CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Venoms and commercial antivenoms

Pooled and lyophilized crude venoms of *Naja naja* (NnV), *Bungarus caeruleus* (KV), *Daboia russelii* (RvV) and *Echis carinatus* (EcV) were procured from Irula Snake Catchers Co-Operative, Tamil Nadu. Pooled and lyophilized crude venom of Indian monocled cobra, *Naja kaouthia* (NkV), was collected from Kamrup district of Assam, North-East India (NEI), under the permission from the Assam State Biodiversity Board, Guwahati (ABB/Permission/2021/114). Pooled and lyophilized venom of *Mesobuthus tamulus* (Indian red scorpion) of western Indian (Maharashtra state) origin was a gift from Premium Serum and Vaccine Pvt. Ltd. (PSVPL), Pune, India.

Lyophilized commercial equine anti-snake antivenom (PAV) was procured from PSVPL, Pune, India (batch no.: ASVS(I)ly-015). Lyophilized equine anti-scorpion antivenom (ASA) was obtained from PSVPL, Pune, India (batch No.: SS170401).

3.1.2 Synthetic custom peptides and polyclonal antibodies

Custom peptides designed based on the antigenic epitopes of *Naja naja* venom PLA_2 toxin (CP1, PVDDLDRCCQVHDGGGGNACAASVCDCDRLAAICFAG), *Daboia russelii* venom PLA_2 toxin (CP2, TDRCCFVHDCCYGNLGGGGENRICECDKAAAICFR), *Echis carinatus* venom Echicetin toxin (CP3, EEILVDIVVSGGGFRSYEIAIRYSECFVLEKQSVFRTWVATP), *Bungarus caeruleus* venom basic PLA_2 β-bungarotoxin (CP4, PIDALDRCCYVHDNCYGGGRRTIICYGAAGTCARIVCDCDRTAALCFGD) and *Bungarus caeruleus* venom basic PLA_2 KPA2 toxin (CP5, PVDELDRCCYTHDGGGGADTCARFLCDCDRTAAICFASA) were synthesized by S Biochem, Thrissur, Kerala, India. Sequences of these peptides are shown in Chapter IV. Custom peptides designed based on the antigenic epitopes of *Mesobuthus tamulus* venom Na^+ channel toxin (α-neurotoxin) (CPS1, CWWVPYGVVSWSEDLPTPVP; CPS2, YISTFNNYSHALSTDC) and K^+ channel toxin (Tamapin) (CPS3, SNLRRSELSSRSLGLLGKC; CPS4, DVKSISSQESWIASKKVC) were synthesized

by S Biochem, Thrissur, Kerala, India. Sequences of these peptides are shown in Chapter V.

Polyclonal antibodies against these custom peptides were produced in rabbits by BioBharati Pvt. Ltd., Kolkata, India.

3.1.3 Animals

The animals used in this study were laboratory-inbreed, pathogen-free Wistar strain albino rats of both sexes aged 2-3 months (180-220 g) purchased from M/s Chakrabarty Enterprise, Kolkata. All the animal experimentations were carried out after approval from the Institutional Animals Ethics Committee of Institute of Advanced Study in Science and Technology, Guwahati, Assam, India (IASST/IAEC/2022/09). The CCSEA (Committee for Control and Supervision of Experiments on Animals) guidelines were followed for the maintenance and use of the Wistar strain albino rats. The rats were acclimatized at 22 ± 3°C with a relative humidity of 30-70% and fed with a standard diet of "Amrut" procured from Krishna Valley Agrotech LLP, Pune, Maharashtra, India, and water *ad libitum*. They were maintained in a 12:12 h light-dark cycle.

Further, BioBharati Pvt. Ltd. antibody production was carried out using white New Zealand rabbits (*Oryctolagus cuniculus*), which were maintained and used according to the guidelines of Institutional Animals Ethics Committee and approved by CCSEA (Registration number of BioBharati Pvt. Ltd. animal house facility: 2309/PO/Rc/S/2024/CCSEA). The rabbits were kept under a 12:12 hour light-dark cycle, with room temperature regulated between 17-21°C and relative humidity maintained at 30-70%.

The present works have been applied for two patents (Application numbers: 202331015487 and 202431065376).

3.1.4 Other fine chemicals

Pre-stained protein molecular markers were bought from BioRad (#16103777; 2–250 kDa). Anti-horse IgG HRP (horse radish peroxidase) conjugated secondary antibodies were purchased from Sigma-Aldrich, USA. Polyvinylidene fluoride (PVDF) membrane, Immobilon®-P PVDF Membrane with 0.45 µm pore size was purchased

from Millipore, Merck, Germany. Other analytical grade reagents, Tris-buffer, phosphate buffer saline, solvents like acetonitrile, methanol, SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) and others were purchased from Sigma-Aldrich, USA or Merck, Germany.

3.2 Methods

3.2.1 Identification and designing of the antigenic epitopes from snake and scorpion toxins

3.2.1.1 Antigenic epitopes from principal toxins of NnV, KV, RvV, EcV and MTV

The amino acid sequences of the major toxins of 'Big Four' venomous snakes of India (PLA_2 toxin of NnV, PLA_2 toxin of RvV, Echicetin of EcV, and β-bungarotoxin and Basic PLA_2 toxin of KV and, two major toxins of MTV, viz. Na^+ and K^+ ion-channel toxins) were retrieved from the National Centre for Biotechnology Information (NCBI) (https://ncbi.nlm.nih.gov) and UniProt (https://uniprot.org) servers. After that, these sequences were submitted to the Immunomedicine Group: Predicted Antigenic Peptides online server (http://imed.med.ucm.es/Tools/antigenic.pl) for antigenic region determination [1]. Predictions of antigenic regions are based on the occurrence of amino acid residues in segmental epitopes known experimentally. The epitopes with antigenic propensity greater than 1.0 were determined as antigenic regions for raising antibodies [1]. Therefore, antigenic peptides with antigenic propensity >1 with amino acid residues on the surface of the toxin were selected and modified to raise polyclonal antibodies in rabbits.

Custom peptides CP1-5 from the snake venom toxins were designed by combining two antigenic epitopes identified for each toxin sequence to increase the antigenic propensity and raise polyclonal antibodies in rabbits (sequences in Chapter IV). Protein-protein BLAST (BLASTp) (https://blast.ncbi.nlm.nih.gov/) searches were performed against a nonredundant protein database under default settings using the custom peptide sequences to determine the sequence identity of CP sequences with *N. kaouthia* PLA_2.

For the custom peptide designed based on the MTV toxins, CPS1, the peptide sequence was initiated from the Trp residue, and a terminal Cys residue was added before the Trp residue. In the case of CPS2 and CPS4, additional terminal Cys residues

were included. For CPS3, a Ser residue was added, while the terminal Cys residue remained unchanged. These alterations were implemented in the peptide design to account for the peptide synthesis, purification procedure, and subsequent Keyhole Limpet Hemocyanin (KLH) conjugation of peptides.

3.2.2 Raising polyclonal antibodies against the toxin-epitope-specific custom peptides

3.2.2.1 KLH conjugation of the custom peptides

The custom peptides were conjugated with KLH as described by [2]. Briefly, m-maleimidobenzoyl-Nhydroxysuccinimide ester (MBS) was dissolved in 200 µL dimethyl formamide (DMF), and the solution was gently stirred at room temperature for 30 min after adding 70 µL of this solution to a 10 mg/mL KLH solution prepared in 10 mM phosphate buffer, pH 7.0. Sephadex G25 size exclusion chromatography was used to remove the free crosslinker (MBS-DMF). Firstly, column equilibration was done in 50 mM phosphate buffer (pH 7.0). The same buffer was used to load the KLH reaction mixture into the column and then elute it. The KLH with a molecular weight of ~400 kDa was eluted in the void volume. The custom peptides dissolved in 100 µL of DMF were rapidly mixed with the purified KLH/MBS. The mixture was vortexed, and 2N NaOH was used to adjust the pH to 7.0-7.2. The mixture was stirred overnight at 4°C to allow the peptides to conjugate to KLH. The next day, the solution was lyophilized after adding 0.1 M ammonium bicarbonate. The coupling efficiency of the synthetic peptides was determined using the Cysteine Standard Assay [3]. The lyophilized conjugates were outsourced to BioBharati Pvt. Ltd. to generate peptide-specific polyclonal antibodies against snakes and *M. tamulus* venoms in rabbits.

3.2.2.2 Raising and purifying custom peptide-specific antibodies by immunizing rabbits with KLH-conjugated custom peptides

Briefly, 2 mL of blood was drawn as pre-immune serum from the white New Zealand rabbits. After drawing the pre-immune serum, primary immunization was performed 24 h later by injecting ~200 µg of the KLH-conjugated peptides in Freund's complete adjuvant subcutaneously. A first booster dose of 100 µg KLH-conjugatedpeptides in Freund's incomplete adjuvant was administered to the rabbits and kept under observation 15 days later.

On the 10th day after administration of the first dose, a second booster dose (in Freund's incomplete adjuvant) was injected into the rabbits. Again, on the following 10th day, a test bleed was done from the ear vein; serum was separated, and an ELISA was performed to check the antibody titre. On the 10th day post the second booster dose, a third booster dose in Freund's incomplete adjuvant was administered to the rabbits. After that, a test bleed was done from the ear vein again, and ELISA was performed with the separated serum to determine the antibody titre.

For the ELISA, 100 µL solution of 2 µg/mL native peptide (non-KLH conjugated custom peptide) in phosphate buffer saline (PBS) (200 ng/well) was used to coat the microtitre wells and left overnight at 4°C. Afterwards, 250 µL of 1% BSA in PBS was added and incubated for 1 h at room temperature. After washing the wells thrice with wash buffer (PBS with 0.05% Tween 20), diluted serum samples (100 µL) were incubated for 30 min at room temperature. The wells were washed thrice with wash buffer again, and 100 µL of Protein A-HRP (Zymed, USA) was diluted to 1/60,000 in blocking buffer (PBS with 5% BSA). The wells were incubated for 30 min at room temperature, washed with wash buffer, and then incubated in the dark with 100 µL of TMB/H_2O_2 substrate solution at room temperature for 30 min. Lastly, 50 µL of 2M H_2SO_4 was used to stop the reaction, and then absorbance was recorded at 450 nm (primary wavelength) and 630 nm (reference wavelength) against the reagent blank.

Affinity chromatography was employed to purify each polyclonal antibody from the rabbit antisera [4-6]. Dry CnBr-activated sepharose 4B resin was swelled and activated using 100 mM carbonate buffer (pH 8.5). The native peptide (2-5 mg) was separately dissolved into the 100 mM carbonate buffer for coupling. Each peptide was added to the resin separately and incubated 16 h at 4°C. Any unreacted peptide was washed from the resin using 100 mM carbonate buffer (pH 8.5). The peptide-coupled resin was incubated with 100 mM Tris-Cl buffer pH 8 for 2 h at room temperature. The resin was washed thrice using PBS. After that, 5 mL of antisera was mixed with 5 mL PBS (1:1) and incubated with the peptide-coupled resin for 16 h at 4°C. The resin was collected and washed thrice using PBS. Then, the bound antibody was eluted using 10mM Glycine, pH 2.5, and after collection, the pH was neutralized using 3M Tris-Cl pH 8.8.

The immune reactivity of the purified polyclonal antibody (PAb) against their respective custom peptide (against which they were raised) and KLH was assessed by dot blot analysis. Briefly, the polyvinylidene fluoride (PVDF) membrane was activated with 100% methanol and then equilibrated with 1X TBS (tris buffer saline) with 0.05% tween-20 (TBS-T). After that, 2 µg (1 µL) of the KLH conjugated custom peptide/KLH was spotted onto the activated membrane and air-dried. Non-specific binding was blocked by 5% fat-free skimmed milk and incubated at room temperature for 1 h, with gentle shaking. The membrane was then washed with TBS-T and incubated with the PAbs (1 µg/µL PAbs) at a dilution of 1:1000. Anti-rabbit HRP-conjugated secondary antibody at 1:2000 dilutions were used to detect the primary antibodies (PAbs). The blot was developed using Enhanced Chemiluminescence (ECL) substrate (Cat no. 1705060, Bio-Rad) using the ChemiDoc imaging system with Image Lab software (Bio-Rad, USA), and the intensity of the dots was measured using ImageJ software (National Institute of Health USA, http://imagej.nih.gov/ij).

For dot intensity analysis using ImgaeJ software, the images were background corrected using the *Process/Subtract Background* command of ImageJ and setting the rolling ball radius to 25 pixels. Post-background correction, "Integrated Density" was enabled using *Analyze/Set Measurements* command. Then, the circular selection tool was chosen, moved over the first dot, and measurement was done by *Analyze/Measure* command. The whole image of the blot was inverted using *Edit/Invert* command for the correct calculation of integrated density.

Five PAbs were raised against CP1-5 from the snake venom toxins, viz. PAb 1, 2, 3, 4 and 5. On the other hand, four PAbs were raised against CPS1-4 from the *Mesobuthus tamulus* venom toxins, viz. PAb 6, 7, 8 and 9.

3.2.3 Determination of *in vitro* immune cross-reactivity of individual PAbs, PAb formulations and commercial anti-snake PAV towards snake venoms

3.2.3.1 Dot blot analysis

For the dot blot analysis, the antibodies were spotted on the activated PVDF membrane, with venom samples sandwiched between the spotted capture antibody and primary antibody). Briefly, PVDF membranes were activated with 100% methanol for 2 min and washed with TBS-T for 15-30 min. The activated membranes were spotted

with 2 µg individual PAbs, PAb formulations and commercial anti-snake PAV, and non-specific binding was blocked with 5% BSA in TBS-T solution for 1 h. The membranes were incubated with the snake venom-spiked rat plasma (1 pg/µL) and *Mesobuthus tamulus* venom-spiked rat plasma (0.3 ng/µL) for 30 min at room temperature and washed thrice with TBS-T. Post-washing, the membranes were incubated for 45 min with individual PAbs, PAb formulations and commercial anti-snake PAV as the primary antibodies at 1:1000 dilution. The HRP-conjugated anti-rabbit IgG antibody and HRP-conjugated anti-horse IgG antibody detected the individual PAbs and PAb formulations and commercial anti-snake PAV at 1:2000 dilutions. The immunoblots were developed using the ECL substrate described above. The dot intensities were analyzed using ImageJ software. The Process/Subtract Background command was used to adjust the pictures' backgrounds to perform dot blot intensity analysis in ImageJ, and the rolling ball radius was set to 25 pixels. After background correction of the photos, the "Integrated Density" option was activated in the Analyze/Set Measurements command, the circular selection tool was dragged over the dots, and measurements were taken using the Analyze/Measure command. The Edit/Invert command inverted the whole picture of the blot in order to calculate integrated density correctly. The PAb formulations prepared by combining the PAbs in different combinations are mentioned in Table 3.1.

Table 3.1. List of PAb formulations comprised of PAbs mixed in different combinations and ratios

PAb formulation	**Combination and ratio**
FPAb	PAb 1+2+3+4+5 (1:1:1:1:1, w/w/w/w/w)
PAbE	PAb 1+4+5 (1:1:1, w/w/w)
PAbV	PAb 2+3 (1:1, w/w)

Since we used the same antibody for capture and detection, another dot blot assay was done, in which we let the secondary antibody directly bind to the capture antibody (control without antigen). The dot intensities of the blots obtained from the above sandwich assay were normalized against the dot intensities of the control without antigen. The analysis was performed in triplicates.

Due to the better immune recognition demonstrated by the PAb formulation FPAb compared to the individual PAbs and other PAb formulations, the subsequent experiments were performed only with FPAb and commercial anti-snake PAV as antibodies.

3.2.3.2 Western blot analysis

Western blot analysis was performed as per previous protocols to evaluate the immunorecognition of the Indian snake venoms (NnV, KV, RvV, EcV, and NkV) against FPAb and commercial anti-snake PAV [7,8]. Briefly, 80 µg (protein content) of snake venoms were separated in a 12.5% SDS-PAGE reduced using β-mercaptoethanol and dithiothreitol (DTT) according to the method described by Laemmli [9]. Resolving and stacking gels were prepared as follows –

A. 12.5% resolving gel (10 mL) -

1. 1.5 M Tris-HCl (pH 8.8) : 2.5 mL

2. 30% acrylamide-bisacrylamide solution : 4.0 mL

3. Type I water : 3.3 mL

4. 10% SDS : 100 µL

5. 10% ammonium persulphate : 100 µL

6. TEMED (Tetramethylethylenediamine) : 10 µL

The abovementioned solution mixture was poured into a gel cassette and allowed to polymerize at room temperature.

B. 4% stacking gel (5 ml) –

1. 0.5 M Tris-HCl (pH 6.8) : 2.5 mL

2. 30% acrylamide-bisacrylamide solution : 0.6 mL

3. Type I water : 1.5 mL

4. 10% SDS : 50 µL

5. 10% ammonium persulphate : 50 µL

6. TEMED : 10 µL

The separated proteins on the gel were then transferred to a methanol-activated PVDF membrane using a Trans-Blot SD semi-dry cell (Bio-Rad). The transfer efficacy of the proteins to the PVDF membrane was determined using 0.5% Ponceau-S red. Post-overnight blocking of the membrane with 5% BSA in TBS-T at 4°C to prevent non-specific binding of the antibodies, the membrane was washed thrice with TBS-T. The membrane was then incubated with FPAb and commercial anti-snake PAV as primary antibody (1:1000 dilution) at room temperature for 2 h. The membrane was incubated with HRP-conjugated anti-rabbit IgG antibody for FPAb and HRP-conjugated anti-horse IgG antibody for commercial anti-snake PAV, at 1:4000 dilution for 1 h at room temperature. The membranes were then washed thrice with TBS-T. The immunoblot was developed using ECL substrate (Cat no. 1705060, Bio-Rad) using the ChemiDoc imaging system with Image Lab software (Bio-Rad, USA). The aggregate band intensities were computed utilizing ImageQuant™ analysis software (Cytiva, USA). The lanes in the blots were selected and treated with Subtract Background command to diminish background noise, employing a rolling ball radius of 50 pixels. The bands were detected in the lanes, and quantified.

3.2.3.3 Spectrofluorometric analysis to determine the venom-antibody interactions

Spectrofluorometric analysis was performed using the methodology described previously [8,10]. In brief, a fixed concentration of each snake venom (0.01 mg/mL) was incubated with different concentrations of PAb formulation (FPAb)/commercial anti-snake PAV (0.01-1.28 mg/mL). For analysis of the reaction mixture, the excitation wavelength was set at 280 nm, temperature at 25°C, emission slits at 5 nm, and emission spectra were recorded from 300 to 500 nm using the Varioskan LUX Multimode Microplate reader (Thermo Fisher Scientific, Denmark). The fluorescence spectra obtained of only venom/only FPAb or commercial anti-snake

PAV were considered as control, and the relative fluorescence spectra intensity (λ_{max}) of snake venom-FPAb/commercial anti-snake PAV interactions were determined by comparing to the control. Change in relative fluorescence spectra intensity ($\Delta\lambda_{max}$) was plotted against the FPAb/commercial anti-snake PAV concentration (mg/mL) to obtain a graph using GraphPad Prism 5.0 software [11,12], and the K_D values were calculated from the graph.

3.2.4 Determination of *in vivo* immune cross-reactivity of the FPAb and commercial anti-snake PAV towards snake venoms in the plasma of envenomed animal model

3.2.4.1 Envenomation of animal model

Albino Wistar strain rats were used to stimulate the experimental envenomation, as shown in a schematic diagram (Fig. 3.1), and a description of methods is mentioned below.

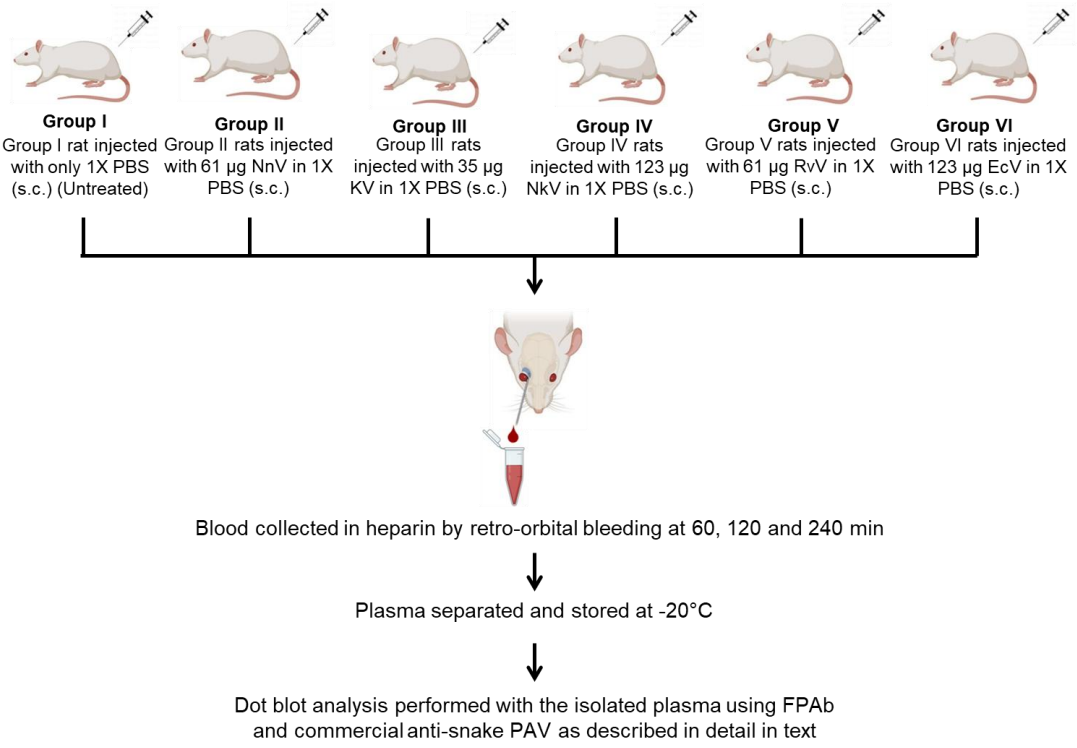

Fig. 3.1 Flow diagram of a methodology for NnV, KV, NkV, RvV and EcV envenomation simulation of Wistar rats by subcutaneous (s.c.) routes and detection of venom in the plasma of envenomed rats. Schematic representations were generated using Biorender (©BioRender: biorender.com).

The experimental rats were divided into six groups (n=30). Individual groups of albino Wistar strain rats (220 ± 10 g, n=5) were subcutaneously injected with different dosages of each venom. Attention was given to the animal's minimum pain during the entire experiment. Group 1 rats (n=5) were injected with subcutaneous injections of 1X PBS and served as controls. Further, five groups of rats (groups 2, 3, 4, 5, and 6, n=5) were injected subcutaneously with the respective venoms (NnV, KV, RvV, EcV, and NkV) dissolved in 200 µL of 1X PBS.

The groups of rats (n=5) used herein are as mentioned: - Group 1: Subcutaneous (s.c.) injection of 1X PBS; Group 2: s.c. injection of NnV (0.28 mg/kg); Group 3: s.c. injection of KV (0.16 mg/kg); Group 4: s.c. injection of RvV (0.28 mg/kg); Group 5: s.c. injection of EcV (0.56 mg/kg); Group 6: s.c. injection of NkV (0.56 mg/kg).

3.2.4.2 Determination of immune-reactivity of the FPAb and commercial anti-snake PAV towards Indian snake venoms in the plasma of envenomed animal model

From each animal of the control and envenomed rat groups, blood collection was done from their retro-orbital plexus at the intervals of 60 min, 120 min, and 240 min, post-venom injection, in tubes containing heparin as an anticoagulant (5% of the total volume of blood collected). The blood collected in the tubes was centrifuged to separate the plasma on a fixed rotor centrifuge at 4°C with 4300 rpm for 15 min. The plasma was stored at -20°C and used within seven days.

The plasma samples were analysed using dot blot analysis, as mentioned above. Briefly, 2 µg of the FPAb/commercial anti-snake PAV (2 µg/µL) were spotted onto the activated PVDF membranes, followed by incubating with 5% BSA solution in TBS-T for 1 h at room temperature to prevent non-specific binding. After washing the membranes with TBS-T, they were treated with 10 µL of the plasma at room temperature for 15 min. Further, the membranes were incubated with FPAb/commercial anti-snake PAV for 45 min, at 1:1000 dilution. The FPAb/commercial PAV were detected by the HRP-conjugated anti-rabbit IgG/anti-horse IgG at 1:2000 dilution. The resulting immunoblots were developed using an ECL substrate. The dot intensities were analysed using ImageJ software, and graphs were prepared using SigmaPlot.

3.2.5 Gold nanoparticle-based detection of snake venom in the plasma of envenomed animals

3.2.5.1 Synthesis of gold nanoparticles (AuNPs)

AuNP synthesis was carried out using the sodium citrate reduction method described previously [13,14]. Briefly, 50 mL chloroauric acid solution ($HAuCl_4$) (1 mM) was heated to reflux under stirring conditions at 100 rpm, and then 5 mL of 38 mM sodium citrate solution was added promptly. The solution was heated until the solution's colour transformed from pale yellow to wine red and then cooled down to room temperature with continuous stirring. The solution was stored in dark amber bottles at 4°C.

The AuNPs synthesized were characterized by UV-Vis (ultraviolet–visible) spectrophotometer, Transmission electron microscope (TEM), Zetasizer, Fourier-transform infrared spectroscopy (FTIR), and Atomic force microscopy (AFM) [15-18]. Optical measurements were carried out using a Shimadzu UV spectrophotometer UV-2600 to obtain the UV-vis spectra of the synthesized AuNPs in the range of 400-700 nm. The chemical bonds after AuNP preparation were recorded using the Nicolet 6700 FTIR instrument with KBr pellets. The surface zeta potential of the AuNPs was determined using the Zetasizer Nano ZS, 90 (Malvern, UK) instrument. The TEM images of the citrate-capped AuNPs were taken in a JEOL TEM-2100 plus model by the drop-casting method on a carbon-coated copper grid and analyzed using ImageJ software. AuNPs were dried on glass slides at room temperature and analyzed by atomic force microscope (NTEGRA PRIMA, NT-MDT technology) in the non-contact mode [19]. The images obtained were analyzed using Gwyddion software, and the heights of the particles were calculated using Origin software with Gaussian function.

3.2.5.2 Synthesis of AuNP-FPAb conjugates

The FPAb was conjugated to surface-modified AuNPs by covalent coupling [17], with slight modifications in the next step. The surface of AuNP was functionalized with a thiolated mercaptoundecanoic acid linker (MUA), with 11 carbon atoms between the COOH and SH groups. Briefly, 1 mL of 10 mM MUA was added to 5 mL AuNPs and incubated under stirring conditions at 100 rpm at 35°C for 24 h. After washing the surface-modified AuNPs thrice by centrifugation (10000 rpm, 30 min), they were

suspended in ultrapure water. The PAbF was conjugated to the surface-modified AuNPs via EDC/NHS [EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS: N-Hydroxysuccinimide] coupling chemistry, where the PAbF forms a pre-activated carboxylic acid amide bond by covalently binding to surface-modified AuNPs via their amino groups. Briefly, 2 mL of the surface-modified AuNPs was added to 200 µL EDC (50 µM) and incubated under stirring conditions for 30 min at 150 rpm. After that, 200 µL of NHS (75 µM) was added to the mixture and stirred for 30 min. Subsequently, 20 µg of FPAb (2 µg/µL) was allowed to react with the reaction mixture at room temperature for 30 min. The reaction mixture was left overnight at 4°C, and the next day, it was centrifuged at 10000 rpm for 30 min. The pellet obtained was suspended in 100 µL of phosphate buffer (10 mM, pH 7.4) after adding 1% (w/v) BSA to functionalize the non-antibody coated areas, forming the AuNP-FPAb conjugate. Protein estimation was done using the Bradford method, using BSA as a standard to determine the concentration of unbound antibodies remaining in the supernatant after the conjugation. The binding efficiency of the FPAb was calculated using the following equation (1),

$$\text{Efficiency (\%)} = ([\text{FPAb}]_0 - [\text{FPAb}])/ [\text{FPAb}]_0 * 100 \quad \text{….. (Equation 1)}$$

where $[\text{FPAb}]_0$ is the initial concentration of FPAb added to the AuNP solution and [FPAb] is the concentration from the supernatant after three consecutive washes.

The AuNP-FPAb conjugates were also characterized by UV–Vis spectrophotometer, FTIR, Zetasizer, TEM, and AFM.

3.2.5.3 Detection of snake venom using AuNP-FPAb conjugate

Different snake venom (NnV, KV, NkV, RvV and EcV) spiked rat plasma concentrations were incubated with 10 µL of AuNP-FPAb for 10 min. The colour changes in the solutions were observed in the transparent centrifuge tubes upon adding AuNP-FPAb. The mobile phone-based assay was performed by capturing photographs of the tubes using a smartphone and analysing them using ImageJ software. The images were captured with the Motorola Edge 40 Neo, featuring a 50-megapixel camera and a resolution of 1080 x 2400 pixels. The settings for image capture were configured to auto white balance, ISO 400, normal exposure, and the image format was set to JPEG. The average R (red), G (green) and B (blue) colour values from the photographs were

measured with the ImageJ image processing toolbox. Photographing of the samples and ImageJ processing of the photographs were carried out in triplicates. The RGB colour values obtained from ImageJ processing were converted to logarithmic scale to obtain colour intensity per the Lambert-Beer law equation in MS Excel 2021 (Fig. 3.2).

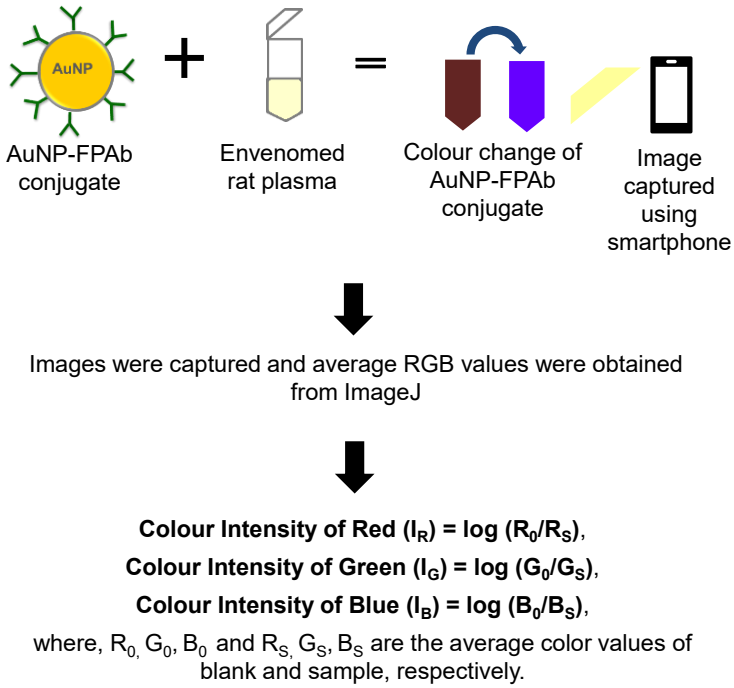

Fig. 3.2 Schematic illustrating the colorimetric assay by determining colour intensities using RGB values from the smartphone images of interaction between AuNP-FPAb conjugate and venom-treated plasma. Prepared using MS PowerPoint 2021.

For actual *in vivo* sample analysis, the plasma collected from the retro-orbital veins of the envenomed rats (s.c.) were treated with 10 µL of AuNP-FPAb for 10 min. The observed colour changes in the solutions were photographed, and the images were analyzed as mentioned above.

In order to learn more about how the AuNP-FPAb conjugate and envenomed snake plasma samples interact, a UV-Vis spectrophotometer (400-700 nm) was used. Additionally, the concentration of venom detected in the envenomed plasma was determined by constructing calibration curves using absorbances recorded from rat plasma samples spiked with snake venom incubated with the AuNP-FPAb conjugates [20].

3.2.6 Determination of *in vitro* immune cross-reactivity of individual PAbs, PAb formulations and commercial anti-scorpion antivenom (commercial ASA) towards MTV

3.2.6.1 Indirect ELISA

The indirect ELISA was performed per the protocol [8,21], followed by some modifications. The PAb formulations prepared by combining the PAbs in different ratios are mentioned in Table 3.2. Briefly, MTV (100 ng protein) were coated onto 96 well microtitre plates (in triplicate) and incubated overnight at 4°C. The next day, the wells were washed thrice with 1X PBS wash buffer and then incubated at room temperature for 2 h with individual PAbs as primary antibodies in 1:4, 1:10, 1:20, 1:40, and 1:60 (MTV: PAb, protein: protein) ratio, followed by washing with wash buffer. Next, the wells were incubated at room temperature for 2 h with anti-rabbit IgG HRP conjugated secondary antibody of 1:2000 to detect the PAb. After three consecutive washes with wash buffer, the wells were incubated with 100 µL 1X TMB/H_2O_2 for another 30 min in dark conditions, and 50 µL of 2M H_2SO_4 was added to stop the reaction. Absorbance was measured at 492 nm in MultiskanGO (Thermo Scientific, USA) microplate reader against reagent blank. The analysis was performed in triplicates.

Table 3.2. List of PAb formulations comprised of PAbs mixed in different combinations and ratios

PAb formulation	Combination and ratio
PAbF	PAb 6+7+8 (1:1:1, w/w/w)
PAbF 2	PAb 6+7+8+9 (1:1:1:1, w/w/w/w)
PAbF 3	PAb 6+7 (1:1, w/w)
PAbF 4	PAb 6+8 (1:1, w/w)
PAbF 5	PAb 6+9 (1:1, w/w)
PAbF 6	PAb 7+8 (1:1, w/w)
PAbF 7	PAb 7+9 (1:1, w/w)
PAbF 8	PAb 8+9 (1:1, w/w)

PAbF 9	PAb 6+7+9 (1:1:1, w/w/w)
PAbF 10	PAb 8+9+6 (1:1:1, w/w/w)
PAbF 11	PAb 8+9+7 (1:1:1, w/w/w)

Subsequently, based on the results obtained, another indirect ELISA was performed with the PAb formulation (PAbF) having the best immune-reactivity (containing a lesser number of PAbs in combination) and commercial ASA as primary antibodies in 1:40 (MTV: PAbF/commercial ASA, protein: protein) ratio. The primary antibodies were recognized by anti-rabbit IgG HRP/anti-horse IgG conjugated secondary antibody at 1:2000 dilution.

3.2.6.2 Dot blot analysis

Dot blot analysis was performed as described in section 3.2.3.2 above. The activated PVDF membranes were spotted with 2 µg individual PAbs, PAb formulations and commercial ASA, followed by blocking of non-specific binding using 5% BSA in TBS-T solution. After that, the membranes were incubated with MTV (0.3ng/µL) spiked rat plasma for 30 min at room temperature. This concentration of MTV was calculated considering that an adult *M. tamulus* can inject 1.5 mg of venom into an adult human with approximately 5 L of blood; therefore, the concentration of MTV in blood comes out to be 0.3 ng/µL [22].

After washing, the membranes were incubated with PAbs, PAb formulations and commercial ASA at 1:1000 dilutions for 45 min. The anti-rabbit IgG HRP detected the primary antibody conjugated (for PAbs and PAb formulations)/anti-horse IgG HRP conjugated (for commercial ASA) secondary antibody (1:2000 dilutions), and the blots were developed by ECL substrate as described above. ImageJ software was used to analyze the dot intensities. Since we used the same antibody for capture and detection, we performed another dot blot assay where we let the secondary antibody directly bind to the capture antibody (control without antigen). The dot intensities of the blots obtained from the above sandwich assay were normalized against the dot intensities of the control without antigen. The analysis was performed in triplicates. In another set of experiments, the potency of PAbF (1 µg/µL) to recognize the different concentrations of

MTV (0.3, 0.15, and 0.075 ng/ µL) spiked rat plasma was studied by dot blot assay. The analysis was performed in triplicates.

3.2.6.3 Western blot analysis

Western blot analysis evaluated immunorecognition of MTV against PAbF, and the protocol was adapted from previous reports [7,8,23]. Briefly, 80 µg MTV proteins were separated in a 15% SDS-PAGE under reduced conditions, and the proteins were transferred to a methanol-activated PVDF membrane. The 15% resolving gel was prepared below, and the 4% stacking gel was prepared per the protocol mentioned in section 3.2.3.1.

15% resolving gel (10 mL) –

1. 1.5 M Tris-HCl (pH 8.8) : 2.5 mL
2. 30% acrylamide-bisacrylamide solution : 5.0 mL
3. Type I water : 2.3 mL
4. 10% SDS : 100 µL
5. 10% ammonium persulphate : 100 µL
6. TEMED (Tetramethylethylenediamine) : 10 µL

Post-transfer of the proteins, transfer efficacy was determined by staining the PVDF membrane with 0.5% Ponceau-S red. The membrane was incubated overnight with 5% BSA in TBS-T at 4°C (to prevent non-specific binding of PAbF). The next day, after washing the membrane thrice with TBS-T, PAbF was added (1:1000 dilution) as the primary antibody and incubated at room temperature for 2 h. After three consecutive washes, the membrane was incubated with anti-rabbit IgG-HRP conjugated antibody (1:4000 dilutions), and the immunoblot was developed with ECL substrate as described above.

3.2.6.4 Spectrofluorometric analysis to determine the venom-antibody interactions

The methodology used to study the MTV-PAbF and MTV-commercial ASA interaction by spectrofluorometric analysis was described above in section 3.2.3.3.

Briefly, graded concentrations of PAbF and commercial ASA (0.01-1.28 mg/mL) were incubated at room temperature with a fixed concentration of MTV (0.01 mg/mL). The analyses of the reaction mixtures were conducted in the Varioskan LUX Multimode Microplate reader (Thermo Fisher Scientific, Denmark) by setting the excitation wavelength at 280 nm, temperature at 25°C, emission slits set at 5 nm, and emission spectra was monitored from 300 to 500 nm. Fluorescence spectra of MTV/PAbF and MTV/commercial ASA were determined as a control and compared with the relative fluorescence spectra intensity (λ_{max}) of MTV-PAbF and MTV-commercial ASA interaction. The change in intensity ($\Delta\lambda_{max}$) was plotted against the PAbF/commercial ASA concentration (mg/mL) using GraphPad Prism 5.0 software, and the K_D value was calculated. The K_D value obtained for MTV-PAbF interaction was compared with the K_D value of MTV-commercial ASA interaction under identical conditions.

3.2.7 Determination of *in vivo* immune cross-reactivity of the PAbF and commercial ASA towards MTV in the plasma of envenomed animal model

The detailed protocol of MTV determination in the plasma of envenomed rats is shown in a schematic diagram (Fig. 3.3), and a description of methods is mentioned below.

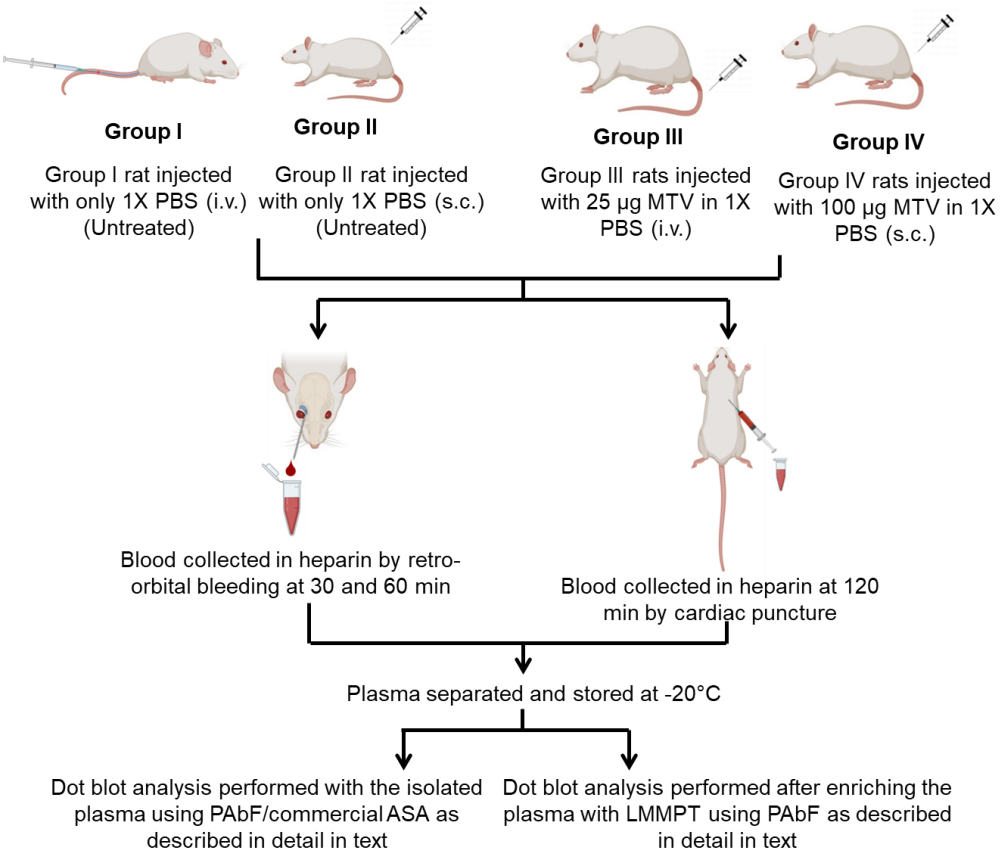

Fig. 3.3 Flow diagram of a methodology for MTV envenomation simulation of Wistar rats, by intravenous (i.v.) and subcutaneous (s.c.) routes, and Detection of MTV in the plasma of envenomed rats. Schematic representations were generated using Biorender (©BioRender: biorender.com).

3.2.7.1 Envenomation of animal model

Envenomation of albino Wistar strain rats were simulated by adopting the method described in section 3.2.4.1 above. The albino Wistar strain rats were divided into four groups (220 ± 10 g, n = 20). As already explained, an adult of an average 60 kg weight can receive a maximum of 1.5 mg MTV in a sting equivalent to 25 µg/kg (5 µg/200 g). Therefore, the group II rats (n = 5) were intravenously injected with 25 µg (approximately five times higher than the amount of MTV entering the bloodstream of an average adult human in one sting) of MTV dissolved in 200 µL of 1X PBS. Meanwhile, group IV rats (n = 5) were subcutaneously injected with 100 µg MTV dissolved in 200 µL of 1X PBS (four times higher than the venom injected

intravenously). Group I (n = 5) and group II (n = 5) were intravenously and subcutaneously injected with only 1X PBS (untreated control), respectively**.**

3.2.7.2 Determination of immune-reactivity of the PAbF and commercial ASA towards MTV in the plasma of envenomed animal model

Blood was collected from the retro-orbital veins of groups I-IV rats at 30 min and 60 min after venom injection in tubes containing heparin as an anticoagulant (5% of total blood volume collected). Blood was collected by cardiac puncture after 120 min of venom injection from all the groups of rats. The tubes were centrifuged on the fixed rotor centrifuge at 4°C with 4300 rpm for 15 min (Eppendorf refrigerated Centrifuge 5804 R) to separate the plasma and stored in aliquots at -20°C until use. Nevertheless, the storage duration never exceeded seven days.

Dot blot analysis was performed by spotting the PAbF/commercial ASA on the activated PVDF membrane, with envenomed and control plasma samples sandwiched between the PAbF/commercial ASA as the capture antibody and the PAbF/commercial ASA as the primary antibody as described in section 3.2.4.2. Briefly, the activated PVDF membranes spotted with the PAbF/commercial ASA (4 µg) were incubated at room temperature with 10 µL of the plasma (group I-IV) for 15 min. The envenomed plasma was immune-recognized by PAbF/commercial ASA as the primary antibody at 1:500 dilutions for 45 min, which was further detected by the anti-rabbit/anti-horse HRP-conjugated antibody (1:1000). The blots were developed by ECL substrate, and their intensities were measured using ImageJ software. The dot intensities obtained were normalized against those obtained from control without antigen. The analysis was performed in triplicates.

3.2.7.3 A process for enriching low molecular mass peptides in plasma

A slightly modified acetonitrile (ACN) precipitation method may effectively precipitate large abundant proteins and disrupt the binding of low molecular mass MTV toxins to their carrier proteins. It may help enrich the plasma with only our desired proteins [24-26]. A modified method was developed for isolating low molecular mass peptide toxins (LMMPT) to provide a more sensitive diagnosis of MTV in the envenomed plasma samples. The envenomed plasma samples were collected 60 min

after MTV injection (i.v. and s.c). ACN was added to the plasma at a ratio of 1:2.5 (envenomed plasma:ACN, v/v) and incubated in ice for 30 min. The mixture was centrifuged at room temperature at 10000 rpm for 5 min in a microcentrifuge (Eppendorf MiniSpin). The supernatant containing low molecular mass peptides was collected and concentrated in a water bath at an optimized temperature of 60C. The semi-solid portions left behind (having LMMPT) were reconstituted in 30 µL of phosphate buffer, pH 7.4. Determination of MTV toxins in the non-enriched envenomed plasma and reconstituted LMMPT by PAbF was done by dot blot and sandwich ELISA following the abovementioned procedures. All the analyses were performed in triplicates.

3.2.7.4 Proteomics analysis to determine the presence of MTV major toxins in LMMPT fraction of envenomed plasma

Proteomic analysis was done to validate the presence of Na^+ and K^+ channel toxins in the LMMPT-enriched MTV-treated rat plasma. The control and MTV-treated plasma were treated with ACN (section 3.2.7.3), and isolated peptides were identified for Na^+ and K^+ channel toxins. Briefly, 40 µg of the LMMPT-enriched peptides of control and MTV-treated plasma underwent reduction with 10 mM dithiothreitol (DTT) and alkylated with 55 mM iodoacetamide (IAA) at room temperature in dark conditions. After reduction and alkylation, the peptides were incubated at 37°C with proteomics-grade trypsin for about 16 h. The ZipTip C18 (EMD Millipore) tips were used to desalt and concentrate the tryptic peptides. Data acquisition was done by Orbitrap Fusion™ mass spectrometer (Thermo Fisher Scientific, Inc.) coupled to an EASY-nLC™ 1200 nano-flow LC system (Thermo Fisher Scientific, Inc.) equipped with EASY-Spray column (50 cm × 75 µm ID; PepMap C18 column). The MS and MS/MS spectra were acquired at 375–1500 m/z [7,8,23].

The raw MS/MS data were analyzed using Proteome Discoverer software (version 2.2; Thermo Fisher Scientific, Inc) with a false discovery rate (FDR) set to <1%. Two missed cleavages, and at least one unique peptide per toxin entry were allowed. The raw data were searched against the nonredundant NCBI databases' Buthidae family (Taxonomy ID: 6856) and *M. tamulus* (Taxonomy ID: 34647) protein entries. Precursor and production tolerances were 10 ppm and 0.05 Da, respectively. Further, the oxidation of methionine residues was marked as a flexible modification, and

carbamidomethylation of cysteine was kept fixed [7,8,23]. The coverage percentages of identified sequences ≤5% have not been considered for protein identification. The amino acid sequences of the proteins and peptides identified were aligned by multiple sequence alignment to find conserved residues and confirm the presence/absence of the targeted MTV toxins.

3.2.8 Gold nanoparticle-based detection of MTV in the plasma of envenomed animals

3.2.8.1 Characterization of AuNPs and AuNP-PAbF conjugate

AuNPs were synthesized per the protocol described in section 3.2.5.1, and PAbF was covalently conjugated to the AuNPs as per the protocol described in section 3.2.5.2. The AuNP and AuNP-PAbF conjugate particles were characterized by UV-Vis spectrophotometer, FTIR, Zetasizer, TEM, and AFM by the above methods (section 3.2.5.1 and 3.2.5.2).

3.2.8.2 Determination of selectivity of the AuNP-PAbF conjugate with the MTV spiked rat plasma (*in vitro*) and LMMPT-enriched plasma from envenomed animals (*in vivo*)

Detection sensitivity of the AuNP-PAbF conjugate was determined by adding 10 µL of this conjugate to 30 µL of MTV spiked rat plasma (0.3 ng/µL) and incubated for 10 min. Only rat plasma (untreated) was used as a control, and *Naja naja* venom (NnV) and *Daboia russelii* venom (RvV) samples (50 ng/µL) spiked rat plasma was treated as negative controls. UV recorded the LSPR peak intensity-Vis spectrophotometer (400-700 nm).

Under *in vivo* conditions, to determine the detection sensitivity of the AuNP-PAbF conjugate, 10 µL of this conjugate was added to the 30 µL of the reconstituted LMMPT enriched plasmas (control and envenomed) and incubated for 10 min. Upon interaction with the MTV toxins in plasma, LSPR peak shifting was determined by recording peak intensity using a UV-Vis spectrophotometer (400–700 nm). To analyze the sensitivity of the AuNP-PAbF conjugate-based LSPR, MTV spiked rat plasma (1–5 ng/µL) was incubated with 20 µL AuNP-PAbF conjugate for 5 min. Based on the LSPR

peak absorbance, a calibration curve was obtained and used to quantify the amount of MTV detected in LMMPT-enriched MTV-treated plasma.

3.2.9 Statistical analysis

All data have been represented as mean ± standard deviation (S.D.) of independent triplicate experiments. The significance of differences between control and test values was analysed by the Student's t-test in Sigma Plot 11.0 for Windows (version 10.0). For more than two groups, the significance of differences was studied with a one-way analysis of variance (ANOVA) followed by Tukey post hoc analysis in GraphPad Prism software. The $p \leq 0.05$ and $p \leq 0.01$ values were considered statistically significant.

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