STUDY ON THE EFFECT OF PARTIALLY PURIFIED RUSSELL’S VIPER (EASTERN REGION, INDIA) VENOM ON HUMAN SPERM

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ABSTRACT
The specific aim of the present study is to investigate the in vitro effect of partially purified Russell’s viper venom (Eastern region of India) on human sperm motility and viability. Russell’s viper venom was subjected to gel filtration chromatography on Sephadex G-75 column and resolved peaks were pooled and subjected to caseinolytic, hemolytic and trypsin inhibitor activity. Sperms were collected from healthy donors and checked for advanced motility and samples less than 60% motility was not used for study. The sperms were incubated in vitro in phosphate buffer, pH 7.0 containing various concentrations of venom 0-80 mg at 37ºC for different time intervals between 0-60 minutes. Peak I showed caseinolytic, peak II showed indirect hemolytic and peak III protein devoid of trypsin inhibitor activity. Further, the results showed that, the percentages of sperm motility were decreased and the percentages of dead sperms were increased in a time and concentration-dependent manner. Our results revealed that Russell’s viper venom induced a concentration and time-dependent action on sperms motility and viability, which may be due to the alteration in spermatozoa plasma membrane and mitochondrial dysfunction.

KEYWORDS: Russell’s viper venom, sperm, motility.

1. INTRODUCTION
Snake envenomation is a major health problem in tropical and subtropical countries.[1] In India approximately 15,000 to 20,000 people are estimated to die annually.[2] The pathology
of snakebite includes both local and systemic effects such as neurotoxicity, myotoxicity, cardiotoxicity, coagulant, hemostatic, hemorrhagic, hemolytic and edema forming activities.\(^3\) However the toxicity of venom depends on the geographical origin and composition of the venom.

*Russell’s viper* is one of the predominant venomous snakes distributed across many parts of Southeast Asia.\(^4\) It occurs in most of the regions of India, Bangladesh, Srilanka, Bhutan and certain parts of Nepal and Pakistan.\(^2\) The clinical evidence suggests that *Russell’s viper* bite result in the pituitary infraction, generalized capillary permeability, intravascular hemolysis and reduced blood coagulability, renal failure, incoagulable blood with bleeding, diathesis and hemolysis and hemorrhage in lungs.\(^5\) The hydrolytic enzymes and toxic peptides present in the *Russell’s viper* venom such as phospholipases are responsible for their pharmacological effects.

Phospholipase A\(_2\) (PLA\(_2\)) is an esterolytic enzyme which hydrolyzes glycerophospholipids at the sn-2 position of the glycerol backbone releasing lysophospholipids and fatty acids. They occur ubiquitously in nature as both intracellular and extracellular forms and hydrolyze various phospholipids. However, in contrast to mammalian PLA\(_2\) enzymes, many snake venom enzymes are toxic and induce a wide spectrum of biological activities.\(^6,7\) Moreover, few researchers reported the effect of snake venoms on testicular tissues in experimental paradigm.\(^8,9\) The result showed altered testicular tissue, in which there was a reduction of spermatogenesis which lead to decrease in the fertility. Alteration in the plasma membrane, mitochondrial dysfunction, which includes inhibition of ATP production and motility system deformation were responsible for inducing infertility of sperms.\(^10\)

Anti-venom therapy is a specific antidote to the venom action, neutralizing the circulating venom. However the antivenom prepared against specific regional venom is suggested to be ineffective or partially effective against the toxicity of various regions of snake venoms.\(^11\) Accumulated evidence suggests that antivenom therapy fails to neutralize the venom fixed to target organs such as platelets and renal systems. Testis seen to be one of the organ prone to venom action reported by Hemmaid *et al.*\(^12\) Hence the purpose of this study is to investigate direct effect of *Russell’s viper* venom on human sperm motility by *in vitro* incubation at different concentrations and different time intervals.
2. MATERIALS AND METHODS
Lyophilized Russell’s viper (eastern region) venom was obtained from Hindustan snake park, Kolkata, India. Venom reconstituted in distilled water for the preparation of the concentrations required in the experiment. Sephadex G-75 was purchased from Sigma Chemicals (St. Louis MO, USA). Sperms are collected from healthy donors (Mediwave IVF and infertility research hospital, Mysore).

2.1 Sephadex G-75 column chromatography of Russell’s viper venom
The column (0.5 x 95cm) was equilibrated with 0.05 M phosphate buffer (pH 7.0) and Russell’s viper venom (50 mg dissolved in 0.5 ml of the phosphate buffer) was loaded on to the column. Elution was carried out with pre-equilibrated buffer at a flow rate of 10 ml/h and 1 ml fractions were collected. Protein elution was monitored at 280 nm using a spectrophotometer. The venom resolved into three peaks. Individual fractions of the protein peaks were pooled, stored at 4 °C and protein content was estimated.

2.2 Hemolytic activity
Indirect/direct hemolytic activity was assayed as described by Bowman and Kalletta et al.[13] The substrate for direct lytic activity was prepared by suspending 1 ml of packed fresh human red blood cells (RBC) in 9 ml of saline. The substrate for indirect hemolytic activity was prepared by suspending 1 ml of packed fresh human RBC and 1 ml of fresh hen’s egg-yolk in 8 ml of Phosphate buffer saline (PBS). The suspension (1 ml) was incubated with 10-20 µg of each chromatographic fraction for 45 min at 37 °C, and the reaction was stopped by addition of 9 ml of ice-cold PBS. The positive control received 20 µg of fraction alone. The suspension was centrifuged at 2000 rpm for 20 min, and then the released hemoglobin was read at 530 nm.

2.3 Caseinolytic activity
Caseinolytic activity was determined according to the method of Satake et al.[14] using casein as the substrate. A 30 µg of sample was incubated with 0.4 ml of casein (2%) buffered with 0.2 M Tris-HCL, pH 8.5 for 2h at 37 °C. The reaction was stopped by adding 1.5 ml 0.44 M trichloroacetic acid (TCA), and the undigested casein was allowed to precipitate for 30 min and then centrifuged. The supernatant (1 ml) was treated with 2.5 ml of sodium carbonate (4%) and 0.5 ml of 1:2 diluted FC (Foline-Ciocalteis phenol) reagents. The mixture was allowed to stand at room temperature for 20 min. The blue color developed was measured at 660 nm.
2.4 Trypsin inhibitory activity
Trypsin activity was assayed according to the method of Hummel et al.\textsuperscript{[15]} using p-tosyl-L-arginine methyl ester (TAME) as substrate. 5 µg of trypsin was incubated with 2 ml of 0.5 mM TAME in 0.2 M Tris-HCL, pH 8.5. The change in absorbance was recorded at 250 nm using spectrophotometer (UV-1601 UV/VIS Spectrophotometer). One unit of activity is defined as an increase in 0.01 OD at 250 nm. To determine the trypsin inhibitory activity of the samples (Peak I, II and III), trypsin (5 µg) was pre-incubated with various concentrations (1-20 µg) of venom samples for 30 min at room temperature. The extent of trypsin inhibition was determined by measuring the residual trypsin activity as described above. One unit of inhibitory activity is defined as the amount of venom required to cause the inhibition of trypsin activity by 10%.

2.5 Electrophoresis
Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (10%) was performed according to the method of Laemmli et al.\textsuperscript{[16]} under non-reducing conditions. The gels were stained with 0.25% Coomassie brilliant blue R-250.

2.6 Test of motility and viability of sperms
Sperms are collected from healthy donors (Mediwave IVF and infertility research hospital, Mysore) and checked for advanced motility and which has sperm count of around 80 milion/ml were used, samples less than 60% motility were discarded. Sperm advanced motility (%) and smears for viability of sperms were determined by using light microscope using a special fertility slide.

2.7 In vitro treatment
The sperms are incubated with different concentrations of Russell’s viper venom 0,2,4,6,8,10,12,14,16,18,20,40 and 80 mg in phosphate buffer and incubated at 37°C with different time intervals 0, 10, 20, 30, 40, 50 and 60 minutes.

3. RESULTS
Russell’s viper venom upon gel filtration chromatography on Sephadex G-75 column was resolved into three distinct peaks (Figure 1). Peak I showed caseinolytic, peak II showed indirect hemolytic and peak III protein devoid of trypsin inhibitor activity (Table 1).
3.1 Determination of motility and viability of sperms

Peak I, II & III showed a decrease sperm motility in vitro in a concentration dependent manner (Figure 3) whereas Peak I, II & III increases death sperms in the concentration dependent manner (Figure 4). The sperm motility decreases as the incubation time increase (Figure 5) and sperm death increases with the increase in incubation time (Figure 6).

![Figure 1: Sephadex-G-75 column chromatography of Russell’s viper venom from eastern regions of India.](image1)

The column (0.5x95cm) was equilibrated with 0.05 M phosphate buffer pH 7.0, 1ml fractions were collected at the flow rate of 10 ml per hour. The column showing three peaks, peak I, II and III.

![Figure 2. Electrophoresis - Non Reducing SDS PAGE](image2)

Russell’s viper whole venom showing three positive bands
Figure 3: Percentage of sperm motility decreases with increase in the concentration of venom.

Figure 4: Percentage of dead sperm increases with the increase in the concentration of venom.

Figure 5: Percentage of sperm motility decreases with increase in the incubation time
Figure 6. Percentage of dead sperm increases with the increase in incubation time

Table 1: Summary of Partial Purification and Characterization of Russell’s viper venom proteins

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Total Protein mg$^1$</th>
<th>Hemolytic Activity</th>
<th>Caseinolytic activity$^2$</th>
<th>Trypsin Inhibition$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Direct lytic activity (50 µg)</td>
<td>Indirect lytic activity (50 µg)</td>
<td></td>
</tr>
<tr>
<td>Dobia</td>
<td>Peak I</td>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>8 Units</td>
</tr>
<tr>
<td>russelii</td>
<td>Peak II</td>
<td>10</td>
<td>NA</td>
<td>78%</td>
<td>NA</td>
</tr>
<tr>
<td>Venom Sephadex -G-75</td>
<td>Peak III</td>
<td>15</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, no activity

1Total protein was estimated in the pooled peaks.

2One unit of activity is defined as the amount of enzyme required to cause an increase in OD by 0.01 at 660 nm per minute.

3One unit of trypsin inhibitor activity is defined as the amount of protein required to cause the inhibition of trypsin (5 mg) activity on TAME by 10%.

4. DISCUSSION

The adverse action of snake venom affects various organs which depend on the region, type of the toxin present in the venom and the amount of venom released. It is believed that the testis seems to one of the organs prone to venom action. However blood test is barrier protects spermatogenesis from direct contact with blood and could prevent most blood toxins from entering spermatogenic cells and spermatozoa.$^{[17,18]}$ In this current study direct incubation of human sperm with Russell’s vipers showed decreases in sperm motility and increases in sperm death with increased incubation time and venom concentration. These
results are in agreement with Rahmy et al.\textsuperscript{[19]} in which in vitro action of cobra venom on goat spermatozoa was studied. In line, few in vivo studies have been reported the effect of venom on atrophy in rat and rabbit testis and altered spermatogenesis.\textsuperscript{[8,12]} These changes may be due to severe alterations of spermatozoa plasma membranes, mitochondrial dysfunction and motility system deformation.

*Rusell’s viper* phospholipase may be attributed to membrane alterations. The mechanism by which phospholipases alters plasma membrane includes hydrolysis of fatty acid ester linkage in the lipid bilayer of membrane phospholipids and lytic synergism with phospholipases on the cell membrane,\textsuperscript{[20,21]} thus resulting in a decrease in sperm motility. Recently a toxin complex called Reprotoxin, containing PLA\textsubscript{2} was found to induce atrophy of testis, which leads to changes in plasma membrane composition and eventually leads to apoptosis.\textsuperscript{[22]} Also, few evidence suggests that, incubation with high venom concentration damages acrosomes by disturbing acid phosphatase, which is a component of acrosome which finally leads to removal and lysis of sperm plasma membrane.\textsuperscript{[23]} Rahmy et al.\textsuperscript{[19]} reported the in vitro action of venom on goat spermatozoa with various concentrations and with different incubation time. High concentration of venom showed swollen irregular plasma membrane of spermatozoa heads after one hour incubation; membrane rupture, disintegration and wrinkling in spermatozoa after two hour incubation; lysis and removal of the plasma membrane external surface after three hour incubation. These findings coincide with our result where the percentages of sperm motility were decreased with increased incubation time.

Nitric oxide may be one of the other major contributing factor to decrease in sperm viability. Nitric oxide is a reactive oxygen species that have been implicated in a variety of physiologic cell signaling mechanisms in many tissues and is recognized as a molecule that importantly regulates the biology and physiology of reproductive function.\textsuperscript{[24]} A rapid increase in NO level following venom treatment responsible for germ cell apoptosis.\textsuperscript{[25,26]} It was proposed that the NO induced apoptosis might be mediated by peroxy nitrite which is generated by the reaction between NO and superoxide.\textsuperscript{[27]} In addition, salgueiropagadigorria\textsuperscript{[28]} and coworkers revealed that venom cytotoxic effect lead to the inhibition of mitochondrial ATP production and also causes disturbance of ATPase and succinate dehydrogenase. As the mitochondrial enzyme system provides the energy required for sperm motility, these alterations in a mitochondria lead to decrease in sperm motility.\textsuperscript{[29]} Disturbance of mitochondrial function
was also associated with deformation in the motility system indicated by axoneme disorganization, damage and irregularities of tail course fibers. It could also be participate directly in the reduction of sperm motility which was observed in our study.

In conclusion, our investigation demonstrates Russell’s viper venom from the eastern region of India induced a direct action on sperm motility and viability in a time and concentration dependent manner in vitro. This could indicate an expected reduction in fertility of Russell’s viper victims. Further studies are required to identify the underlying mechanism.

5. REFERENCES


