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Original Article

PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST INDIAN PAPER WASP ROPALIDIA MARGINATA VENOM TOXINS AND THEIR EFFICACY IN THE REVERSAL OF TOXIC EFFECTS

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ABSTRACT

Objective: In this study, albino mice were injected with a sub-lethal dosage of purified wasp *Ropalidia Marginata* venom toxins to assess the effectiveness of polyclonal anti-venom antibodies.

Methods: To neutralize the toxic effects, polyclonal antibodies were generated by immunizing albino mice. The antibody underwent partial purification using ammonium sulphate treatment and octanoic acid precipitation. To detect the presence of antibodies in the antiserum, an immunodouble diffusion test was conducted using Ouchterlony's method (1962). This involved allowing both antigens and antibodies to diffuse radially towards each other from their respective wells. When they reached an equivalence zone, a precipitation complex of antigen and antibody became visible as a concentric band, indicating the development of the combination. To quantitatively determine the amount of antibodies in the antiserum, the equivalency zone approach was used.

Results: Experimental mice were injected with a combination containing 400, 800, and 1200 µg of pure antibody, which had been treated serum biomolecules, including metabolic enzymes, completely reversed in the experimental with 40% of the LD50 of wasp venom the elevated serum parameters were glucose, pyruvic acid, lipid, protein and free amino acid, reached to normal (100%) in the treated with 40% of LD50 of the venom and polyclonal treated after 6 h of administration. Anti-serum treatment also successfully normalized the alteration in serum enzyme just after 4h.

Similarly, anti-serum treatment also successfully normalized the alteration in serum enzyme just after 4h treated with 40% of LD_{50} of the venom. Serum ACP content was obtained as 125.35% after 40% of LD_{50} venom injection, which was get normalized up to 102.81% after 4 h of the anti-venom treatment. Serum ALP content of 114.8% elevation was reversed back to 102.40% after anti-venom treatment. The GPT level significantly reversed up to 102.5%, while it was 130% in the venom-treated mice. A complete reversal was obtained in GPT level, which was obtained as 104.54% in the venom-treated animal. Similarly, LDH which was elevated up to 112.45 % in venom-injected mice was successfully reversed up to 100.25% after anti-venom treatment. Similarly, Ache concentration was fully recovered after anti-venom treatment 6 h, all animals (group B-E) that had received 40% of the LD50 of venom treated with pure antiserum.

Conclusion: The venom-injected group showed a complete restoration of serum protein, free amino acid, uric acid, cholesterol, pyruvic acid, total lipid, and glucose level in experimental mice.

Keywords: Polyclonal anti-venom antibody, Wasp toxins, Antiserum, Immunotherapy and reversal effect

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INTRODUCTION

Wasps belong to the Hymenoptera; its sting causes local and systemic allergic reactions and even life-threatening anaphylaxis. The main components of wasp venom are peptides and proteins, although it also contains a complex mixture of other natural substances such as alkaloids, terpenes, polysaccharides, biogenic amines (histamine), organic acids (formic acids), and amino acids [1, 2]. Highly active chemicals found in wasp venom [3] cause edema, skin inflammation, swelling, headaches, weakness, exhaustion, and dizziness following a sting [4]. Visible signs include diarrhoea [5], hypotension [6], and nausea [7, 8]. Wasp venom causes severe harm to the body's vital organ systems, including the vascular, renal, and respiratory systems, and can cause both local and systemic reactions [9, 10]. Additionally, it may have damaged the hepatocytes and increased blood levels of glycogen [11]. They target ion channels as well and bind them strongly. Wasp venom has an effect on voltage-gated K+ channels, suppresses the body's immune system [12], and, in the event of repeated stings, quickly results in death [13]. In addition, it modifies the expression of the Ca++-activated conductance and induces severe innervation and denervation [14]. These also impact numerous cells' Na+ and K+ channels as well as the brain's synaptic processes [15]. Wasp venoms is a mixture of various enzymes i.e. hyaluronidase, phospholipase A2, Metallo endopeptidase, and several biologically active proteins, peptides and allergens.

This study shows the effectiveness of polyclonal anti-venom antibodies in reversing the toxic effects of paper wasp venom. This venom causes allergic reactions and changes the enzymes and bio-molecules in the body, leading to molecular toxicity. Albino mice were immunized to generate polyclonal antibodies, which were partially purified using octanoic acid precipitation and ammonium sulfate treatment. An immunodouble diffusion test confirmed the presence of antibodies in the antiserum. The study injected a mixture of the venom and purified antibodies into experimental mice, while another group received only venom with saline as a control. The results showed that the venom-injected group had increased levels of free amino acids, uric acid, cholesterol, pyruvic acid, total lipid, and glucose, as well as serum enzymes ACP, ALP, GPT, GOT, and LDH. In contrast, the group that received the venom and was incubated with pure antiserum showed a complete recovery of these anomalies in the content of serum bio-molecules and enzymes.

MATERIALS AND METHODS

Collection of *Ropalidia marginata* toxins

The living specimens of paper wasp *Ropalidia marginata* were collected from rural areas of the Gorakhpur district by using a nylon net. The collected wasps were immobilized by quick freezing at-20 °C. Insects were dissected for taking out sting glands; its homogenate was prepared in phosphate buffer saline (50 mm, pH 6.9) with the help of a power homogenizer. The homogenate was centrifuged at 10,000 rpm at 4 °C for 10 min and the supernatant was used as crude venom.

Preparation of homogenate

Ropalidia marginata sting glands 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. Homogenate was centrifuged at 12,000 rpm in the cold for 30 min and the supernatant was separated out. Total protein contents were estimated in the different supernatants, according to Lowry (1951) [19].

Purification of venom protein of *Ropalidia marginata*

Proteins were eluted on a Sepharose CL-6B-200 a double cavity gel filtration column with a sintered disc filtered in the bottom having a height of 1 meter in 25 mm diameter. A known volume i.e., 5 ml of toxin proteins solubilized in PBS was loaded on the column and the flow rate was maintained between 5 ml/min by using a continuous buffer supply in a cold room illusion of the venom proteins through a gel filtration column was done in a cold room at 40 °C at the flow rate of 5 ml/min. The protein concentrations in the various eluted fractions were plotted on the graph, which was created from 121 regular fractions that were collected using a Pharmacia fraction collector at a predetermined time interval. Each fraction's absorbance was measured at 280 nm using a Shimadzu spectrophotometer (UV 2001 PC). After estimating the amount of protein using the Lowry method, the absorbance of the identical fractions was measured at 640 nm [19].

Spectrophotometric analysis of proteins eluted fractions

The protein content eluted in each fraction was determined at a wavelength of 280 nm. A graph was plotted between absorption at 280 nm by using absorbance obtained in continuous fractions. Proteins/toxins eluted in the same faction numbers were determined at 640 nm by using the Lowry method [19]. Values were also plotted for preparing the graph.

Molecular weight determination of purified venom proteins

The range of molecular weight of different proteins/toxins in the purified paper wasp *Ropalidia marginata* toxins/proteins was determined by running the proteins of known molecular weight through the Sepharose CL-6B gel column as done previously at the same flow rate. A calibration curve was drawn between Ve/Vo log M and with the help of the calibration curve, the range of molecular weight of different proteins in the purified *Ropalidia marginata* protein/toxins was determined.

Lyophilization of eluted venom protein

The eluted fractions containing venom proteins were pooled and lyophilized to a desired concentration of venom toxins/proteins. Biological activity of the purified venom protein biological activity testing of *Ropalidia marginata* toxins was determined in albino mice serially known volumes of the purified toxins were injected intra-peritoneal.

Determination of lethality of *Ropalidia marginata* venom toxins

For determination of toxic effects, purified *Ropalidia marginata* venom toxins were administered to the albino mice with sub-lethal doses 40% and 80% of the LD50 of purified venom toxins. After the injection, the mice were sacrificed 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.

(A) Determination of blood bio-molecules

Isolation of blood serum

Both control and tested albino mice were bled at the same time to obtain blood serum. Freshly drawn blood was taken directly into a clean glass test tube without adding any coagulants.

Determination of serum total protein

Estimation of the total protein in the serum was carried out by Lowry's method (1915) [19].

Determination of total free amino acid

Changes in the level of free amino acids in the blood serum of albino mice were determined according to the method of Spies (1957) [20].

Determination of serum glucose

Changes in serum glucose levels were measured according to the method of Mendel *et al.*, (1954) [21].

Determination of serum pyruvic acid

Changes in the level of pyruvic acid were determined according to the method of Freidman and Haugen (1943) [22].

Determination of serum uric acid

Changes in serum uric acid levels were determined by the cyanide-free method of Folin (1933) [23].

Determination of serum cholesterol

Changes in serum cholesterol levels were measured according to the method of Abell *et al.,* (1952) [24].

Determination of glycogen in the tissue of the liver, heart and gastrocnemius muscles

Glycogen contents were measured according to the method of Dubois *et al.,* 1956 [25].

Effects of purified wasp venom on certain serum enzymes (*in vivo*) on blood serum

Determination of alkaline phosphatase

Changes in alkaline phosphatase levels were determined according to the method of Andrech and Szeypiaske (1947) and modified by Bergmeyer (1967) [26].

Determination of acid phosphatase

Changes in acid phosphate levels were determined according to the method of Andrech and Szeypiaske (1947) and modified by Bergmeyer (1967) [26].

Determination of serum glutamate pyruvate transaminase (GPT)

Changes in the serum glutamate pyruvate transaminase (GPT) level were measured according to the method of Reitman and Frankel (1957) [27].

Determination of serum glutamate oxaloacetate transaminase (GOT)

Changes in the serum glutamate oxaloacetate transaminase (GOT) level were measured according to the method of Rietman and Frankel (1957) [27].

Determination of serum lactic dehydrogenase

Changes in the activity of serum lactic dehydrogenase were measured according to the method of Annon (1984) [28].

Determination of serum acetyl cholinesterase (AChE)

Changes in the activity of the acetyl cholinesterase (AChE) were measured according to the method of Ellman (1961) [29].

Production of polyclonal antibodies

Choice of animals

For the production of antibodies against was pvenom protein, Albino mice (*Mus musculus*) weighted 65±0.05 gm was used for immunization. These animals were reared in laboratory conditions with proper care and feeding and provided treatment humanly for nursing.

Immunization

Purified wasp venom toxins were used as an immunogen after mixing with an equal amount of Freund's adjuvant. For primary immunization 50 µl (700µg) wasp venom protein and 50 µl of complete Freund's adjuvant were mixed well and injected into the

body of albino mice. Immunization was done intra-peritoneal (Photograph 1).

Boosting

After the 7th day of primary immunization, experimental mice were provided a booster dose (700µg) of venom protein by the same route. Similarly, the mice will be given a second booster dose after the 21st day of primary immunization. After the 28th day of immunization, complete and tested albino mice were ready for antibody production. This study, involving use of animals, was approved by Institutional Ethics Committee, Department of Zoology, Deen Dayal Upadhyaya Gorakhpur University, and Gorakhpur. Four pairs of male and female adults of albino mice were procured from Banaras Scientific Centre, Varanasi. Four albino mouse pairs, one male and one female, were purchased from Banaras Scientific Centre in 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 12.5–13.20 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 a plastic floor and frame with steel net and an opening window. Animals were feed twice a day on corn pellets, and fresh chopped vegetables and food grains. Drinking water supply was given by using an autoclavable water bottle (250 ml) made up of Polypropylene (PP) with a silicone plug and a tube of stainless steel. Bottle plug is made of silicone. Tube was made up of stainless steel.

Collection of blood

Following the second booster's seven-day period, the animals were killed and left 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, the 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 min centrifugation at 1000 rpm to separate the supernatant. In a process akin to the dialysis of purified toxin, 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% sodium azide (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 IDD method of Ouchterlony was followed [30]. 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 to 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. 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 370C for two hours. Infected mice were monitored for up to 7 d. During this time, the mice's entire behavioural activity was documented. After 4 h of treatment, all significant changes in biomolecule and enzyme levels were identified in the mice treated as above.

The experimental albino mice were divided into the following five groups

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

Group B: Received 40% of LD50 of purified *Ropalidia marginata* venom toxins.

Group C: Received 40% of LD50 of purified *Ropalidia marginata* venom toxins pre-incubated with 400 µg of anti-toxin

Group D: Received 40% of LD50 of purified *Ropalidia marginata* venom toxins pre-incubated with 800 µg of anti-toxin.

Group E: Received 40% of LD50 of purified *Ropalidia marginata* venom toxins pre-incubated with 1200 µg of anti-toxin.

Mice were sacrificed 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.

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 [31]. All of the chemicals were analytical grade and were bought from reputable businesses.

RESULTS

Solubilization of *Ropalidia marginata* venom toxins

Before extracting poisonous toxins, it was homogenized and dissolved in different solution buffers, i.e., Triton was isolated by homogenizing Triton X 100 (0.01%), Tris EDTA (0.1 mmol) PBS buffer 78%, TCA 5%, and absolute alcohol, Triton X.-100 (0.1%) proved to be good for solubilization of paper wasp *Ropalidia marginata* venoms because it showed 82.4% dissolution than any other solubilizing buffer used. Higher protein solubility was obtained in the supernatant than in the residue, except in TCA (fig. 1). Solubility in PBS buffer is about 78% (fig. 1).

Purification

For isolation and purification sting glands of *Ropalidia marginata* were taken out and homogenized in 5 ml of PBS (pH 6.9) using a glass-glass

homogenizer with 5 ml of solubilizing buffer. The homogenate was centrifuged in the cold at 4 °C for 30 min at 15,000 rpm and the supernatant was carefully separated. It was loaded onto a Sepharose CL-6B 200 column to separate the venom toxins. The venom toxin homogenate elution pattern showed five major peaks at 280 nm. Immediately after the void fraction, there are three peaks at 37-42, 46-51, and 64-71, while the fourth and fifth peaks were present at 81-97 (fig. 2). In addition, the concentration of *Ropalidia marginata* toxin was determined in each test tube using the method of Lowry (1951). Again, two similar protein peaks were resolved at 640 nm (fig. 2). The first peak was a large peak between 46 and 56, while the second peak was large and located between fractions numbers 61 and 67 (fig. 3). Both peaks were eluted with PBS buffer (pH 6.9). The total yield of poisonous toxins in the eluted fractions was 76.8%.

Molecular weight determination of wasp venom toxins

The molecular weight of *Ropalidia marginata* venom toxins/proteins was determined by Sepharose CL-6B 200 gel column chromatography using standard marker proteins of known molecular weight (fig. 3). The calibration curve shows that the molecular weight of the purified venom proteins is 12.6-63kDa (fig. 4).

Venom fractions

The eluted venom protein fractions were pooled and lyophilized. The toxicity of purified wasp venom toxins from *Ropalidia marginata* toxin was determined in various tissues of albino mice (Mus musculus). Wasp venom proteins obtained by lyophilization caused heavy toxicity in albino mice. The LD_{50} of *Ropalidia marginata* venom protein was found to be 20.6±0.094 mg/kg body weight in albino mice.

In the investigation of the efficacy of purified *Ropalidia marginata* anti-venom protein in the reversal of glucose pyruvic acid, cholesterol, uric acid, and free amino acid level alteration in the serum of albino mice caused by purified paper wasp venom protein. Albino mice were injected with 40% of 24-h LD_{50} of purified paper wasp venom protein toxins pre-incubated with different doses (100 µg, 200 µg, 400 µg, 800 µg) of purified paper wasp venom anti-serum. All antibody physiological alterations were reserved significantly after 4 H of the treatment. The above treatment successfully normalizes the serum protein level up to 96.86%, 98.95%, and 99.30% separately. For comparison, mice were injected with 40% of 24-h LD_{50} of purified paper wasp venom protein toxins, indicating 99.30% of protein level (table 1; fig. 5).

Effects of purified *Ropalidia marginata* venom on various bio-molecules in the blood serum of albino mice were evaluated. Changes in the concentration of certain macromolecules, i.e., proteins, free amino acids, uric acid, cholesterol, pyruvic acid, total lipids, and glucose, were measured after an intraperitoneal 24h LD_{50} injection of 40% and 80% purified *Ropalidia marginata* venom toxins.

The toxic effect of the purified venom toxins of *Ropalidia marginata* was observed in albino mice on serum, liver, and gastrocnemius muscle. The albino mice were treated with 40% and 80% of 24 h LD_{50} of purified wasp toxins and alterations in enzyme activity were measured after 2, 4, 6, 8, and 10 h of treatment. Wasp venom caused a significant increase in the activity of alkaline phosphatase and acid phosphatase activity in serum, liver, and gastrocnemius muscles in treated albino mice in comparison to control mice (Tables 1, 2).

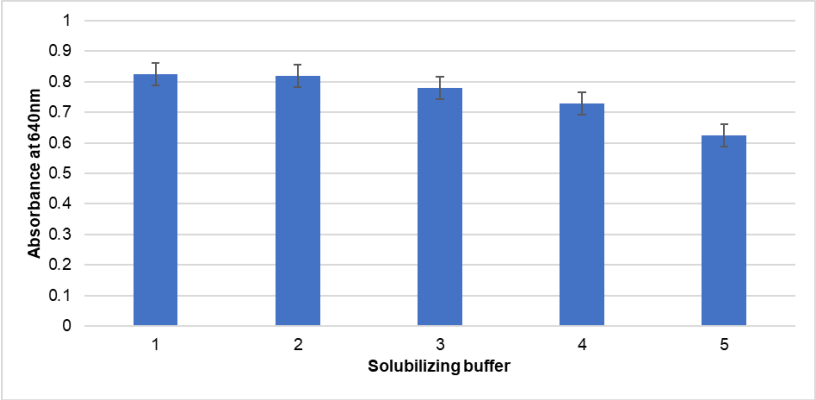

Fig. 1: Solubilization of *Ropalidia marginata* in different buffers. Absorbance of solubilized protein was taken at 640 nm. Solubilizing buffers on X-axis are (1) Triton X 100 (0.01%), (2) Tris+EDTA (0.1 Mm) (3) PBS buffer (4) TCA 5% and (5) Absolute alcohol

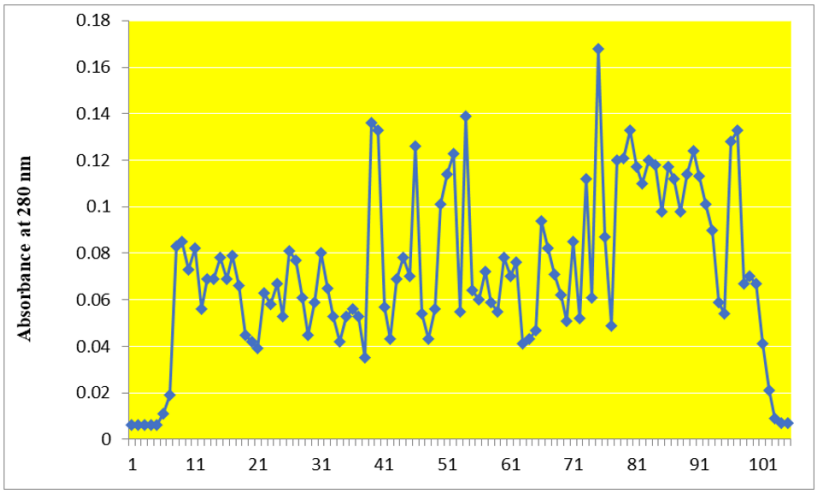

Fig. 2: Elution pattern of PBS extractable proteins of *Ropalidia marginata wasp* chromatographed on a sepharose CL-6B 200 column. Absorbance was determined at 280 nm

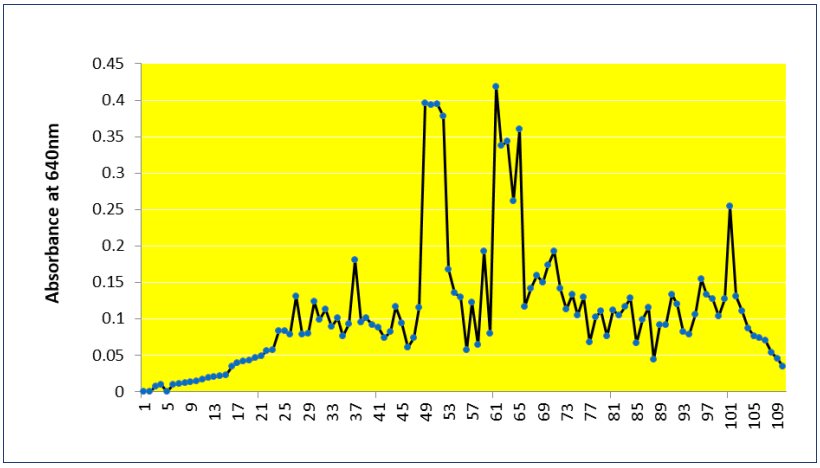

Fig. 3: Elution pattern of PBS extractable proteins of *Ropalidia marginata* wasp chromatographed on a sepharose CL-6B 200 column. Absorbance was determined at 280 nm

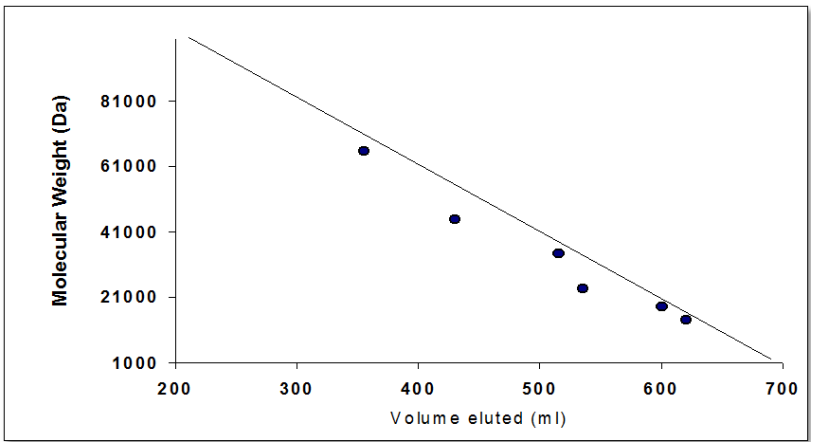

Fig. 4: Standard proteins chromatographed on Sepharose CL-6B 200 column for determining the molecular weights peptides isolated from *Ropalidia marginata.* Proteins used were bovine albumin mol. wt. 66,000, egg albumin mol. wt. 45,000, pepsin mol. wt. 34,700, trypsinogen mol. wt. 24,000, beta-lactoglobulin mol. Wt. 18,400 and lysozyme mol

Immunotherapy and neutralization of wasp venom toxin effects

Purification of polyclonal antibodies from antiserum

For this, octanoic acid- partially purified polyclonal antibodies were produced against Ropalidia marginata 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. The overall yield was 63.5 percent.

Detection of antibodies in the antiserum

The presence of antibodies was detected by an immunodouble diffusion test (Ouchterlony, 1962). For this purpose, both antigens (in peripheral wells) and antibodies (in central well) were allowed to diffuse radially from their corresponding wells towards each other; finally, a concentration gradient was established as their concentration gradients reached an equivalence zone, a visible concentric band of precipitation complex of antigen-antibody was formed (fig. 1). This precipitation band represented the formation of the antigen-antibody complex.

Quantitative determination of antibodies in the antiserum

For the quantitative determination of antibodies in the antiserum, the equivalence zone method was applied. For this purpose, partially purified antiserum that contained 4.95 µg/µl polyclonal antibodies was used. In the first set of the experiment, varying concentrations of antibody ranges were used to interact with the fixed volume of antigen, while in the second set of the experiment, a fixed volume of antibody and variable doses of antigen were taken. On both the sets, antigen and antibody interaction was allowed to be incubated at room temperature for 30 min. After which both sets were spun at 10,000 rpm for 15 min in the centrifuge. Supernatant was separated out and the pellet was dissolved in 200 µl of PBS for protein estimation. Further, the PBS-soluble pellet was treated with isopropanol to take out the non-precipitating material from the tube. Protein contents in the tubes were determined by Lowry's method. From the equivalence rule, 2.764 µg antigen/µg antibody was calculated (fig. 1) but after isopropanol treatment of antigen-antibody complex, the binding ratio was obtained approximately as 1.01 µg antigen/µg antibody.

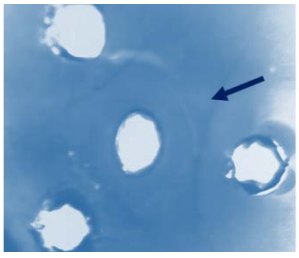

Fig. 5: This fig. shows antigen-antibody interactions in immune double diffusion test. Central well contains antiserum and peripheral antigen. Arrows indicate precipitation band of antigen-antibody complex

Reversal of bimolecular alterations by purified *Ropalidia marginata* anti-venom

Experimental mice injected with 40% of 24 h LD_{50} of purified paper wasp venom toxins were pre-incubated with different doses (400, 800, 1200µg) of the purified paper wasp anti-serum. All metabolic alterations were reversed significantly after 4 h of the treatment. The elevated serum glucose content was successfully reversed at its normal value in the control group (100%), while it was merely increased in mice treated with 40% LD_{50}. Paper wasp anti-venom made 100% reversal in the serum pyruvic acid as it was increased up to 106.66% after the 40% of the LD_{50} venom injection (table 1, fig. 6). It was also given a significant reversal in serum uric acid up to 102.38% while it was 149.11% in the venom-treated mice (table 1). After antiserum treatment, cholesterol, which was marginally elevated in the venom-injected mice was also reversed fully at low concentrations of the anti-venom (table 1, fig. 6). The lipid level was reversed up to 102.70% and that obtained in the venom-treated mice was 125% (table 1, fig. 6). In mice treated with only the venom serum protein, the content obtained was 89.69% which was reversed up to 91.28% after the antiserum treatment (table 1). A similar treatment gave 100% reversal of free amino acid, which was increased to 99.30% in the mice treated with 40% of LD_{50} of the venom (table 1, fig. 6).

The lipid level was reversed up to 102.70%, and that obtained in the venom-treated mice was 125% (table 1). In mice treated with only the venom serum protein, the content obtained was 89.69%, which was reversed up to 91.28% after the antiserum treatment (table 1). A similar treatment gave 100% reversal of free amino acid, which was increased to 99.30% in the mice treated with 40% of LD_{50} of the venom (table 1, fig. 6).

Anti-serum treatment also successfully normalized/reversed the alteration in serum enzyme just after 4h. Serum ACP content was obtained as 125.35% after 40% of LD_{50} venom injection, which was get normalized up to 102.81% after 4 h of the anti-venom treatment (table 2, fig. 7). Similarly, Serum ALP content of 114.8% elevation was reversed back to 102.40% after anti-venom treatment (table 2, fig. 7). Also, the GPT level was significantly reversed up to 102.5% while it was 130% in the venom-treated mice (table 2, fig. 7). A complete reversal was obtained in GPT level, which was obtained as 104.54% in the venom-treated animal (table 2, fig. 7). Similarly, LDH which was elevated up to 112.45 % in venom-injected mice was successfully reversed up to 100.25% after anti-venom treatment (table 2, fig. 7). Paper wasp venom showed an 80% decrease in the Ache concentration, which was fully recovered after anti-venom treatment (table 2, fig. 7).

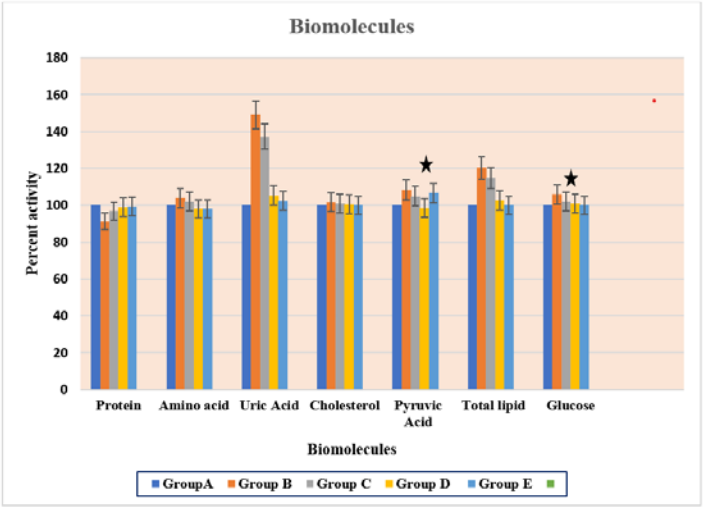

Fig. 6: Reversal of level of certain biomolecules i.e. total protein, free amino acids, uric acid, cholesterol, pyruvic acid, total lipid, and glucose, in blood serum of albino mice after injection with toxinspre–incubated with purified *Ropalidia marginata* anti-venom. The data represent means+SD of three independent experiments. *P<0.05 represents a significant difference in toxin-treated and untreated mice

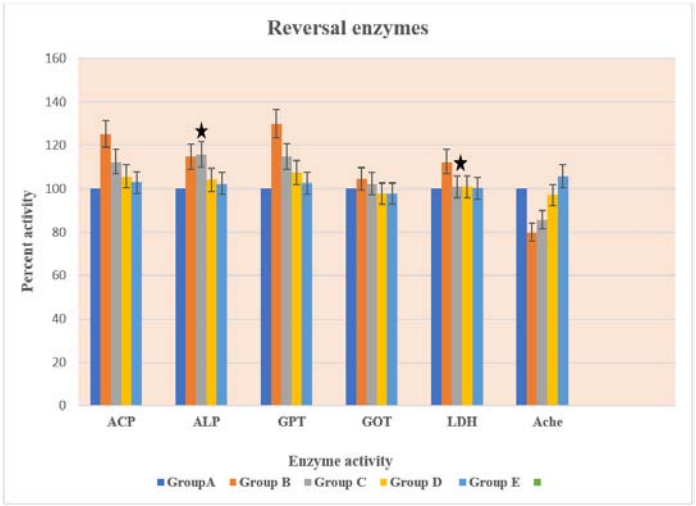

Fig. 7: Reversal of level of certain enzymes i.e. acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, lactic dehydrogenase and acetylcholinesterase in blood serum of albino mice after injection with the saliva toxins pre-incubated with purified *Ropalidia marginata* anti-venom. The data represent means+SD of three independent experiments. *P<0.05 represents a significant difference in toxin-treated and untreated mice

Table 1: Reversal of level 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 *Ropalidia marginata* venom anti-toxins

Bio-molecules	Group A	Group B	Group C	Group D	Group E
Protein	5.74±0.0288 (100)	5.24±0.0288 (91.28)	5.56±0.0288 (96.86)	5.68±0.0288 (98.95)	5.70±0.0288 (99.30)
Free amino acids	0.051±0.0288 (100)	0.053±0.028*8 (103.92)	0.052±0.0288 (101.96)	0.0505±0.0288* (98.03)	0.050±0.0288 (98.03)
Uric acid	00.845±0.0288 (100)	1.26±0.0288 (149.11)	1.16±0.0288 (137.27)	0.89±0.0288 (105.32)	0.86±0.0288 (102.38)
Cholesterol	3.81±0.0288 (100)	3.87±0.0288 (101.57)	3.84±0.0288 (100.78)	3.83±0.0288 (100.52)	3.81±0.0288 (100)
Pyruvic acid	0.60±0.0288 (100)	0.65±0.0288 (108.35)	0.63±0.0288 (105)	0.60±0.0288 (98.63)	0.60±0.0288 (106.66)
Total lipid	0.37±0.0288 (100)	0.445±0.0288 (120.27)	0.425±0.0288* (114.86)	0.38±0.0288 (102.70)	0.37±0.0288 (100)
Glucose	0.0925±0.0288 (100)	0.098±0.0577 (105.94)	0.0945±0.0288 (102.16)	0.092±0.0577 (101.08)	0.0925±0.0288 (100)

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)

Table 2: Reversal of level of certain enzymes i.e. acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, lactic dehydrogenase and acetylcholinesterase in blood serum of albino mice after injection with the saliva toxins pre-incubated with purified *Ropalidia marginata* venom anti-toxin

Enzymes	Group A	Group B	Group C	Group D	Group E
ACP	0.355±0.0288 (100)	0.455±0.0288 (125.35)	0.405±0.0288 (112.5)	0.375±0.0288 105.63)	0.365±0.0288* (102.81)
ALP	2.08±0.0577 (100)	2.4±0.0577 (114.8)	2.31±0.0577 (115.86)	2.165±0.0577 (104.08)	2.13±0.0577 (102.40)
GPT	0.040±0.0577 (100)	0.051±0.0577 (130)	0.046±0.0577 (115)	0.043±0.0577* (107.5)	0.041±0.0577 (102.5)
GOT	0.44±0.0577 (100)	0.46±0.0577 (104.54)	0.45±0.0577* (102.27)	0.43±0.0577 (97.72)	0.43±0.0577 (97.72)
LDH	7.95±0.0577 (100)	8.94±0.0577* (112.45)	8.02±0.0577 (100.88)	8.02±0.0577 (100.88) *	7.97±0.0577 (100.25)
ACHE	0.035±0.0577 (100)	0.028±0.0577 (80)	0.030±0.0577 (85.71)	0.034±0.0577 (97.14)	0.035±0.0577 (105.71)

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/h/mg prot, Glutamate oxaloacetate transaminase (GOT): Units of Glutamate oxaloacetate transaminase activity/h/mg protein. Lactic dehydrogense (LDH): µ moles of pyruvate reduced/45 min/mg protein. Acetylcholinesterase (AchE): µ moles 'SH' hydrolyzed/min/mg protein.

DISCUSSION

Hymenopteran insects, mainly bees, paper wasps, yellow jackets and ants possess stings. Stinging by these insects is very painful and is of major clinical significance. If the patient is stung by a group of wasps or bees, venom toxins generate more severe multiple pathophysiological effects, i.e. immediate hypersensitivity reactions, anaphylaxis, and death of the patient. It has been largely observed in nonallergic individuals who face both severe systemic reactions and toxic effects, which later on become a cause of death of patients [32].

Envenomation is a very serious problem for the beekeepers, passersby, and children playing near its colony. Wasps and bees suddenly attack at a little disturbance and inflict poisonous venom by means of the stinger into victims [33, 34]. This crude venom infliction breaks down the plasma membrane integrity, believing, rounding, and swelling and causes cellular injury [35]. Venom toxins bind to G-protein-dependent receptors [36]. Paper wasp venom toxins cause cardio-respiratory failure and systemic anaphylactic shock [37]. It results in sudden death of animals and patients [38].

All these toxic effects and disruption of the plasma membrane is done by low molecular weight toxin peptides, mainly phospholipase A2, and mellitin found in wasp venoms. These venom enzymes cause highly allergic and toxic in nature heavy cell lysis [39]. Wasp venom damages the nerve cell plasma membrane, loose its integrity [40] and impose intense myotoxic effects in victims [41]. In the present study wasp venom toxins were isolated and purified on a gel filtration column. These are low molecular proteins and trigger immune response in experimental mice (fig. 1-4). These were mixed with Freund's adjuvant used as the immunogen. For the preparation of complete immunogen, purified paper wasp venom toxins were mixed with Freund's adjuvant and injected into the experimental mice intra-peritoneal. After seven days, a booster dose was provided. Similarly, a 2nd booster was also given to the mice after 21 d (fig. 1). The presence of polyclonal antibodies in the antiserum was detected by the IDD method developed by of Ouchterlony. Due to antigen and antibody interaction, an equivalence zone was formed, which appeared in the form of a visible concentric band. This precipitation band represented the formation of the antigen-antibody complex (fig. 5).

The technique of Ouchterlony (1962) was used to identify the presence of polyclonal antibodies in the antiserum. An apparent concentric ring known as the equivalency zone was created as a result of the interaction between the antigen and the antibody (fig. 5). The development of the antigen-antibody combination was indicated by this precipitation band. Further, polyclonal antibodies were purified by octanoic acid followed by ammonium sulfate precipitation. It was found that the treatment of anti-paper wasp venom polyclonal antibodies with octanoic acid removed lipoproteins from the reaction mixture. Further, treatment of the anti-paper wasp venom polyclonal antibodies with ammonium sulfate precipitated the antibodies out of the solution and concentrated the antibodies in the form of a pellet. In this investigation, neutralization of paper wasp venom toxins was also observed. For this purpose, different concentrations (400 µg, 800 µg, 1200 µg) of polyclonal antibody were mixed and incubated with 40% of 24 h LD_{50}. This pre-incubated mixture was injected into the experimental mice. It was found that all the physiological effects were normalized and anti-venom reversed the oxidative stress and other adverse effects such as muscular paralysis, hypotension, and allergic responses in the experimental mice. Further, metabolism and enzyme alterations in the blood serum of the mice were also found reversed after 4 h of antibody treatment. Similarly, the efficacy of paper wasp anti-venom was also tested by Jones *et al.* (1999) [42].

Experimental mice injected with 40% and 80% of 24 h LD_{50} of purified paper wasp venom toxins were pre-incubated with different doses (400, 800, 1200µg) of the purified paper wasp anti-serum. More specifically, paper wasp anti-venom completely reversed the serum pyruvic acid; it was exceeded up to 106.66% after the 40% of the LD_{50} venom injection (table 1, fig. 6). It was also given a significant reversal in serum uric acid up to 102.38% while it was 149.11% in the venom-treated mice (table 1, fig. 6). After antiserum treatment, cholesterol which was marginally elevated in the venom-injected mice was also reversed fully at low concentrations of the anti-venom (table 1). The lipid level was reversed up to 102.70%, and that obtained in the venom-treated mice was 125% (table 1, fig. 6). In mice treated with only the venom serum protein, the content obtained was 89.69% which was reversed up to 91.28% after the

antiserum treatment (table 1, fig. 6). A similar treatment gave 100% reversal of free amino acid, which was increased to 99.30% in the mice treated with 40% of LD_{50} of the venom (table 1, fig. 6).

Experimental mice injected with 40% of 24 h LD_{50} of purified paper wasp venom toxins were pre-incubated with different doses (400, 800, 1200µg) of the purified paper wasp anti-serum. All metabolic alterations were reversed significantly after 4 h of the treatment. The elevated serum glucose content was successfully reversed at its normal value in the control group (100%), while it was merely increased in mice treated with 40% LD_{50}. Similarly, protein level was found completely restored after 4 h as it was noted as 96.8640%, 98.9540%, 99.30 40% in C, D and E experimental groups of mice. Total lipids were restored in all the treatment groups i.e. 100-120.27. Similarly, cholesterol level was fully restored 100.78 after 4H (table 1, fig. 6).

Neutralizing antibodies restored the level of metabolic enzymes in experimental animals. Reversal of enzymatic alterations by purified paper wasp anti-venom Anti-serum treatment also successfully normalized the alteration in serum enzyme just after 4h. Serum ACP content was obtained as 125.35% after 40% of LD50 venom injection, which was getting normalized up to 102.81% after 4 h of the anti-venom treatment (table 2, fig. 7). Similarly, Serum ALP content 114.8% elevation was reversed back to 102.40% after anti-venom treatment (table 2). Also, the GPT level was significantly reversed up to 102.5% while it was 130% in the venom-treated mice (table 2 fig. 7). A complete reversal was obtained at GPT level, which was obtained as 104.54% in the venom-treated animal (table 2). Similarly, LDH, which was elevated up to 112.45 % in venom-injected mice, was successfully reversed up to 100.25% after anti-venom treatment (table 2, fig. 7).

The neutralization efficacy of paper wasp anti-venom was tested by giving injections of venom toxins pre-incubated with antibodies [43]. In most cases, detoxification or toxoid production requires the neutralization of toxins, but at the same time, it shows maximum immunogenicity [44]. Thus, for the generation of more effective antiserum adjuvants are used as immunogen. For commercial production of anti-serum, pharma industries use rabbits, goats, and sheep [45]. Similar activity is reported in sheep and equine commercial anti-venoms [46]. Normally, sheep present very high tolerance both to Freund's adjuvant and other adjuvants with no local lesions. However, for neutralization of toxin-induced effects of wasp venom, antiserum was also added to purified toxins for *in vitro* incubation. It was found quite effective and observed as a successful treatment in beekeepers [47].

Paper wasp venom immunotherapy is an efficient treatment for wasp venom allergy and toxicity. It does not make any side effects and successfully restore physiological effects [48, 49]. In response to the venom toxins, B-lymphocytes release a group of immunoglobulins each recognizing different epitopes, which bind to venom toxins [50-52]. Venom immunotherapy develops peripheral tolerance that is mediated by the production of blocking IgG/IgG4 antibodies that may inhibit IgE-dependent reactions through both high affinity (FcεRI) and low affinity (FcεRII) IgE receptors on mast cells, basophils, and B cells [53]. The generation of antigen-specific regulatory T cells produces IL-10 and suppresses Th2 immunity and the immune responses shift toward a Th1-type response. B regulatory cells are also involved in the production of IL-10 and the development of long-term immune tolerance.

During VIT the number of effector cells in target organs also decreases, such as mast cells, basophils, innate type 2 lymphocytes, and eosinophils [54]. VIT was found to be associated with changes level of certain proteins i.e., fibrinogen alpha chain, complement C4-A, complement C3, filamin-B, kininogen-1, myosin-9 and inter-alpha-trypsin inhibitor heavy chain H1 (Venom immunotherapy of patients leads to an increase in allergen-specific IgG, it shows clinical improvement of the patient after administration of antiserum.

IgG is the major antibody that is synthesized by plasma B-cells and is much safer to be used in wasp venom immunotherapy. It may be used as the universal safety kit for the cure of toxin envenomation [55]. After treatment of patients with antitoxins generated against bee venom hypersensitivity, specific IgE antibodies get decreased from 24 to 6 kW/l, while specific IgG increased after VIT immunotherapy finished the systemic reactions generated due to allergens. In the present study, the efficacy of antiserum was explored in the form of reversed metabolic reactions and restoration of the physiology of various biomolecules, including enzyme level. In VIT-treated experimental mice anti-venom has successfully restored the level of serum proteins. Further, cardiopulmonary resuscitation allowed survival in a case of near-fatal. In the present study anti-toxins (polyclonal antibodies) synthesized against purified wasp venom toxins successfully reversed the pathophysiological alterations. It was noted that after 4 h of the level of bio-molecules and enzymes get reversed or restored. Behavioral effects were also found revered in experimental mice after anti-toxin treatment [56].

CONCLUSION

Hymenoptera venom is a complex mixture of many substances such as toxins, enzymes, growth factor activators, and inhibitors. These bioactive agents impose deleterious effects on cells and tissues after venom infliction. These enzymes disrupt cellular membranes and induce hypersensitive reactions, including life-threatening anaphylaxis. Polyclonal antibodies successfully neutralize 80 to 95% of toxic effects in patients within 4-5 h. Therefore, it can be concluded that the anti-venom produced against paper wasp venom has wider clinical and therapeutic applications for the retardation of all physiological alterations caused by paper wasp venom toxins. In the present study anti-toxins (polyclonal antibodies) synthesized against purified wasp venom toxins successfully reversed the pathophysiological alterations. It was noted that after 4h of time, bio-molecules and enzymes get reversed or restored. Behavioral effects were also found revered in experimental mice after anti-toxin treatment.

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AUTHORS CONTRIBUTIONS

Ravi Kant Upadhyay and Simran Sharma were responsible for the conception, experiments, writing and revising the manuscript.

CONFLICT OF INTERESTS

Declared none

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