mountains) and failure to collect wild neonates from this population could negatively affect the results, which mandates a cautious interpretation of our findings.

5. Conclusion

Our study revealed that the biological and toxic effects of wild and captive *G. caucasicus* venoms were nearly similar. The minor differences

in toxic activities of venom of captive and wild specimens may be related to their living conditions and age. In this regard, it is relevant to argue that the antivenom obtained from captive vipers' venom has the capacity to efficiently neutralize the toxic effects of wild vipers' venom, and thus shows a great potential for treatment of snakebites in humans. Therefore, there will be no need to capture wild vipers from their wild populations for venom milking, which could subsequently heighten their extinction risk. Further comprehensive studies and better understanding of the biological and toxic characteristics of venoms and antivenoms are essential in clinical monitoring of snakebites in patients and for effective therapy.

Declarations

Author contribution statement

M. Kaboli and D. Shahbazzadeh: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

F. Rasoulinasab and M. Rasoulinasab: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A. Asadi: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e05717.

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