International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 15, Issue 11, 2023

Original Article

PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST INDIAN CATTLE TICK *RHIPICEPHALUS MICROPLUS* SALIVA TOXINS AND ITS EFFICACY IN REVERSAL OF TOXIC EFFECTS IN ALBINO MICE

NIDHI YADAV, RAVI KANT UPADHYAY*

Department of Zoology, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur U. P. India
***Corresponding author: Ravi Kant Upadhyay; *Email: rkupadhya@yahoo.com**

Received: 23 Jul 2023, Revised and Accepted: 25 Sep 2023

ABSTRACT

Objective: This study focuses on the generation of polyclonal antibodies against tick saliva toxins and its use to reverse the toxic effects in albino mice.

Methods: Polyclonal antibodies were generated by immunizing albino mice were immunized with saliva toxins mixed with incomplete Freund's adjuvant. Experimental mice were treated with antiserum (polyclonal antibodies) and pre-incubated with tick saliva toxins in five different groups for observation of reversal of toxic effects, i.e. levels of bio-molecules and enzymes. For detection of polyclonal antibodies in the antiserum immune double diffusion (IDD) test of Ouchterlony was followed.

Results: By employing a step-by-step octanoic acid and ammonium sulphate precipitation process, IgG antibodies were separated from antiserum. A crescent band and precipitation band was obtained due to the interaction of antigen and antibodies in wet agarose gels (1%). When these antibodies were injected in albino mice, these have been successfully reversed the levels of acid phosphatase (ACP), alkaline phosphatase (ALP), glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), lactic dehydrogenase (LDH) and acetylcholinesterase (AchE). Alkaline phosphate levels in the serum of albino mice injected with polyclonal antibodies were found to be 122.64%, 107.849%, and 104.71%, respectively. Glutamate pyruvate transaminase (GPT) has been reversed in mice treated with polyclonal antibodies up to 94.59%, 86.48% and 78.37% in the serum, while it was found to be 116.21% at 40% of 24-h LD_{50} dose in comparison to control respectively.

Similarly, level of lactic dehydrogenase was restored and found i.e. 104.55%, 103.82%, and 102.20% in the serum of albino mice. Respectively, in comparison to control, while mice injected with 40% of 24-h LD_{50} of the purified saliva toxins demonstrated 117.20% of lactic dehydrogenase (LDH) level in comparison to control.

Conclusion: Polyclonal antibodies administered for serotherapy reversed the toxic effects and all biochemical parameters become normal after 6 h of treatment in albino mice in comparison to control.

Keywords: Ticks, Salivary proteins toxins, Disease pathogens, Morbidities, Antiserum, Immunotherapy

DOI: https://dx.doi.org/10.22159/ijpps.2023v15i11.48954. Journal homepage: https://innovareacademics.in/journals/index.php/ijpps.

INTRODUCTION

Ticks are obligatory ectoparasites who rely on blood meals to meet their metabolic requirements [1]. During feeding ticks discharge salivary secretion or a cocktail of various molecules in the host's bloodstream. Tick saliva contains anti-coagulatory molecules, specifically salivary proteins, which slow down blood clotting and help in uninterrupting blood feeding by rupturing host skin [2].

Tick saliva contains anti-coagulatory molecules, specifically salivary proteins, which slow down blood clotting and help in uninterrupting blood feeding by rupturing host skin [2]. Ticks are the main vectors of a number of pathogenic agents, i.e. Viruses, Rickettsia, spirochetes and bacteria, fungi, protozoa, and wired nematodes in humans, cattle, and wildlife. Ticks easily transmit various disease pathogens in the host's body during blood feeding [3, 4].

Numerous illnesses and morbidities are brought on by these viruses in people, cattle, and wild animals [5]. The primary causes of tick parasitism include ticks' hematophagous nature, host immunological condition, age, breed of cattle, and local environment [6]. Globally, the developers of parasitism and the quick reproduction cycles of ticks have an impact on the well-being and survival of domestic and wild animals [7]. Ticks make livestock anaemic and generate huge losses for dairy farmers.

During blood feeding, ticks produce iron-bound proteins, specifically ferritins [8, 9]. Lipocalines are abundant proteins in soft and hard tick saliva. *Ixodes ricinus* lipocalin (LIR) was also related to the modulation of inflammation [10]. Tick saliva proteins play a role in many physiological functions, including egg development, protein transportation, immunity and anti-microorganism, anti-coagulant, and adhesion. Thus, tick saliva not only controls host homeostasis and wound healing but also subverts the host immune response to avoid tick rejection that creates a favorable niche for the survival and propagation of diverse tick-borne pathogens.

The pharmacological and immunological activities of the tick's saliva are characterized by the presence of many proteins that exhibit strong pharmacological and immunological activities. These proteins are primarily utilized in host invasion for blood feeding and are known as evasions. Ticks may have the ability to feed for longer than 8-10 d without being detected by the host animal due to the presence of salivary proteins. These inhibit the secretion of the host chemokine and prevent painful inflammation. Therefore, after seeing the pathogenic effects and morbidities and lifelong blood-sucking habit, it was assessed that there must be the production of toxin-neutralizing antibodies for immunotherapy and fast recovery of patient/diseased animal. Immunotherapy is considered an appropriate method to treat patients with a disease that may be an antibody/antiserum [11, 12].

Clinicians highly recommend and seek for anti-venom medications because of their great mortality decrease. Only when venoms are extremely poisonous or provide a significant risk of toxicity is anti-venom serums advised [13]. Antiserum's immunoglobulins efficiently counteract the pathological consequences brought on by venomous bites and stings [13]. For the treatment of the venom of several venomous animals, species-specific anti-venoms are

necessary. These are being provided by injection and are being targeted [13]. The harmful effects of the tick saliva toxins on experimental albino mice were successfully neutralized in the current investigation using polyclonal antibodies produced against *Rhipicephalus microplus* tick saliva toxins. Albino mice showed that all biochemical parameters had returned to normal or were restored after 6 h of therapy.

MATERIALS AND METHODS

Animal culture

This study, involving the use of animals, was approved by the Departmental Ethics Committee (RC/FSC/ZOO/2019-20/26/22) Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur. Four pairs of male and female adults of albino mice were procured from the Banaras Scientific Centre, Varanasi. Animals were acclimated to the laboratory conditions for two weeks. These were reared in our animal house facility according to strict guidelines of CPCSEA and allowed to breed in pairs in separate cages. Young ones were kept with their mother in clean and natural environmental conditions Temp 24–25 °C; Photoperiod 12:12. h; Relative humidity 40–45%). Animals were housed in plastic cages laboratory mice bin cage (size 50x30x30 cm) (Spectrum Marketing, Mumbai, Maharashtra). Each cage had plastic floor and frame with steel net and opening window. Animals were fed twice a day on corn pellets and fresh chopped vegetables and food grains. Drinking water supply was given by using autoclavable water bottle (250 ml) made up of Polypropylene (PP) with silicone plug and tube of stainless steel. Bottle plug is made of silicone. The tube was made up of stainless steel.

Chemicals and reagents

Triton X-100, PBS buffer (pH 6.9), TCA, Tris-EDTA and, sodium azide, NaCl, ammonium sulphate were purchased from Merck and CDH. From High media, the purchase of Absolute ethanol, octanoic acid, Sepharose CL-6B-200, Freund's adjuvant, and Agarose was made.

Isolation and purification of tick saliva toxin

The live *Rhipicephalus microplus* was collected in the rural regions of Gorakhpur district. The collected ticks were immobilized by rapid freezing to-20 °C. The whole-body homogenate was prepared in a saline phosphate buffer (50 mm, pH 6.9) using a power homogenized. The homogenate was centrifuged at 10 000 rpm at 4 °C for 10 min and the supernatant was used as a raw salivary toxin.

Preparation of homogenate

Rhipicephalus microplus were homogenized properly in a glass-glass homogenizer in 5 ml of different solubilizing buffers such as Triton X-100, PBS buffer (pH 6.9), 10% TCA, Tris-EDTA and Absolute ethanol separately. After centrifuging at 12000 rpm in the cold for 30 min, the homogenate was separated into the supernatant by centrifugation. The total protein content was estimated in the various supernatants [14].

Purification of saliva protein of *Rhipicephalus microplus*

Proteins were extracted from a double cavity gel filtration column named Sepharose CL-6B-200 using a sintered disc filtered in the bottom with a height of 1 meter and 25 mm diameter. A known volume i.e., 5 ml of toxin proteins solubilized in PBS was loaded on the column. Flow rate was maintained between 1 ml/minute by a continuous supply of buffers through a gel filtration column in a cold room. Eluted fractions were collected at a fixed time interval using a Pharmacia fraction collector and the values of protein concentration in different eluted fractions were plotted on a graph. Absorbance in each fraction was determined at 280 nm using a Shimadzu spectrophotometer (UV 2001 PC). Same fractions were also analyzed for protein estimation by using Lowry's method [14]. The absorbance of the same fractions was determined at 640 nm for protein estimation.

Fraction collection

Elution of the saliva toxin proteins through the gel filtration column were done at a constant flow rate of 5 ml/minutes at a fixed time interval. Total 140 fractions were collected and each eluted fraction was analyzed for the detection of the presence of saliva protein at a wavelength of 280 nm. The protein content eluted in each fraction was determined by using the method of [14]. A graph was plotted between absorption at 620 nm and fraction numbers to show the elution pattern of *Rhipicephalus microplus* saliva protein/toxins.

Molecular weight determination of purified saliva proteins

Range of molecular weight of different proteins/toxins in the purified tick saliva toxins/proteins was determined by running the proteins of known molecular weight through Sepharose CL-6B gel column as done previously at the same flow rate. The range of molecular weight of various proteins in the isolated *Rhipicephalus microplus* saliva protein/toxins was identified using the calibration curve between Ve/Vo log M.

Lyophilization of eluted saliva proteins

In order to obtain the appropriate concentration of saliva toxins, the eluted fractions were combined and lyophilized.

Biological activity of the purified saliva protein/toxins

In order to investigate the biological activity of *Rhipicephalus microplus* saliva protein/toxins, albino mice were serially injected with predetermined volumes of the purified saliva toxins.

Determination of lethality of *Rhipicephalus microplus* saliva toxins

Known amounts of the pure saliva toxins were serially administered intra-peritoneally into albino mice to investigate the biological activity of *Rhipicephalus microplus* saliva protein/toxins. The LD_{50} was calculated every 24 h after the albino mice were subcutaneously injected with purified saliva toxins at various serial concentrations. Neurotoxic consequences and deformities, including paralysis were also noted. To calculate the LD_{50}, six albino mice were serially injected with the saliva toxins. The formula proposed by Abbot was used to calculate mortality. The LD_{50} values-the points at which half the test animals perished were calculated. The formula proposed by Abbott was used to calculate mortality. Calculated LD_{50} values were those at which half of the test animals perished. The doses-mortality regression line was drawn using the log Probit method's to establish the fatal concentration of 40% and 80% of the LD_{50} [15]. The confidence limits were calculated at 95% probability levels.

Dialysis of lyophilized saliva toxins

A considerable amount of 2% (w/v) sodium bicarbonate and 1 mmol EDTA (pH 8.0) was used to boil a cellulose membrane dialysis bag for 10 min before thoroughly rinsing it in distilled water. After that, the membrane was cooled and kept at 4 °C. Before use, the membrane underwent a second cleaning with distilled water both inside and out. To remove the excess salt from the lyophilized saliva toxins protein solution, the dialyzing bag was filled with the lyophilized saliva toxin protein and dialyzed against three changes of phosphate buffer (50 mmol, pH 6.9).

Determination of biomolecules

Determination of serum glucose

Changes in serum glucose level were measured according to Mendel *et al.* [16].

Determination of serum pyruic acid

Changes in levels of serum pyruvic acid were determined according to the method of Friedman and Haugen [17].

Determination of serum uric acid

Changes in serum uric acid level were determined by the cyanide-free method of Folin [18].

Determination of serum cholesterol

Changes in serum cholesterol level were measured according to the method of Abell *et al.* [19].

Determination of serum total lipid

Changes in the serum total lipid level were estimated according to method of Floch *et al.* [20].

Determination of serum total protein

Estimation of protein in the serum was carried according to the method of Lowry [14].

Determination of free amino acids

Changes in the level of free amino acids in blood serum of albino mice were determined according to the method of Spies [21].

In vivo

Purified *Rhipicephalus microplus* saliva protein/toxins were administered to the albino mice in sub-lethal dosages (40% and 80% of 24-h LD_{50}). After the injection, the mice were slaughtered 2, 4, 6, 8, and 10 h later, and blood was drawn to obtain the serum. Mice treated just with PBS buffer were slaughtered and used as a baseline for comparison. The previously outlined procedure was used to collect blood and isolate serum.

Determination of acid phosphatase

Changes in acid phosphatase level were determined according to the method of Andrech and Szeypiaske [22].

Determination of alkaline phosphatase

Changes in alkaline phosphatase level were determined according to the method of Bergmeyer [23].

Determination of serum glutamate pyruvate transaminase

Changes in serum glutamate pyruvate transaminase (GPT) level were measured according to the method of Reitman and Frankel [24].

Determination of serum glutamate oxaloacetate transaminase activity

Changes in glutamic-oxaloacetate transaminase (GOT) level were measured according to the method of Reitman and Frankel [24].

Determination of lactic dehydrogenase

Changes in serum lactic dehydrogenase activity were measured by the method of Annon [25].

Determination of acetylcholinesterase

Changes in serum acetylcholinesterase (AchE) level were measured according to the method of Ellman *et al.* [26].

Production of polylconal antibodies

Animal culture and immunization

Albino mice (*Mus musculus*) weighted 65±0.015 gm was used for immunization. These animals were reared in the laboratory according to standard laboratory methods (in 16 X 16 X 16 cages) with proper care feeding and provided treatment humanly for nursing. Purified *Rhipicephalus microplus* saliva toxins were combined with an equivalent amount of incomplete Freund's adjuvant to create the immunogen. A fine needle was adjusted to spread the water phase while the emulsion was repeatedly drawn into and discharged from a syringe for fine emulsification. Only tick saliva toxins were given as an immunogen for boosting.

For the animal's initial injection, 100 µl (281.25 µg) of saliva protein and 100 µl of Complete Freund's Adjuvant were thoroughly mixed. Immunogen was administered intra-peritoneal. The release of the immunogen emulsion was done slowly and cautiously. The injection site was squeezed after the needle was removed. Each mouse received a single injection.

Booster dose

Each experimental mouse received a booster dose (281.25 µg) of saliva toxins via the same method after 7 d following the original immunization. After 21 d from the original immunization, the mice received a second booster dose in a similar manner.

Collection of blood

Following the second booster's seven-day period, the animals were killed and let to bleed to obtain the serum. To acquire clear serum, the mice were bled before being fed. By puncturing the heart, blood was drawn. A beaker with a wire grid bottom and cotton that had been wet with chloroform was used for this purpose. A petri dish served as the lid for the beaker's top. Alcohol was used to disinfect the sedated animal's chest area. In order to get as close to the sternum as feasible, a fine sterilized needle connected to a 2.0 ml syringe was placed between the left third and fourth intercostal muscle spaces. The needle was inserted at an angle intended to puncture the right ventricle of the heart, moving in the direction of the right shoulder. The blood was sucked up when it appeared in the syringe.

Without using an anticoagulant, freshly obtained blood was collected in a clean glass tube. In the cold, the blood was permitted to coagulate. The serum was separated in a new tube after the clot was very carefully detached from the side walls. To obtain clear antiserum, it was centrifuged at 10,000 rpm for 20 min to remove any particle material.

Partial purification of antibodies and storage

Octanoic acid precipitation

Utilizing the octanoic acid precipitation method, anti-serum was partially purified. For this, two volumes of sodium acetate buffer (60 mmol, pH 4.0) and one volume of antiserum were added at room temperature. For every 10 ml of the original antiserum, 1 ml of n-octanoic acid was added. After 30 min of thorough mixing, the contents underwent a 20-minute centrifugation at 1000 rpm to separate the supernatant. In a process akin to the dialysis of purified saliva, the supernatant was dialyzed against the proper buffer.

Ammonium sulphate precipitation

Antiserum was also precipitated using 1.82 M ammonium sulphate solution in addition to octanoic acid. For this, the mixture was incubated for 30 min while being constantly stirred, centrifuged for the same amount of time at 3,000 rpm, and the precipitate was collected. With 1.82 M of 40% saturated ammonium sulphate, the precipitate was washed. The process was repeated after centrifuging the suspension once more. The precipitate was dialyzed against 150 mmol NaCl with 0.1% sodiumazide (w/v) after being dissolved in distilled water (about 2.0 ml distilled water per 10 ml antiserum). Purified antiserum was kept at 40 °C after dialysis. By employing a step-by-step octanoic acid and ammonium sulphate precipitation process, IgG antibodies were separated from antiserum. To stop microbial development, partially purified antibodies were combined with sodium azide, a bacteriostatic chemical, and held at 4 °C Aqueous aliquots of antibodies were frozen at 0 °C in sterile plastic tubes.

Detection of antibodies in antiserum

For detection of antibodies in the antiserum Ouchterlony's immune double diffusion (IDD) method was followed [27]. A thin film of 0.1% agar was applied to a clean tiny glass plate and left to dry. Now, 1% Agarose in phosphate buffer-acid solution was applied onto the previously incubated slide. On the Agarose-coated slide, three 3 mm diameter peripheral and one central well were created. In the center well, 40 µl (112.5 g) of antiserum was supplied, and the peripheral wells received 20 µl (56.25 µg) of antigen (purified tick saliva). This slide was now incubated overnight in a humid chamber. The glass plate was immersed in 0.15 M NaCl for two hours after the appearance of the precipitation band to remove the non-precipitating protein. By soaking the glass plate in distilled water, the salts were eliminated. After drying, the glass plate was photographed.

Serotherapy

Albino mice were used to test the effectiveness of the anti-toxin. 400µg, 800µg, and 1200µg of a purified polyclonal antibody were combined with 40% of the 24-h LD50 for this purpose. Early-aged experimental mice with similar body weights of 65±0.015 g were injected with this mixture after it had been incubated at 37 °C for two hours. Infected mice were monitored for up to 7 d. During this time, the mice's entire behavioral activity was documented. After 4 h of treatment, all significant changes in bio-molecule and enzyme levels were identified in the mice treated as above.

Statistical analysis

Results are presented as the mean and standard error of three replicated estimations. One-way ANOVA, the statistical probability of $p<0.05$, and the student's t test were used to analyze the data. To find significant changes, Student's t-test and analysis of variance were used [28]. All of the chemicals were analytical grade and were bought from reputable businesses.

Experimental protocol for determination of the efficacy of *Rhipicephalus microplus* anti-toxin

The experimental albino mice were divided into the following five groups:

Group A: Received phosphate buffer only (Control group).

Group B: Received 40% of LD_{50} of purified *Rhipicephalus microplus* saliva toxin.

Group C: Received 40% of LD_{50} of purified *Rhipicephalus microplus* saliva toxin pre-incubated with 400µg of anti-toxin.

Group D: Received 40% of LD_{50} of purified *Rhipicephalus microplus* saliva toxin pre-incubated with 800µg of anti-toxin.

Group E: Received 40% of LD_{50} of purified *Rhipicephalus microplus* saliva toxin pre-incubated with 1200µg of anti-toxin.

Mice were scarified and bled out for serum 4 h after the injection. The biomolecules and enzymes identified earlier in this study were examined in these serum samples.

RESULTS

Purification of polyclonal antibodies from antiserum

For this, octanoic acid- partially purified polyclonal antibodies were produced against *Rhipicephalus microplus* saliva toxins. It eliminated lipoproteins from the anti-toxin. Such a procedure could not, however, concentrate the antibodies; they remained in solution. Ammonium sulphate was also used to treat the antiserum, which helped the antibodies gently precipitate out of the solution. Overall yield was 67.75 percent.

Detection of antibodies in antiserum

Detection of antibodies in antiserum was done by following Immunodouble Diffusion Test (IDD) [27]. To achieve this, the antigen was filled in peripheral wells and antibodies in the central well of the manually prepared agarose gel on a small glass plate. After 24 h both antigen and antibodies were found to diffuse radially from their respective wells in the direction of one another. After concentration gradients were established, both the antigen and the antibody reached an equivalence zone and precipitated an apparent crescent-shaped band of antigen-antibody complex. Fig. 1 shows this precipitation band that symbolizes the antigen-antibody complex formation.

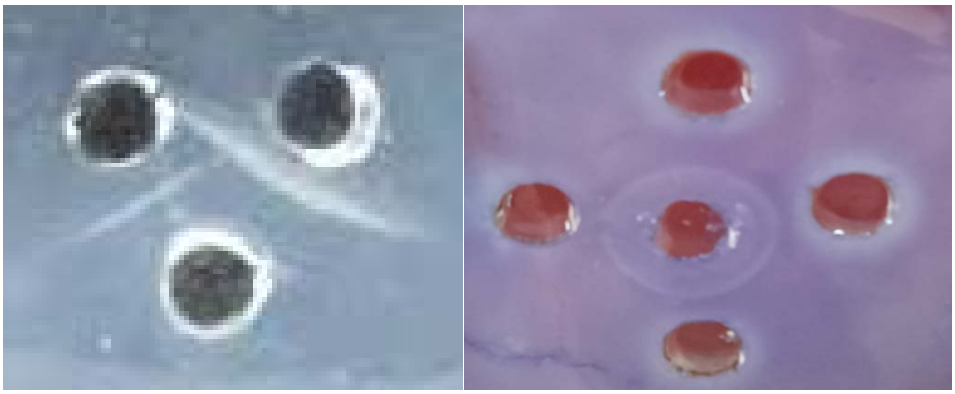

Fig. 1: Showing antigen-antibody interactions in immune double diffusion test. Central well contains antiserum and peripheral antigen. Arrows indicate precipitation band of antigen-antibody complex

Reversal effect of polyclonal antibodies on bio-molecular alterations

In the serum of albino mice, the levels of total protein, free amino acids, uric acid, cholesterol, total lipids, pyruvic acid, and glucose were reversed by purified polyclonal antibodies (anti-toxins) produced against *Rhipicephalus microplus* saliva toxins. For this, purified *Rhipicephalus microplus* saliva toxins were pre-incubated with various doses (400 µg, 800 µg, and 1200 µg) of purified *Rhipicephalus microplus* anti-serum and then injected into albino mice at 40% of the 24-hour LD_{50}. After 4 h of treatment, all metabolic changes were significantly reversed.

The aforementioned treatment successfully returns the serum protein level to normal levels of 90.31%, 96.31%, and 94.18%, respectively. For comparison, mice were given injections of purified *Rhipicephalus microplus* saliva toxin at a concentration of 40% of the LD_{50}, which resulted in a protein level of 87.4% (table 1, fig. 2). Similar to how the albino mice responded to the same treatment, the amino acid level was also discovered to have successfully recovered. Mice injected with purified saliva toxins only showed 122.72% of free amino acid after 4 h of treatment, while mice injected with pre-incubated saliva toxins showed 106.81%, 95.45%, and 97.72% of free amino acid (table 1, fig. 2).

Similar to how *Rhipicephalus microplus* anti-toxin reversed uric acid levels, which were noted to be 108.85%, 102.33%, and 98.6% respectively in comparison to controls, mice injected with only purified *Rhipicephalus microplus* saliva toxins showed a uric acid level of 124.10% in comparison to controls (table 1, fig. 2).

Similarly, mice injected with only purified saliva toxins showed 98.97% of cholesterol level in comparison to control, while mice injected with *Rhipicephalus microplus* anti-toxin showed 97.95%, 96.92%, and 96.24% of cholesterol level in serum, respectively, in comparison to control (table 1, fig. 2).

In addition, *Rhipicephalus microplus* anti-toxin reversed the level of serum pyruvic acid, as noted by 103.84%, 100%, and 96.51%, respectively, while mice only given purified *Rhipicephalus microplus* saliva toxins showed a higher level of pyruvic acid than controls (table 1, fig. 2). Similar to the complete reversal of total lipid level, mice given 40% of the 24-h LD_{50} of the purified saliva toxins experienced a 136.36%, 150%, and 113.63% increase, respectively, in their blood serum when compared to the control. Compared to control, albino mice given a polyclonal antibody injection had their glucose levels fully restored after 4 h (table 1, fig. 2).

Similar to this, in group B mice, only purified saliva toxins were injected, it showed an increase in serum glucose level of 114.28% when compared to control, while serum glucose levels recovered up to 103.57%, 97.61%, and 98.80% after anti-toxin treatment (table 1, and fig. 2).

Table 1: Reversal of levels of certain bio-molecules i.e. total protein, free amino acids, uric acid, cholesterol pyruvic acid total lipid and glucose in blood serum of albino mice after injection with the venom toxins pre-incubated with purified *Rhipicephalus microplus* anti-toxin

Bio-molecules	(Group A)	(Group B)	(Group C)	(Group D)	(Group E)
Protein	5.16±0.0725 (100)	4.51±0.029 **(87.4)**	4.66±0.021* (90.31)	4.97±0.01414 (96.31)	4.86±0.0216 (94.18)
Free Amino Acid	0.44±0.00216 (100)	0.54±0.0043 (122.72)	0.047±0.014 (106.81)	0.042±0.0163* (95.45)	0.043±0.00294 (97.72)
Uric Acid	0.98±0.0081 (100)	1.22±0.0141 (124.10)	1.07±0.008 (108.85)	1.006±0.0216* (102.33)	0.97±0.01414 (98.66)
Cholesterol	2.93±0.0141 (100)	2.90±0.00816 (98.97)	2.87±0.01414* (97.95)	2.84±0.01414 (96.92)	2.82±0.01414 (96.24)
Pyruvic Acid	0.52±0.0141 (100)	0.57±0.01414 (109.61)	0.54±0.01414* (103.84)	0.52±0.01414 (100)	0.50±0.01414 (96.51)
Total lipid	0.22±0.0294 (100)	0.33±0.01414 (150)	0.30±0.0282 (136.36)	0.33±0.0216* (150)	0.25±0.0496 (113.63)
Glucose	0.84±0.0534 (100)	0.96±0.01414 (114.28)	0.87±0.0454 (103.57)	0.82±0.0216* (97.61)	0.83±0.00816 (98.80)

Values (mg/100 ml blood serum) are mean±SE of three replicates, Values in parentheses indicate percent level with control taken as 100%, *Significant ($p<0.05$, Student t-test), Group A: Without envenomation (control), Group B: After injection of 40% of 24-h LD_{50} of purified venom toxin, Group C: After injection of 40% of 24-h LD_{50} pre-incubated with 400µg of anti-venom, Group D: After injection of Group C40% of 24-h LD_{50} pre-incubated with 800µg of anti-venom, Group E: After injection of Group C40% of 24-h LD_{50} pre-incubated with 1200µg of anti-venom

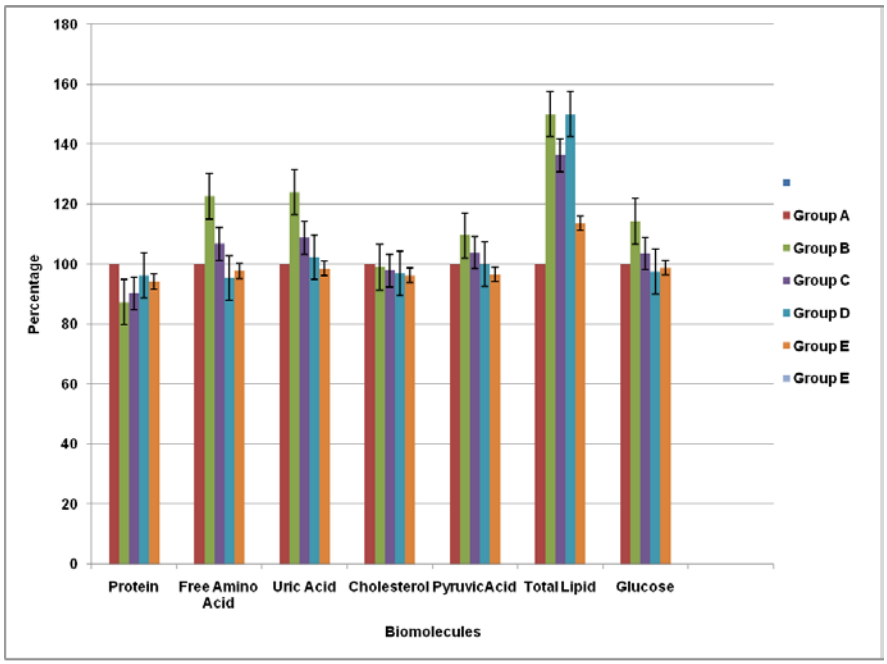

Fig. 2: Reversal of levels of certain biomolecules i.e. total protein, free amino acids, uric acid, cholesterol, pyruvic acid, total lipid, and glucose, in blood serum of albino mice after injection with toxins pre–incubated with purified *Rhipicephalus microplus* anti-toxin (2.0µg/µl)

In a related procedure, purified *Rhipicephalus microplus* saliva toxins (40 percent of the 24-hour LD_{50}) were injected into albino mice, and after pre-incubation with 400µ, 800µ, and 1200µg of polyclonal antibodies (anti-toxins), all enzyme alterations were discovered to be reversed at 4 h after the procedure. Acid phosphatase (ACP), alkaline phosphatase (ALP), glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), lactic dehydrogenase (LDH) and acetylcholinesterase (AchE) levels in serum samples from albino mice showed this reversal of levels.

When albino mice received a similar anti-toxin treatment, their blood serum levels of acid phosphatase recovered by 142.10%, 131.57%, and 142.10% in group C, Group D and Group E mice compared to the control. In comparison to control, it increased up to 147.36% in mice (group B) given 40% of the 24 h LD_{50} of the purified saliva toxins (table 2, fig. 3).

Similar to this, mice injected with polyclonal antibodies demonstrated 122.64%, 107.849%, and 104.71% of alkaline phosphate levels in the serum, respectively, while mice injected mice treated with 40% of 24-h LD_{50} of the purified saliva toxins demonstrated 141.50% of in comparison to control (table 2, fig. 3).

Similarly, glutamate pyruvate transaminase (GPT) has been reversed in mice treated with polyclonal antibodies up to 94.59%, 86.48% and 78.37% in the serum, while it was found to be 116.21% at 40% of the 24-h LD5 0 dose in comparison to control (table 2, fig. 3).

Similar to this, mice treated with polyclonal antibodies showed complete reversal of glutamate oxaloacetate level. In contrast, mice injected with 40% of the 24-h LD_{50} of the purified saliva toxins showed 94.59% of glutamate oxaloacetate transaminase (GOT) level in comparison to control (table 2; fig. 3). It was 89.18%, 75.67%, and 78.37% in the serum of albino mice in comparison to control, respectively. Similar to this, mice treated with polyclonal antibodies demonstrated reversal of lactic dehydrogenase up to 104.55%, 103.82%, and 102.20% in the serum of albino mice, respectively, in comparison to control, while mice injected with 40% of 24-h LD_{50} of the purified saliva toxins demonstrated 117.20% of lactic dehydrogenase (LDH) level in comparison to control (table 2, fig. 3).

Similar to this, polyclonal antibody treatments caused acetylcholinesterase (AchE) to reverse up to 77.77%, 100%, and 88.88% in the serum of albino mice, respectively, compared to control, while it was reduced 55.55% in mice injected with 40% of 24 h LD_{50} compared to control (table 2, fig. 3).

Table 2: Reversal of levels of certain enzymes i.e. acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, lactic dehydrogenase and acetyl cholinesterase in blood serum of albino mice after injection with the saliva toxins pre-incubated with purified *Rhipicephalus microplus* saliva toxin

Enzymes	(Group A)	(Group B)	(Group C)	(Group D)	(Group E)
ACP	0.19±0.015 (100)	0.28±0.014 (147.36)	0.27±0.0050* (142.10)	0.25±0.0014 (131.57)	0.27±0.014 (142.10
ALP	1.06±0.015 (100)	1.50±0.014 (141.50)	1.30±0.0141 (122.64)	1.15±0.028 (107.849)	1.11±0.014* (104.71)
GPT	0.037±0.001 (100)	0.043±0.00021 (116.21)	0.035±0.0041 (94.59)	0.032±0.0028* (86.48)	0.029±0.0021 (78.37)
GOT	0.37±0.021 (100)	0.35±0.021 (94.59)	0.33±0.0216 (89.18)	0.28±0.0029 (75.67)	0.29±0.016 (78.37)
LDH	6.8±0.021 (100)	7.97±0.021 (117.20)	7.11±0.0078 (104.55)	7.06±0.014* (103.82)	6.95±0.043 (102.20)
AchE	0.027±0.002 (100)	0.015±0.004* (55.55)	0.021±0.0041 (77.77)	0.027±0.0016 (100.00)	0.024±0.0014 (88.88)

Values are mean±SE of three replicates, Values in parentheses indicate percent enzyme activity with respect to control taken as 100%, *Significant ($p<0.05$, Student t-test), Acid phosphatase (ACP) and Alkaline phosphatase (ALP): µ moles of p-nitrophenol formed/30 min/mg protein. Glutamate pyruvate transaminases (GPT): Units of Glutamate pyruvate transaminase activity/hour/mg protein, Glutamate oxaloacetate transaminase (GOT): Units of Glutamate oxaloacetate transaminase activity/hour/mg protein. Lactic dehydrogense (LDH): µ moles of pyruvate reduced/45 minute/mg protein. Acetylcholinesterase (AchE): µ moles 'SH' hydrolysed/minute/mg protein., Group A: Without envenomation (control), Group B: After injection of 40% of 24-h LD_{50} of purified venom toxin, Group C: After injection of 40% of 24-h LD_{50} pre-incubated with 400µg of anti-toxin, Group D: After injection of Group C40% of 24 h LD_{50} pre-incubated with 800µg of anti-toxin, Group E: After injection of Group C40% of 24-h LD_{50} pre-incubated with 1200µg of anti-toxin

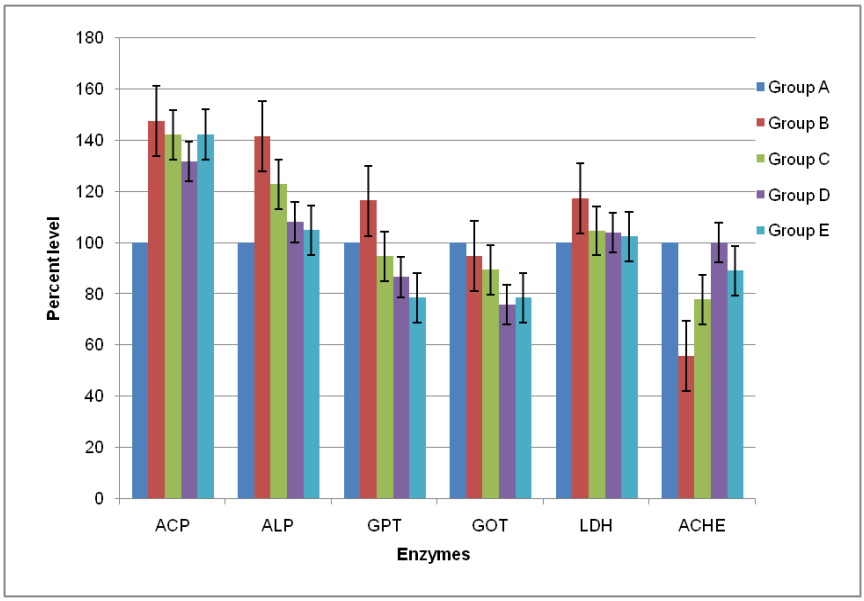

Fig. 3: Reversal of levels of certain enzymes i.e. acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, lactic dehydrogenase and acetyl cholinesterase in blood serum of albino mice after injection with the saliva toxins pre-incubated with purified *Rhipicephalus microplus* anti-toxin (2.0µg/µl)

DISCUSSION

In the current study, a Sepharose CL-6B 200 gel filtration column was used to separate the toxins found in tick saliva. The majority of common low-molecular-weight proteins ranges in size from about 2.0 KD to 18 KD were isolated. When these purified saliva toxins were administered subcutaneously to experimental mice, they showed adverse physiological effects, including hemolytic, hepatotoxic, and paralytic effects, which are very similar to those of insect venom toxins.

In order to neutralize the lethal effects induced by tick saliva toxins, polyclonal antibodies were raised in albino mice by using them and mixed with Freund's adjuvant. Polyclonal antibodies were obtained in form of antiserum were confirmed by using the Ouchterlony method [27]. Furthermore, octanoic acid and ammonium sulphate precipitation were used to separate polyclonal antibodies. It was discovered that the removal of lipoproteins from the reaction mixture by the treatment of polyclonal antibodies against tick saliva toxins with octanoic acid. Additionally, the ammonium sulphate treatment of the polyclonal antibodies to the tick saliva toxins precipitated the antibodies out of the solution and concentrated them into pellet form.

The antigens (antibodies) and antibodies (antigen-antibody complex) were diffused across the gel slice. The interaction of antigens with antibodies results in the formation of the equivalence zone (fig. 1). In bioassays, administration of anti-tick saliva toxin polyclonal antibodies have significantly altered [28] the levels of various biological molecules and metabolic enzymes in experimental mice [29].

Anti-toxin was found to reverse oxidative stress and other negative effects like muscular paralysis, hypotension, and allergic reactions in experimental mice, restoring all physiological effects to normal. A 4 h antibody treatment was also found to reverse metabolic and enzyme changes in the mice's blood serum.

Protein level was also restored 96.31% and 94.18% in group D and group E, but it was lower that other bio-molecules it is clear that structural component will take some more time in their restoration, but level of free amino i.e. 106.81% in group C was restored degradation of proteins might be stopped (table 1, fig. 2). More specifically, the level of cholesterol was also reversed up to normal level, 98.97% in group B in comparison to the control group. In treatment groups from B to E level of glucose concentration was found to be normalized 114.28%, 103.57%, 97.61% and 98.80% after toxin/proteins were allowed to pre-incubate and interact with antiserum (table 1, fig. 2). Similarly, total lipids were

found to normalize 113.63% in group E. Pyruvate level was obtained 109.61%, 103.84%, 100%, 96.51% much similar to control group A (table 1, fig. 2).

In a similar bioassay level of certain enzymes i.e. acid phosphatase (ACP), alkaline phosphatase (ALP), glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), lactic dehydrogenase (LDH) and acetylcholinesterase (AchE) was also reversed in blood serum of albino mice after injection with the saliva toxins pre-incubated with purified *Rhipicephalus microplus* saliva toxins. Saliva toxins in treated mice increased serum acid phosphatase levels up to 147.36% to 142.10% and alkaline phosphatase levels up to141. 50% to 104.71% compared to control, indicating that these enzymes are membrane-bound (table 2, fig. 3). Both ACP and ALP normal levels indicate that membrane functions have been restored because both are membrane-bound enzymes.

The GPT level was found in a range from 78.37% to 116.21% in each group while GOT level was obtained 75.67% to 94.59% (table 2, fig. 3). Additionally, the level of serum lactic dehydrogenase (LDH) rose above control levels by up to117. 20% to 102.20% (table 2, fig. 3). Increased levels of LDH indicate inadequate oxygen delivery, extensive cell lysis, and enzyme leakage into the bloodstream. It amply demonstrates the toxic effects of saliva toxins on the functions of hepatocytes, myocardial muscle cells, and blood cell membranes in albino mice.

In group D, the level of AchE was shown to be 100% recovered (table 2, fig. 3). In contrast, acetylcholinesterase activity decreased up to 55.55% to 88.88% after the saliva toxin injection compared to control (Group B to E) (table 2, fig. 3). The accumulation of acetylcholine molecules at synaptic junctions as a result of this inhibition of acetylcholinesterase activity results in prolonged activation of receptors for acetylcholine. It results in constant stimulation of nerve and muscle cells, which may cause muscle paralysis and ultimately cause animal death. Restoration of levels of various biomolecules was noted normal in behavior of pre and post-injected polyclonal antibodies in albino mice.

However, administration of anti-serum or treatment of patient's immunotherapy is only successful way to treat venom toxin neutralization [29]. Sheep were used to produce an antibody against the entire *Rhipicephalus microplus* toxins, which are also used for immunotherapeutic treatment [30]. Antiserum administration does not produce any allergic and toxic effects [31]. These polyclonal antibodies are IgG, which are much safer to use in bee and wasp venom immunotherapy. These are primarily produced by toxin-induced selected population of plasma B-cells. As a result, B-lymphocytes release a collection of immunoglobulins in response to the toxins, each of which recognizes a different epitope and binds to the venomous toxins [32-34]. These might serve as an all-purpose safety kit for treating poison envenomation [35]. Similar treatment was done by using anti-scorpion venom serum for treatment and reversal of levels of specific bio-molecules in patients stung by a scorpion *Buthus tumulus* [36].

Historically anti-serum was also produced in animals such as mice, rabbit, goat, and sheep which produced high quality serum [37-41]. However, immunization should only be performed on young healthy, disease-free, or long-lived animal strains. Antigen detoxification is also used to reduce the venom toxicity of vaccinated animals. However, the immunogenicity of the venom toxins has to be removed [42]. Therefore, to produce anti-venom in sheep, Freund's adjuvant is added to the toxic mix to address this issue. Sheep with no local lesions showed tolerance to the Freund's adjuvant and other adjuvants. High-titer of high-affinity circulating antibodies protects against foreign antigens, mainly toxins. Antibodies are also naturally produced in beekeepers. They provide a stronger immune response against bee venom. These are the best therapeutic options for neutralizing and neutralizing the toxic effect of saliva toxin in allergic patients. Therefore, we can conclude that immunotherapy more effectively neutralizes the toxic effect of tick saliva toxin and will be very useful in the future.

CONCLUSION

The complete control of the tick population is achieved by inhibiting tick feeding, which prevents host-parasite interaction. For breaking the tick reproduction cycle, nymphs and adults of ticks must be disallowed from feeding. The second way is to have a vaccination of human and animal population to check the pathogen transmission. Polyclonal antibodies produced in albino mice were found to be effective in restoring the metabolic changes caused by tick saliva in the current study. These were administered intravenously or pre-incubated with anti-serum-containing polyclonal antibodies, completely neutralized the toxin-induced effects. They might also directly neutralize the toxic and allergic effects of tick salivary gland secretions and aid in the induction of innate and adaptive immune responses in the host animals. Additionally, new vector and pathogen control methods can be created by functionally analyzing the numerous genes and proteins expressed in tick salivary glands. These could be used as novel antigen to have more effective antibodies. In light of this, it can be said that the polyclonal antibodies created to combat tick toxins are extremely beneficial and have a wide range of clinical and therapeutic applications for delaying all physiological changes brought on by tick saliva toxins.

ACKNOWLEDGEMENT

Nidhi Yadav is thankful to DST for DST/INSPIRE Fellowship/[IF190957] awarded by Department of Science and Technology Ministry of Science and Technology, Government of India, wide letter dated 3rd September 2021. Authors are thankful to H. O. D., Department of Zoology for the research facilities.

AUTHORS CONTRIBUTIONS

Ravi Kant Upadhyay and Nidhi Yadav were responsible for the conception, experiments, writing and revising the manuscript.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Blisnick AA, Simo L, Grillon C, Fasani F, Brule S, Le Bonniec B. The immunomodulatory effect of IrSPI, a tick salivary gland serine protease inhibitor involved in *Ixodes ricinus* tick feeding. Vaccines (Basel). 2019;7(4):148. doi: 10.3390/vaccines7040148, PMID 31614804.
2. Fogaca AC, Sousa G, Pavanelo DB, Esteves E, Martins LA, Urbanova V. Tick immune system: what is known, the interconnections, the gaps, and the challenges. Front Immunol. 2021;12:628054. doi: 10.3389/fimmu.2021.628054, PMID 33737931.
3. Vancova M, Bily T, Simo L, Tous J, Horodysky P, Ruzek D. Three-dimensional reconstruction of the feeding apparatus of the tick Ixodes ricinus (Acari: Ixodidae): a new insight into the mechanism of blood-feeding. Sci Rep. 2020;10(1):165. doi: 10.1038/s41598-019-56811-2, PMID 31932602.
4. Perveen N, Muzaffar SB, Al-Deeb MA. Ticks and tick-borne diseases of livestock in the Middle East and North Africa: a review. Insects. 2021;12(1):83. doi: 10.3390/insects12010083, PMID 33477991.
5. Obaid MK, Islam N, Alouffi A, Khan AZ, da Silva Vaz I Jr, Tanaka T. Acaricides resistance in ticks: selection, diagnosis, mechanisms, and mitigation. Front Cell Infect Microbiol. 2022;12:941831. doi: 10.3389/fcimb.2022.941831, PMID 35873149.
6. Hrnkova J, Schneiderova I, Golovchenko M, Grubhoffer L, Rudenko N, Cerny J. Role of zoo-housed animals in the ecology of ticks and tick-borne pathogens-a review. Pathogens. 2021;10(2):210. doi: 10.3390/pathogens10020210, PMID 33669161.
7. Feng LL, Liu L, Cheng TY. Proteomic analysis of saliva from partially and fully engorged adult female rhipicephalus microplus (Acari: Ixodidae). Exp Appl Acarol. 2019;78(3):443-60.
8. Galay RL, Umemiya Shirafuji R, Bacolod ET, Maeda H, Kusakisako K, Koyama J. Two kinds of ferritin protect ixodid ticks from iron overload and consequent oxidative stress. PLOS ONE. 2014;9(3):e90661. doi: 10.1371/journal.pone.0090661, PMID 24594832.
9. Hajdusek O, Sojka D, Kopacek P, Buresova V, Franta Z, Sauman I. Knockdown of proteins involved in iron metabolism limits

tick reproduction and development. Proc Natl Acad Sci USA. 2009;106(4):1033-8. doi: 10.1073/pnas.0807961106, PMID 19171899.
10. Chmelar J, Kotal J, Kovarikova A, Kotsyfakis M. The use of tick salivary proteins as novel therapeutics. Front Physiol. 2019;10:812. doi: 10.3389/fphys.2019.00812, PMID 31297067.
11. Potet J, Beran D, Ray N, Alcoba G, Habib AG, Iliyasu G. Access to antivenoms in the developing world: a multidisciplinary analysis. Toxicon X. 2021;12:100086. doi: 10.1016/j.toxcx.2021.100086, PMID 34786555.
12. Hamza M, Knudsen C, Gnanathasan CA, Monteiro W, Lewin MR, Laustsen AH. Clinical management of snakebite envenoming: future perspectives. Toxicon X. 2021;11:100079. doi: 10.1016/j.toxcx.2021.100079, PMID 34430847.
13. World Health Organization, Stuart MC, Kouimtzi M, Hill SR. editors. WHO model formulary 2008. World Health Organization; 2009. p. 396-7.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75. doi: 10.1016/S0021-9258(19)52451-6, PMID 14907713.
15. Spier RE. Animal cell technology: an overview. J Chem Technol Biotechnol. 1982;32(1):304-12.
16. Mendel B, Kemp A, Myers DK. A colorimetric micro-method for the determination of glucose. Biochem J. 1954;56(4):639-46. doi: 10.1042/bj0560639, PMID 13159895.
17. Friedemann TE, Haugen GE. Pyruvic acid II. J Biol Chem. 1943;147(2):415-42. doi: 10.1016/S0021-9258(18)72397-1.
18. Folin O. Standardized methods for the determination of uric acid in unlaked blood and in urine. J Biol Chem. 1933;101(1):111-25. doi: 10.1016/S0021-9258(18)75918-8.
19. Abel LL, Levy BB, Brodie BB, Kendall FE. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J Biol Chem. 1952;195(1):357-66. doi: 10.1016/S0021-9258(19)50907-3, PMID 14938387.
20. Folch J, Lees M, Sloane Stanley GHS. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957;226(1):497-509. doi: 10.1016/S0021-9258(18)64849-5, PMID 13428781.
21. Spies JR. 76 Colorimetric procedures for amino acids. In: Colowich SP, Kalpan NO, editors. Methods in enzymology. Academic Press; 1957. p. 467-77. doi: 10.1016/S0076-6879(57)03417-5.
22. Andersch MA, Szczypinski AJ. Use of p-nitrophenylphosphate as the substrate in determination of serum acid phosphatase. Am J Clin Pathol. 1947;17(7):571-4. doi: 10.1093/ajcp/17.7_ts.571, PMID 20256398.
23. Bergmeyer UH. Determination of alkaline phophatase and acid phosphatase was determined by using p-nitrophenyl phosphate, Method of enzymetic analysis. New York Acadmic Press; 1967. p. 1129.
24. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol. 1957;28(1):56-63. doi: 10.1093/ajcp/28.1.56, PMID 13458125.
25. Annon TM. Sigma diagnostic: Lactate dehydrogenase (Quantitative, Colorimeteric determination in serum, urine and cerebrospinal fluid) at 400-500 nm. Procedure No. 500; 1984.
26. Ellman GL, Courtney KD, Andres V Jr, Feather-stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88-95. doi: 10.1016/0006-2952(61)90145-9, PMID 13726518.
27. Ouchterlony O. Diffusion-in-gel methods for immunological analysis. II. Prog Allergy. 1962;6:30-154. doi: 10.1159/000313795, PMID 14482809.
28. Sokal RR, Rohlf FJ. Introduction to biostatistics. San Francisco: W H Freeman and Company, Ltd.; 1973.
29. Banks BEC, Shipolini RA. Chemistry and pharmacology of honeybee venom. In: Piek T, editor. Venoms of hymenoptera. Biochemical, Pharmacological and Behavioral aspects. London: Acadmy Press; 1986. p. 329-16.
30. Jones RG, Corteling RL, Bhogal G, Landon J. A novel Fab-based antivenom for the treatment of mass bee attacks. Am J Trop Med Hyg. 1999;61(3):361-6. doi: 10.4269/ajtmh.1999.61.361, PMID 10497971.
31. Muller U, Akdis CA, Fricker M, Akdis M, Blesken T, Bettens F. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. J Allergy Clin Immunol. 1998;101(6 Pt 1):747-54. doi: 10.1016/S0091-6749(98)70402-6, PMID 9648701.
32. Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. J Immunol. 1998;160(7):3555-61. doi: 10.4049/jimmunol.160.7.3555, PMID 9531318.
33. Paull BR, Jacob GL, Yunginger JW, Gleich GJ. Comparison of binding of IgE and IgG antibodies to honeybee venom phospholipase-a. J Immunol. 1978;120(6):1917-23. doi: 10.4049/jimmunol.120.6.1917, PMID 659884.
34. Kemeny DM, Mackenziemills M, Harries MG, Youlten LJ, Lessof MH. Antibodies to purified bee venom proteins and peptides II. A detailed study of changes in IgE and IgG antibodies to individual bee venom antigens. Journal of Allergy and Clinical Immunology. 1983;72(4):376-85. doi: 10.1016/0091-6749(83)90503-1.
35. Lipps BV, Khan AA. Antigenic cross-reactivity among the venoms and toxins from unrelated diverse sources. Toxicon. 2000;38(7):973-80. doi: 10.1016/S0041-0101(99)00214-7.
36. Natu VS, Murthy RK, Deodhar KP. Efficacy of species-specific anti-scorpion venom serum (AScVS) against severe, serious scorpion stings (Mesobuthus tumulus concanesis Pocock)-an experience from rural hospital in Western Maharashtra. J Assoc Phys India. 2006;54:283-7.
37. Egner W, Ward C, Brown DL, Ewan PW. The frequency and clinical significance of specific IgE to both wasp (Vespula) and honey-bee (Apis) venoms in the same patient. Clin Exp Allergy. 1998;28(1):26-34. doi: 10.1046/j.1365-2222.1998.00176.x, PMID 9537776.
38. Rawat S, Laing G, Smith DC, Theakston D, Landon J. A new antivenom to treat eastern coral snake (Micrurus fulvius fulvius) envenoming. Toxicon. 1994;32(2):185-90. doi: 10.1016/0041-0101(94)90107-4, PMID 8153957.
39. Russell FE, Lauritzen L. Antivenins. Trans R Soc Trop Med Hyg. 1966;60(6):797-810. doi: 10.1016/0035-9203(66)90232-x, PMID 6005778.
40. Russell FE, Timmerman WF, Meadows PE. Clinical use of antivenin prepared from goat serum. Toxicon. 1970;8(1):63-5. doi: 10.1016/0041-0101(70)90175-3, PMID 5465748.
41. Sjostrom L, Al-Abdulla IH, Rawat S, Smith DC, Landon J. A comparison of ovine and equine antivenoms. Toxicon. 1994;32(4):427-33. doi: 10.1016/0041-0101(94)90294-1, PMID 8052997.
42. Khan SA, Humayun MZ, Jacob TM. A sensitive radioimmunoassay for isopentenyladenosine. Anal Biochem. 1977;83(2):632-5. doi: 10.1016/0003-2697(77)90067-7, PMID 603045.
43. Schumacher MJ, Schmidt JO, Egen NB, Lowry JE. Quantity, analysis, and lethality of European and Africanized honey bee venoms. Am J Trop Med Hyg. 1990;43(1):79-86. doi: 10.4269/ajtmh.1990.43.79, PMID 2200291.
44. Fernandez J, Soriano V, Mayorga L, Mayor M. Natural history of hymenoptera venom allergy in Eastern Spain. Clin Exp Allergy. 2005;35(2):179-85. doi: 10.1111/j.1365-2222.2005.02169.x, PMID 15725189.