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Research Article

Physiology Monitoring as a Tool of Effective Venom Research

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ABSTRACT

The commercial availability of the snake venom antiserum (SVA) significantly improved the chances of survival of snakebite victims and eventually, the research on phytotherapeutics for snake envenomation declined. India is the capital of snakebite deaths and needs safer molecules for treatment. Systematic investigations on phytotherapeutics are carried out on many animals, and most of them are sacrificed. The study evaluates the possibility of reducing the number of animals sacrificed in venom research by monitoring physiological parameters. Respiratory and electrocardiographic (ECG) monitoring was done in anaesthetized rats after administration of *Naja naja* (neurotoxic) and *Daboia russelii* (cytotoxic) venoms separately. Anti-venom action of *Woodfordia fruticosa* (Lythraceae) extract was evaluated against these venoms and SVA was used as the positive control. Physiological parameters were recorded with the LabChart® program and PowerLab® system coupled with 3 electrode ECG bioamplifier and a respiratory flow-head with a custom-designed mask. Sinus bradycardia was a major cardiac effect imparted by both venoms. The absence or inverted appearance of P-waves, PR prolongation, changes in QRS configuration and QT prolongation were induced by venoms. Significant respiratory depression was observed with *N. naja* venom and significant ECG changes were observed with *D. russelii* venom. *W. fruticosa* extract significantly prevented envenomed animals from developing sinus bradycardia ($P < 0.001$) for both venoms comparable to the action of SVA. *W. fruticosa* extract reversed severe respiratory depression induced by *N. naja* venom up to 70% and *D. russelii* venom up to 91%. Prolongation of PR and QT intervals induced by both the venoms was significantly reversed by *W. fruticosa* extract ($p < 0.001$). Development of RSR' configuration in ECG and changes to cardiac axis induced by *D. russelii* venom were reversed by *W. fruticosa*. Possible mechanisms of venom toxicity and their reversal can be studied with such well-designed methods, and using sub-lethal venom doses would reduce animal sacrifices. Correlating prospective clinical case studies of snakebite victims with these controlled animal studies can generate base data for future venom research.

Introduction

India is a land of snakes and probably has the highest number of snakebites as compared to any other country.[1] Even today, many cases of snakebites are not recorded as many patients are taken to traditional healers. Every year, around 45,900 snakebites deaths occur in India[2,3] which might be around 50% of total in the world. Snake venom has a dual role: immobilizing the prey and initiating digestion. This is accomplished by combining peptides that act as toxins and enzymes. Venom simultaneously acts on different tissues throughout the body of the animal and no isolated tissue preparation can precisely predict its pharmacological impact on the body as a whole.[4]

Commercially available snake venom antiserum (SVA) is the only approved therapy in India for treating snake bites and most of the available SVAs are of equine origin.[5] SVA presents significant challenges, such as anaphylactic reactions and the need for critical monitoring during administration. Moreover, local tissue damage or other

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systemic conditions like haemorrhage or nephrotoxicity arising due to snake envenomation are not addressed by SVA.[6,7] Many plant-based therapies have been described in the Indian traditional literature and very few have been investigated pharmacologically.[8-14] Aqueous extract of leaves of *Woodfordia fruticosa* (L.) Kurz. was earlier investigated for its anti-snake venom potential and was found effective against elapid and viperid venoms. *In mice, W. fruticosa exhibited around 20 and 5 fold neutralization of venom lethality in vitro and in vivo*.[15] *W. fruticosa* also neutralized the activity of phospholipase A2 and acetylcholinesterase enzymes from snake venoms *in vitro*.[15]

The current investigation was initially planned to know the possible mechanism of action of plant derivatives as anti-venom. Knowing changes in physiological parameters and correlating them to a particular event through well-designed experiments would create a guiding pathway in further venom research. Venom research involves using a large number of animals to have a statistical correlation. Most of the animals used are needed to be sacrificed at the end of the experiment to avoid suffering. It is hypothesized that, by monitoring animal physiology and the use of sub-lethal venom doses, these animal sacrifices and be reduced.

The majority of land snakes have venom that is either neurotoxic (elapids) or cytotoxic (viperids). Neurotoxic venoms disrupt nerve signal transmission, resulting in respiratory paralysis and death. Cytotoxic venoms cause hemorrhages, coagulopathies and cardiotoxicity that lead to the prey's death. To understand the characteristics of the venoms, respiratory and cardiac (ECG) monitoring in rats was planned. Recording Although ECG in rodents is simple, its interpretation is not. There are no set reference criteria of ECG parameters in rodents. Human ECG differs from rodent ECG in many ways. Having said this, rodent ECG is still a choice in basic cardiovascular research.[16] Venomous snake bites impart cardiac complications and are more frequent than reported.[17] Though rodents might not be perfect models for human lung diseases, they are chosen due to their flexibility and various advantages. The pulmonary function can be easily measured in rodents with spirometry using invasive or non-invasive models.[18]

The current study is designed to generate data of the effect of representative elapid and viperid venoms on vital physiological parameters in well-designed and controlled animal models. Effect of *W. fruticosa* extract as test substance and SVA as positive control would establish reversal of the venom effects. This data can be used in further venom research to use sub-lethal venom doses and reduce overall number of animals used. This data also can be used to establish correlation between changes in vital physiological parameters manifested by snake venoms in humans and rodents.

Materials and Methods

Venoms

Neurotoxic venom of *Naja naja* and cytotoxic venom of *Daboia russelii* were procured from Irula Snake Catchers' Industrial Co-operative Society, Kancheepuram, Tamil Nadu, India. These lyophilized venoms were stored in the desiccated environment at 4°C. During experimentation for reconstitution into solutions, physiological saline was used as a medium and the concentrations were expressed as dry weight of the venoms. From researchers' earlier experiments on these venoms, the median lethal dose (LD_{50}) of the *N. naja* and *D. russelii* venoms were found to be 0.625 and 4 mg/kg, respectively in murine models.

Snake Venom Antiserum (SVA)

As a reference standard and positive control comparator, an antiserum of equine origin, manufactured by Vins Bioproducts Limited, India was used. This polyvalent antiserum has the potential to neutralize the venoms of the 'big four' snake species found in India.[19] While using this lyophilized antiserum, it was reconstituted into solution as per manufacturer's guidelines but in double strength by adding half the volume of water than mentioned. SVA was expressed quantitatively in terms of reconstituted volume. As per the original label claim, 1-mL of reconstituted antiserum could neutralize 0.6 mg each of *N. naja* and *D. russelii* venoms by dry weight.

Plant Product

The plant *W. fruticosa* (L.) Kurz. (Lythraceae) was collected personally from the hillside, adjacent to Kusgaon village near Lonavala, Maharashtra, India. These hills are part of the vivid biodiversity areas of the *Western Ghats* of Maharashtra. Plant species were authenticated at Botanical Survey of India, Western Regional Centre, Pune, Maharashtra, India vide voucher specimen number ADDWOF3. The leaves of the plant were shade dried and coarsely powdered with a pulveriser (Gem Pharma, Navi Mumbai, India). 300 ml of distilled water was used as an extractant for 25 g of leaves powder and was subjected to soxhlation (Borosil Soxhlet extractor coupled with Allihn condenser) at 100°C. The extracted material was then subjected to lyophilization (Alpha 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) to obtain the powdered extract. 2.66 ± 0.02 g of extract was yielded from one batch, which was then stored in the desiccated environment at 4°C until further use. While dosing, the extract was reconstituted with the physiological saline solution and the concentrations were expressed as dry weight of the extract. Researchers' earlier experiments confirmed that this extract did not present any significant toxicity and was safe up to 2000 mg/kg dose in murine models when determined by OECD 423 method.[20]

Experimental Animals

Male Wistar rats (180 ± 10 g) used for experimentation were procured from Haffkine Biopharmaceutical Corporation, Mumbai, India and were maintained under standard laboratory conditions.[21] To reduce the hormonal interference, only males were used for the experiment. The animals had free access to food and water during housing. Before the initiation of the experiment, the animals had fasted for two hours. After experimentation, animals with better health were allowed to heal and maintained on a standard diet. Moribund and/or traumatized animals were humanely sacrificed. All protocols involving animal experimentation (SIPS/IAEC/2011-12/03, SIPS/IAEC/2012-13/04, SIPS/IAEC/2013-14/07) were approved by the Institutional Animal Ethics Committee (Reg. No. IAEC - 962/c/06/CPCSEA).

Anesthesia

A plain Ketamine solution was used as an anesthetic agent and was administered via the intraperitoneal route. With the initial dose of 30 mg/kg, the Ketamine solution was administered to the rats until a sufficient level of anesthesia was reached. The animals were monitored for the level of anesthesia at regular intervals by examining reflex responses and additional volumes of the Ketamine solution were administered as needed.

Electrocardiogram (ECG) and Respiratory Monitoring

The PowerLab® 26T data acquisition system with the LabChart® 7 software (AD Instruments, Australia) was used. A bio-amplifier with a 3 electrode ECG attachment was used for recording the ECG. The needle electrodes were inserted below the limb skin to record the lead II ECG in each rat. PR interval, QRS complex, ST interval were monitored and heart rate was recorded by the cyclic measurements from ECG.

For recording the respiratory cycles, a spirometer pod attached to a flow-head (operation range of 1 L/min) was used. Unlike the most popular method of tracheotomy and cannulation used for respiratory measurements in the rats, a custom-designed low density polyethylene (LDPE) head mask was used. This mask covered a rat's entire head, and a gel strip facilitated proper fixation of the mask at the rat's neck. Residual air in the mask was removed and the assembly was observed from time to time for any leakage. A small tube attached to the mask was connected to the respiratory flow-head of the spirometer pod. The other side of the respiratory flow-head was left open. This arrangement was meant only for measurements of respiratory cycles and no volumetric measurements were done. Fig. 1 represents the schematics of the monitoring assembly. Real-time recording of the respiratory cycles and the ECG was done in two channels on LabChart® software which was analysed further.

Animal Grouping, Dosing, Venom Lethality and Inhibition

After stabilizing the animals on anesthesia, normal state ECG and respiratory cycles were recorded in each rat. Eight treatment groups with 6 animals in each of the groups were formed. The animals in groups 1-4 received a $2LD_{50}$ dose of *N. naja* venom, whereas the animals in groups 5-8 received a $2LD_{50}$ dose of *D. russelii* venom. The animals in groups 1 and 5 were not treated further by any agents and were negative control comparators for the respective venoms. The effects of the venoms on ECG and respiration were recorded until the animals reached the moribund stage in these two groups. Soon after subcutaneous venom dosing, the animals in groups 2 and 6 received a 40 mg/kg dose of the *W. fruticosa* extract, the animals in groups 3 and 7 received 60 mg/kg dose of the *W. fruticosa* extract, the animals in group 4 received 1 mL/kg SVA and the animals in group 8 received 6.5 mL/kg SVA in divided doses as necessary at different subcutaneous sites than venom dosing. Groups 4 and 8 were positive control comparators for the respective venoms. ECG and respiration in these animals were recorded till the completion of 4 hours post venom dosing. ECG and respiratory monitoring were performed on a few surviving animals at random for up to a few days as needed, and these animals were anesthetized again only at the time of physiology recording. Researchers' earlier experiments had established that co-administration of *W. fruticosa* extract provided 5.2 fold protection from *N. naja* venom and 5.4 fold protection from *D. russelii* venom *in-vivo* in murine models.

Statistical Analysis

The numbers for heart rate, respiratory cycles, PR/QRS/QT intervals were expressed as mean ± standard error of the mean (SEM). The significance of difference was determined by the application of paired samples

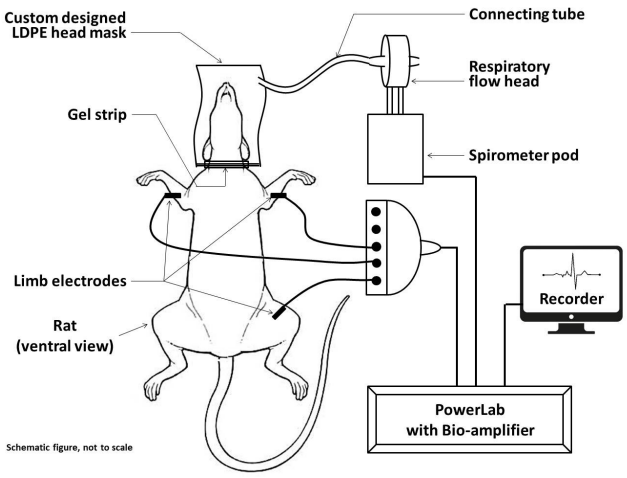

Fig. 1: Schematic representation of the assembly for the measurement of ECG and respiratory cycles in rats.

t-test between reference and test groups, where group 1 was a reference for test groups 2-4 and group 5 was a reference for test groups 6-8. P-value <0.05 was considered significant.[22]

Results

Anesthesia

With the starting dose of 30 mg/kg, on average, a 50 mg/kg dose of plain Ketamine was sufficient to induce anesthesia in most animals. Preliminary studies on anesthesia indicated that a relatively high starting dose of Ketamine was used, the onset of anesthesia was quick, but the animal died within a short period due to anesthetic. Thus smaller and gradual doses of Ketamine were used for induction of anesthesia. In some rare episodes, seizures were observed post Ketamine dosing in rats. Except for a few initial odd events, overall induction and maintenance of anesthesia were smooth.

ECG and Respiratory Monitoring Post *N. naja* Envenomation

Animals in groups 1-4 received a 1.25 mg/kg dose of *N. naja* venom and group 1 was the negative control. Before venom administration, normal ECG and respiration were recorded as a baseline. For group 1, the mean heart rate (HR) was 340 beats per minute (b/min) and the respiratory rate was around 151 cycles per minute (c/min). PR, QRS and QT intervals were 42, 14 and 77 ms, respectively (Table 1). Fig. 2A represents pre-dose normal ECG recorded for one of the animals in this group. One hour post envenomation, heart rate dropped to around 219 b/min indicating bradycardia. The PR, QRS and QT intervals were 48, 17 and 76 ms, respectively which were higher than earlier but still within the normal ranges. Fig. 2B represents 1-hour post-dose ECG recorded for one of the animals in this group. The P-waves appeared on a regular interval but with slightly lower amplitude. QRS-complexes had typical Rs configuration as Q-waves are generally not detected in rat ECG.[16] The respiratory rate was reduced to 109 c/min. This deterioration in animal health continued further as time passed and after around 4 hours, the average heart rate was reduced to 54 b/min and respiratory cycles were not measurable due to slow and shallow breathing. Fig. 2C represents 4 hours post-dose ECG recorded for one of the animals in this group. There were significant changes observed in ECG. The occurrence of the P-wave was highly irregular and did not appear at regular intervals (Fig. 2D). In the absence of a P-wave, a junctional rhythm was observed. Only R-wave was visible with an average 21 ms interval. Sharp T-waves with an average QT-interval of 154 ms were observed. Whenever P-waves appeared, they were inverted, indicating non-sinus origin and were immediately followed by QRS-complexes (Rs configuration) and broader T-waves (Fig. 2C-D).

Animals in group 2 received a 1.25 mg/kg dose of *N. naja* venom and a 40 mg/kg dose of *W. fruticosa* extract. *W. fruticosa* seemed to neutralize the effects of *N. naja* venom

Table 1: Average heart rate, respiratory cycles and PR/QRS/QT intervals from ECG observed for group 1-4 animals.

		Group 1	Group 2	Group 3	Group 4
N. naja venom		1.25 mg/kg	1.25 mg/kg	1.25 mg/kg	1.25 mg/kg
W. fruticosa extract		--	40 mg/kg	60 mg/kg	--
SVA		--	--	--	1 ml/kg
Pre-dose	HR (b/min)	340 ± 5.01	335 ± 3.94	368 ± 5.53	354 ± 3.81
	RC (c/min)	151 ± 2.60	166 ± 0.97	161 ± 2.38	159 ± 2.54
	PR (ms)	42 ± 1.12	43 ± 1.15	48 ± 1.07	43 ± 1.10
	QRS (ms)	14 ± 0.80	15 ± 0.87	18 ± 0.33	16 ± 0.56
	QT (ms)	77 ± 1.47	80 ± 1.40	76 ± 1.73	82 ± 1.38
1 h post-dose	HR (b/min)	219 ± 4.13	266 ± 3.6 **	295 ± 2.72 **	275 ± 3.30 **
	RC (c/min)	109 ± 2.60	103 ± 2.56 ^	119 ± 1.58 ^	100 ± 2.51 ^
	PR (ms)	48 ± 1.15	53 ± 0.61 *	54 ± 0.75 *	44 ± 0.37 ^
	QRS (ms)	17 ± 0.84	16 ± 0.58 NS	18 ± 0.44 NS	17 ± 0.73 NS
	QT (ms)	76 ± 1.84	104 ± 1.25 **	96 ± 1.44 **	105 ± 1.49 **
4 h post-dose	HR (b/min)	54 ± 2.48	248 ± 4.06 **	299 ± 4.05 **	258 ± 3.49 **
	RC (c/min)	NM	111 ± 2.47 **	124 ± 2.74 **	108 ± 2.59 **
	PR (ms)	74 ± 1.74	57 ± 1.14 **	52 ± 0.72 **	51 ± 1.05 **
	QRS (ms)	21 ± 0.66	18 ± 0.55 ^	17 ± 0.49 **	20 ± 0.85 NS
	QT (ms)	154 ± 1.73	91 ± 1.11 **	89 ± 1.43 **	88 ± 1.39 **

^ P < 0.05, * P < 0.01, ** P < 0.001, NS – Non significant, NM – Not measurable.

and statistically significant positive observations were recorded 1 and 4 hours post-dosing. The average heart rate was significantly higher with a relatively higher number of respiratory cycles as compared to the negative control group (Table 1). Notably, respiratory depression that was observed during the first-hour post-dosing was marginally reversed at the 4 h observation. P-waves were normal with normal amplitude, and QRS complexes retained the typical Rs configuration (Fig. 3W). Animals in group 3 received a 1.25 mg/kg dose of *N. naja* venom and a 60 mg/kg dose of *W. fruticosa* extract. Observations for all study parameters in group 3 were similar to those in group 2 or slightly more positive, indicating dose-dependent correlation.

Animals in group 4 received a 1.25 mg/kg dose of *N. naja* venom and a 1 ml/kg dose of SVA and this group was a positive control comparator. Heart rate, respiratory cycles PR/QRS/QT intervals were comparable to those of group 2 (Table 1). The occurrence of P-waves was normal but with slightly lower amplitude. The QRS complexes retained typical Rs configuration (Fig. 3X). As group 1 was a negative control comparator, all animals reached a moribund state and were humanely sacrificed at the end of the study to avert further suffering. Animals from groups 2, 3 and 4 survived and gradually recovered due to the anti-venom action of *W. fruticosa* and SVA (Table 1 and Figures 2A-C, 3W-X).

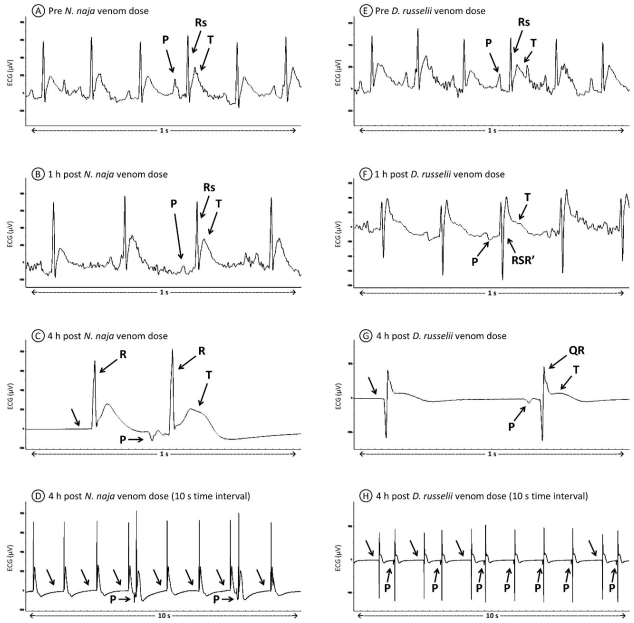

Fig. 2: Representative electrocardiograms (lead II) pre and post envenomation without further treatment. A-D: *N. naja* venom. E-H: *D. russelii* venom. (The unlabelled arrow indicates an absence of a P-wave.)

ECG and Respiratory Monitoring Post *D. russelii* Envenomation.

Animals in groups 5-8 received an 8 mg/kg dose of *D. russelii* venom; group 5 was the negative control. Baseline observations were recorded just before the administration of venom. For group 5, the mean heart rate was 352 b/min and the respiratory rate was around 164 c/min. PR, QRS and QT intervals were 43, 12 and 87 ms, respectively (Table 2). Fig. 2E represents pre-dose normal ECG

Table 2: Average heart rate, respiratory cycles and PR/QRS/QT intervals from ECG observed for group 5-8 animals.

		Group 5	*Group 6*	*Group 7*	*Group 8*
D. russelii venom		8 mg/kg	8 mg/kg	8 mg/kg	8 mg/kg
W. fruticosa extract		--	40 mg/kg	60 mg/kg	--
SVA		--	--	--	6.5 ml/kg
Pre-dose	HR (b/min)	352 ± 5.62	361 ± 2.65	346 ± 2.91	332 ± 5.32
	RC (c/min)	164 ± 1.01	169 ± 2.48	156 ± 2.79	161 ± 2.59
	PR (ms)	43 ± 1.04	41 ± 0.87	46 ± 0.7	43 ± 0.44
	QRS (ms)	12 ± 0.20	14 ± 0.39	16 ± 0.45	13 ± 0.28
	QT (ms)	87 ± 1.87	85 ± 1.88	75 ± 1.74	81 ± 1.15
1 h post-dose	HR (b/min)	195 ± 3.02	217 ± 4.27 *	229 ± 3.9 *	219 ± 4.52 *
	RC (c/min)	127 ± 2.6	139 ± 1.71 ^	154 ± 1.73 **	135 ± 0.98 ^
	PR (ms)	52 ± 0.89	43 ± 0.9 **	46 ± 1.05 *	44 ± 0.28 **
	QRS (ms)	41 ± 1.82	17 ± 0.31 **	14 ± 0.51 **	15 ± 0.28 **
	QT (ms)	110 ± 1.26	96 ± 1.54 **	94 ± 1.07 **	96 ± 1.52 **
4 h post-dose	HR (b/min)	75 ± 2.97	221 ± 3.74 **	250 ± 3.63 **	247 ± 3.16 **
	RC (c/min)	NM	146 ± 1.31 **	151 ± 3.06 **	147 ± 2.87 **
	PR (ms)	60 ± 1.04	47 ± 0.98 **	52 ± 1.24 *	45 ± 0.66 **
	QRS (ms)	37 ± 1.00	14 ± 0.47 **	12 ± 0.44 **	15 ± 0.53 **
	QT (ms)	129 ± 1.43	93 ± 1.2 **	90 ± 1.82 **	92 ± 0.96 **

^ P < 0.05, * P < 0.01, ** P < 0.001, NS – Non significant, NM – Not measurable.

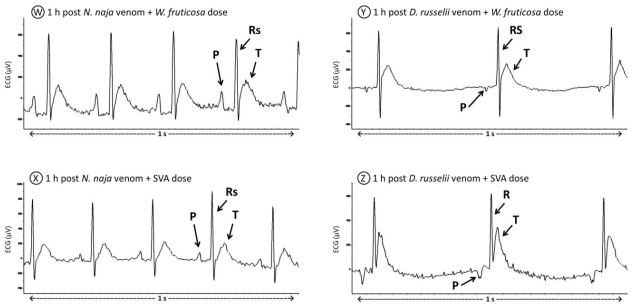

Fig. 3: Representative electrocardiograms (lead II) 1 h post envenomation and treatment with *W. fruticosa* extract or SVA. W-X: *N. naja* venom. Y-Z: *D. russelii* venom.

recorded for one of the animals in group 5. One hour post envenomation, heart rate dropped to around 195 b/min, indicating bradycardia and the respiratory rate was also reduced to 127 c/min. Significant changes in ECG were observed including inverted P-wave and QRS configuration was changed to RSR'. This configuration change visibly differentiated the ECGs of viper envenomation from cobra envenomation (Fig. 2B/2F). The PR interval was 52 ms (longer), QRS duration was 41 ms (longer) and QT interval was 110 ms (longer), still, they were within normal ranges (Table 2). Animal health continued to deteriorate with passing time and after around 4 hours, the average heart rate dropped to 75 b/min and respiratory cycles were not measurable. ECG continued to be unusual. The occurrence of the P-wave was irregular and did not appear at regular intervals (Fig. 2H). Some beats appeared with P-wave and some without that. When P-wave appeared, it was inverted and of lesser amplitude. QRS complex configuration was then changed to QR (Fig. 2G) which was RSR' during post 1 h observation (refer Fig. 2F). The presence or absence of a P-wave did not influence the QRS complex or T-wave (Fig. 2G). Significant prolongation of PR, QRS and QT intervals was observed as compared to typical ECG values (Table 2).

With *D. russelii* envenomation, the reduction in heart rate was profound but the reduction in respiratory rate was lesser compared to that with *N. naja* envenomation after similar time intervals. This observation was sacrosanct with the known effects of elapid and viperid venoms on physiological parameters where elapid venoms act faster and more on the nervous system leading to respiratory paralysis and viperid venoms tend to act more on the cardiovascular and circulatory system.

Animals in group 6 received an 8 mg/kg dose of *D. russelii* venom and a 40 mg/kg dose of *W. fruticosa* extract. In comparison to the observations from group 5, animals in group 6 presented lower toxic effects of envenomation. Reduction in heart rate and respiratory rate was lower (217 b/min and 139 c/min, respectively) and PR prolongation was non-significant but QT-interval was still on the higher side 1-hour post-dose. P-wave appeared consistently inverted with lesser amplitude, but particularly QRS-complex had RS configuration indicating the relatively lesser impact of *D. russelii* venom components (refer Fig. 3Y). Though the average QRS interval is higher, it is still within normal limits.[16] Animals in group 7 received the same treatment as group 6, but the dose of *W. fruticosa* extract was 60 mg/kg and the expression of results (Table 2).

Group 8 was positive control comparator where animals received an 8 mg/kg dose of *D. russelii* venom and a 6.5 mL/kg dose of SVA. 1 h post-dose, average heart rate, respiratory cycles and PR/QRS intervals were nearly similar to those of group 5 animals indicating the similarity of action between the dose of *W. fruticosa* and SVA. However, in group 8, the average QT duration was reduced to 96 ms. QRS complex configuration was qR that indicated more normalcy. P-wave continued to be inverted throughout the observation (Fig. 3Z).

Discussion

Anesthesia

Various chemical agents are used for inducing anesthesia among experimental animals. Being xenobiotics, most of them affect physiological parameters in varying degrees. Some inhalation anesthetics can induce arrhythmia;[23] for example, Urethane significantly lowers heart rate in rats[24] and Pentobarbital causes ventricular arrhythmia.[16] A combination of Ketamine and Xylazine is a popular injectable anesthetic in murine models. Though Xylazine has longer action, it is known to induce cardiac and respiratory depression. Thus it needs close monitoring during both induction and maintenance of anesthesia.[25,26] Almost every animal requires a different dose for induction and maintenance of anesthesia and the degree of physiological depression due to anesthetic is different in each animal. Thus, combining Ketamine and Xylazine as an anesthetic would add more variables and make it difficult to understand the effect of desired agents. To minimize cardiac or respiratory depression, plain Ketamine was used as an anaesthetic agent. Ketamine induced the desired level of anesthesia in all animals and its effects on cardio-physiology were within the ranges described by Konopelski and Ufnal.[16]

Respiration

Many invasive and non-invasive methods have been used and documented by various researchers in the past to record and study pulmonary function in rodents.[18] Many non-invasive techniques, such as head-out body plethysmography and barometric whole-body plethysmography, use conscious rodents rather than anesthetized intubated animals. These individual methods have their own sets of pros and cons.[18] In the current study design, both ECG recording and respiratory measurements were to be done. Invasive methods would have allowed for more precise measurement of pulmonary function, but they would have added surgical trauma

and thus more variables. With non-invasive methods like whole-body plethysmography, immobilization of the animal and insertion of electrodes under the skin at the same time is difficult in the conscious animal. To ease the process and achieve the desired goal, animals were anesthetized and a custom-designed LDPE head mask was used for the recording of respiratory cycles. Since air exchange volume recording was not a necessary parameter of this study, this setup worked well without adding any more variables. The number of respiratory cycles and wave morphology were typical in the pre-dose recordings. 1 h post-envenomation, respiratory depression was observed in all groups, indicating venom action, and the wave morphology was uneven. Intermittently deep breath wave pattern was also observed which might be a compensatory mechanism for hypoxia. In the negative control groups, the respiratory depression intensified over time, and the degree of respiratory depression was much greater with *N. naja* venom than with *D. russelii* venom. This observation is in sync with the generally known action of neurotoxic venoms to eventually cause respiratory paralysis. In the treatment and positive control groups, the numbers of respiratory cycles were improved slightly during the 4 h observation indicating action of agents and their distinctive mechanism of action. With more sophisticated methods of spirometry, volume measurements would facilitate in-depth analysis of the impact of venom on respiration.

Heart Rate

In the current investigation, one major rhythm abnormality confirmed was sinus bradycardia. Both *N. naja* and *D. russelii* venoms induced bradycardia, similar to the clinical observation on 96 snakebite patients in a prospective study conducted by Sunil Kumar and co-workers.[17] The onset and progress of bradycardia were gradual and it was profound in the negative control groups. In contrast to this observation, in some clinical reports available, clinicians have reported sinus tachycardia as a major rhythm abnormality too.[27,28] Differences in venom action on human and murine hearts could be one explanation for this anomaly, or it could be due to post-envenomation anxiety. In the current investigation, as the animals have no impact of such mental trauma, bradycardia can be better correlated to envenomation than tachycardia. *W. fruticosa* and SVA prevented severe bradycardia in animals proving the hypothesis. Additionally, anesthetic is also known to cause bradycardia. But, as the anesthetic is used in all the groups and the effects of venoms and test agents are compared with undosed anesthetized animals, effect of anesthetic causing bradycardia can be ignored.

P-wave

In humans and rodents, P-wave is a positive deflection in ECG and is a reflection of atrial depolarization.[16] In the negative control groups, a retrograde P-wave was observed after a few hours along with the onset of bradycardia. Junctional rhythm and irregular retrograde P-waves were observed as time progressed. Regular positive P-waves were observed 1 h post *N. naja* envenomation in all relevant groups. During the 4 h observation, P-wave appeared inverted and at irregular intervals (Fig. 2C-D). Whenever a retrograde P-wave appeared, it was coupled with uneven ECG deflections, suggesting atrial fibrillation in *N. naja* venom negative control group. P-waves were always positive in the *N. naja* venom treatment groups (Fig. 3W-X). P-waves were always inverted in all groups after *D. russelii* envenomation. In group 5, 1 h post envenomation, inverted P-waves appeared at regular intervals, but after 4 h, they became irregular with severe bradycardia. In the treatment groups (6-7), the P-wave remained inverted for longer and took a few days to return to normal in surviving animals. The appearance of the P-wave can be a point of distinction for identifying *N. naja* and *D. russelii* venoms.

PR-interval

PR-interval is well measurable in both rodents and humans and is an indicator of impulse conduction between the sinus node and AV node. Any conduction delay is marked by prolonged PR-interval. In rodents, however, the normal PR-interval range is wider, making it difficult to distinguish between normal and altered. However, statistically significant alterations within the same group provide a relatively clear idea about pathophysiological changes. In the current study, significant PR prolongation was observed in negative control groups in a time-dependent manner suggesting progressive conduction block. In positive control and treatment groups, slight PR prolongation was seen which was statistically non-significant than normal. PR prolongation was relatively prominent in *N. naja* envenomation than *D. russelii*. Correlating rodent and human PR-intervals might be difficult because of the large variability in rodent PR-interval values. PR prolongation due to venom and its reversal with test agents is a good tool to map conduction blocks caused by cardiotoxicity of snake venoms.

QRS-complex

Propagation of depolarization through the ventricles is distinctly marked on ECG by QRS-complex. It's a short duration, high amplitude structure on ECG that allows monitoring of a few arrhythmias and conductions blocks. In the rat, Q-wave is generally not detectable in normal ECGs; thus, measurements are only based on Rs or RS complexes.[16] As shown in figures 2A-C, and 3W-X, QRS complexes generally seem to retain their structure post *N. naja* envenomation except late-phase where S-wave disappeared (Fig. 2C). The length of the QRS interval increased from an average of 14 ms to 17 ms to 21 ms in the negative control group post *N. naja* envenomation. In the treatment and positive control groups, the duration of the QRS interval was observed to increase but not as

significant as in the negative control group. In the case of *D. russelii* envenomation, QRS-complex presented a wide range of forms. In the negative control group, within 1 h post *D. russelii* envenomation, a distinct RSR' configuration appeared on rat ECG (Fig. 2F) and with passing time it changed to QR configuration (Fig. 2G). This is one of those rare events when a distinct Q-wave appeared on rat ECG. In this ECG a broad R-wave was observed. In the test groups, an RS configuration was observed (Fig. 3Y), with a high amplitude S-wave observed initially, but the amplitude of the S-wave decreased with time. In the negative control group 5, the pre-dose normal duration of QRS-complex was 12 ms. 1 h post-envenomation, RSR' configuration was observed with a significantly longer duration of 41 ms. At 4 h observation, QRS duration was longer still averaging to 37 ms and configuration was changed to QR. These wide QRS complexes were indicators of Right Bundle Branch Block and abnormalities in cardiac impulse conduction. In the treatment and positive control groups, 1 h and 4 h post-dose, the difference in QRS duration was not statistically significant than pre-dose normal. This indicates that both *W. fruticosa* and SVA reverse the action of the *D. russelii* venom component responsible for such dramatic changes in the QRS configuration. This observation can be exploited a lot in *D. russelii* venom research. The changes in the configuration of QRS-complex attribute to changes in cardiac axis from time to time in an envenomed animal. Changes in electrical conduction vectors affect heart function to a major extent and further research and use of multi-lead ECG in animals will shed light on the exact mechanism of these changes. QRS complex in a typical rat ECG had a voltage gradient of around 600 μV between the highest and lowest points of a beat. However, in the negative control groups with severe bradycardia, a voltage gradient of 900-1000 μV was observed. This could be the heart's compensatory mechanism for dealing with bradycardia and pumping more blood throughout the body. This could be due to the increased voltage gradient between epicardial and endocardial cells at the tissue level, suggesting a direct effect on certain membrane channels.[29,30] Both *N. naja* and *D. russelii* venoms presented this anomaly but with *D. russelii* venom this increase in voltage gradient was much higher. The changes in QRS configuration seen in rats have not been reported in clinical observations, and there may be limitations in extrapolating animal experiment results to an actual clinical scenario. However, alterations in QRS configuration due to cardiotoxic venom are so distinct that they can be used effectively to study the action of venom and anti-venom on cardiomyocytes.

T-wave

As a point of distinction from human ECG, T-wave initiates in the continuity of QRS-complex and no ST-segment can be detected in rats.[31,32] Thus a study related to depression or elevation of ST-segment is difficult with the rat model. In the case of *N. naja* envenomation, T-wave mostly retains its normal orientation (Fig. 2B) but in post 4 h observation, broader T-waves are observed. T-wave's appearance is relatively symmetrical in the beats without P-wave; in the beats where a retrograde P-wave is observed, T-wave is broad and asymmetric (Fig. 2C). T-wave appearance is near normal in the treatment and positive control groups of both N. naja and D. russelii venoms (Fig. 3). In the negative control group post *D. russelii* envenomation, the initial part of T-wave merges with QRS-complex as seen in figures 2F-G. In the clinical reports following snake bite, inversion of T-wave was one of the commonly observed scenarios in various reports; [28,29] however, no inversion of T-wave was observed in rat ECG in the current experimental setup.

QT-interval

QT-interval represents total ventricular activity[33] and a prolonged QT-interval is a known indicator of cardiotoxicity[34] and is used as a screening tool for checking cardiotoxic effects of chemical agents in rodents.[35] Weherns et al. reported that anesthesia has no direct effect on QT interval.[32] QT prolongation is observed in the negative control groups of both *N. naja* and *D. russelii* venoms. There is no significant QT prolongation 1 h post envenomation with *N. naja* venom, but *D. russelii* venom results in longer QT-intervals. However, QT prolongation with *N. naja* venom is significantly longer than that of *D. russelii* venom 4 h post envenomation. The QT-interval in the treatment (92-93 ms) and positive control (90 ms) groups is not significantly different from pre-dose normal and is significantly different from the negative control group, indicating that *W. fruticosa* and SVA work as intended. Longer QT intervals can be attributed to conduction defects and repolarization within the heart.[29] Corrected QT interval (QTc) is used in human measurements to nullify the effects of changes in heart rate.[36] There are differences in opinions if using QTc is necessary in rodents as their heart rate is around 4-5 times more than in humans.[37] Various formulae are used to adjust QT with heart rate, like Bazett's formula or Fridericia's formula.[37] All these formulae have some limitations, and to avoid complexity, no corrections were made to QT in the current investigation.

There can be many possible mechanisms of cardiotoxicity induced by snake venoms, but no event can be pinpointed to a reason yet. Direct association of venom toxin or its indirect disturbances in the autonomic innervation of the heart might contribute to toxicity.[29] Altered vascular permeability, vasospasms, alterations in coagulation cascade or combinations of these might be a few of the contributing factors.[17] More specific research in this regard can throw light on the exact mechanism. The morphology of P-wave, prolongation of PR and QT-intervals indicating conduction blocks and ventricular events, and more importantly the peculiar changes in QRS morphology can be significant tools in venom research.

With the availability of specific information, managing clinical cases would be easier. Neurotoxic venoms also contribute to cardiotoxicity, but cytotoxic venoms are much more cardiotoxic, as observed in ECG and rhythm anomalies. Conventionally ECG has been used as a major tool in the identification of drug overdose and poisoning[38,39] and a few drug molecules have been withdrawn from the market due to their cardiotoxicity manifested by the QRS and ST abnormalities.[39]

India is home to various species of venomous snakes and has the highest incidence of snake bites. However, the commercially available SVA is active against only the 'big four' and does not protect against other clinically important snake venoms.[40] This SVA might neutralize a few components of other snake venoms but such cases present a huge challenge to the clinician and saving the victim's life is difficult. Specific research on SVAs and phytotherapeutic agents is much needed to save precious human lives. With further well designed experiments on physiology monitoring, animal sacrifices in research can also be reduced.

Conclusion

From the experimental findings, it was ascertained that cardiac (ECG) and respiratory rate monitoring can be used as effective tools in venom research and help reduce the number of animals used. ECG facilitates the identification of venoms due to the distinct changes imparted by neurotoxic and cytotoxic venoms. Primary and advanced venom research can utilize physiology monitoring as a significant tool, from the venom lethality studies to finding the mechanisms of venom or anti-venom action. *W. fruticosa* has the promising potential of venom neutralization and has comparable results to SVA. Venom research on animals and correlating the same to clinical case studies will prove as a baseline for the future.

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