AN ALTERNATIVE MODEL TO ANALYZE THE EFFICACY OF COMMERCIAL ANTIVENOM AGAINST HEMOTOXIC EFFECT OF INDIAN RUSSELL’S AND SAW-SCALED VIPER VENOMS

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ABSTRACT
Snake envenoming is an under-reported health hazard that leads to fatality, especially among the rural populations. The toxicity of snake venoms differs from species to species and individual to individual also depending heavily on the susceptibility of the victim to these complex mixtures. Among the snakes of India, three kinds of clinical venom toxicity predominate in varying proportions: hemotoxic, neurotoxic and cytotoxic effects. Vipers such as Russell’s (Daboia russelii) and saw scaled (Echis carinatus) are known to cause local and systemic bleeding with regional variations in neurotoxic effects especially noted for the Daboia russelii. Venom-induced hemorrhage and coagulopathy is considered as an outstanding feature of viper snake envenomation. The treatment of snakebite is in need of improvement in order to increase the probability of good outcomes for envenomed people and livestock worldwide. Rodents are the mainstay models of antivenom efficacy testing. Herein we extend knowledge of an alternative method to study the efficacy of antivenom against the hemorrhagic activity
of viper snake venoms, using embryonated chicken eggs between 4 to 7 days of age. The greater use of eggs in venom toxicology studies would result in more humane, cost effective means of improving patient care and reducing the burden on laboratory animals.

KEYWORDS: Hemorrhage, venom, antivenom, Standard Hemorrhagic Dose.

INTRODUCTION
Snakebite is a major health issue that leads to a shocking number of fatalities in India despite the existence of polyvalent antivenoms. There are about 276 clearly identified snake species in India. Among them, at least 62 species are venomous in nature, but antivenoms are generally manufactured to treat only the most common bites of the so-called “Big Four”. The four major venomous snake species in India accounting for 70-80% of bites are the spectacled cobra (*Naja naja*), common krait (*Bungarus caeruleus*), Russell’s viper (*Daboia russelii*) and the saw-scaled viper (*Echis carinatus*).[1] Among these big four venomous snake species, Russell’s viper and saw-scaled viper are the two species that are predominantly hemotoxic in human victims. The bites from these snakes cause local and systemic bleeding as well as tissue destruction with complications such as renal failure and even pituitary infarct. Venom-induced hemorrhage is known to be an essential feature for the clinical diagnosis of envenoming by these snakes.[2,3] Hemorrhagins are a vital constituent of venoms causing damage to blood cells, disruption of coagulation and are primarily snake venom metalloproteases (svMP). It is imperative to re-appraise standard techniques to evaluate the venom induced hemorrhage and the efficacy of antivenom against them while developing newer techniques with broad utility. Most commonly used method for evaluating hemorrhage is that of, of Kondo.[4] Mice are subjected to intradermal injection with the venom of interest and euthanized after 24 hours for necropsy.[5,6] The same technique is then used to test the neutralization efficacy of antivenoms by comparison to control.[4,7] An alternative model system to check the activity of venoms and potential treatments such as antivenom and newer experimental therapies[8,9,10] is desirable since large number of mice are needed to obtain reliable results with the murine model. These models are expensive and take long time periods to complete at the cost of many animals lives.

To supplement or replace the animal models, a novel method using hen’s egg as an alternative to the conventional *in-vivo* rodent assay for antidotes to study the hemorrhagic venoms of snakes such as *Echis leucogaster*, *Echis pyramidum leakeyi*, *Bothrops jararaca*, *Echis carinatus*, and *Echis leucogaster*.
Naja kouthia was previously been proposed.[11] Herein we extend knowledge of the use of the embryonated hen’s eggs technique to study the efficacy of antivenoms against hemorrhagic activity of India’s venomous snakes such as Russell’s viper and saw-scaled viper. These egg-based methods results in a painless procedure because the experiments were performed in the earliest stages of egg development during the first week after laying. During this period, the neural reflex arcs (i.e. those responsible for nociception) are not yet developed.[11] Hence, the early embryonic content will not feel the pain or pain-like experiences during the experiment. This method may be used for venom studies to advance the essential medical science efficiently, economically and promote animal welfare at the same time and without need for high-technology laboratory facilities.

MATERIALS AND METHODS

Source of venom and antivenom

The lyophilized snake venom powders were obtained from Irula snake catchers industrial cooperative society Ltd., Chennai and the antivenom used for the study is a commercially available antisnake venom (ASV) produced by Vins Bioproducts, Hyderabad. Vins ASV is polyvalent and the biomaterial is obtained from immunized horses in the standard fashion. One ml of the re-suspended preparation is capable of neutralizing 0.60mg of dried Russell’s viper venom and 0.45 mg of dried saw-scaled viper venom.

Preparation of stock venom dilution

0.01g of lyophilized venom powder was suspended to 0.5ml of the normal saline, making a concentration of 20 µg/µl.

Preparation of working standard venom solutions: from the stock venom dilution, the following working standard solutions were prepared.

10µl of stock venom solution was mixed with 790µl of saline solution to obtain the concentration of 0.5 µg/2µl. 10µl of stock venom solution was mixed with 390µl of saline solution to obtain the concentration of 1µg/2µl. 10µl of stock venom solution was mixed with 256.60µl of saline to obtain the concentration of 1.5µg/2µl.10µl of stock venom solution was mixed with 190µl of the saline to obtain the concentration of 2µg/2µl. 10µl of stock venom solution was mixed with 123.2µl of saline solution to obtain the concentration of 3µg/2µl. 10µl of stock venom solution was mixed with 90µl of saline solution to obtain the concentration of 4µg/2µl.
From the stock venom solution, 5µl was taken directly to obtain the concentration of 100µg/5µl. From the stock venom solution, 10µl was taken and directly used to get the concentration of 200µg/10µl.

The complete methodology of this experiment is pictorially represented in Figure 1.

Selection and Incubation of Embryonated eggs
With slight modifications, the eggs were prepared using the method of Dunn and Boone. The eggs were collected on day 1 from hens' nests and incubated at 37°C in a humid incubator for approximately 3 to 5 days until blood vessels were visible by candling and prior to neurulation. The embryonated eggs that were selected by candling process and wiped with 70% ethanol to clean the shell surface. A small opening was made at the air sac portion. The eggshell was removed until a clear air sac area with visible blood vessels was obtained. The process was carried in a Laminar hood, aseptically and the partially shell-less egg preparations were kept in sterile Clingfilm-Hammocks. The partially shell-less egg preparations were made for different concentrations of venom and antivenom with duplicates.

Preparation of Clingfilm-hammocks
The Clingfilm-hammocks were prepared by draping the film (cleanwrap, Clingfilm, non-PVC, R.K Hygiene products, Delhi) over a 7x7cm plastic container, securing it at the base with elastic band and covering it with a sterile Petri dish lid (Fig 2) The Clingfilm-hammock was sterilized by ultraviolet (UV) radiation and Petri dish lids were sterilized at 121°C for 15 minutes at 15lb/inch² before use. No water is placed beneath the sling. The incubator is humidified.

Preparation of Filter paper discs
The Whatmann No.1 filter paper was used in preparing the discs, using a hand punch machine. The discs measuring 5mm in diameter were prepared (Fiber glass filters can cause irritation on membrane and nitrocellulose paper may retain some applied proteins with it). The discs were sterilized at 121°C for 15 minutes at 15lb/inch² in a Petridish. A sample disc was taken to test its holding capacity of venom solution. The appropriate concentration of venom dilutions were impregnated on to the discs using a sterile micropipette and the disc was aseptically placed on the yolk sac membrane on the major bilateral blood vessel of the embryonated egg kept in Clingfilm-hammock. The eggs
Measurement of Hemorrhage

A corona of hemorrhage surrounding the disc impregnated with the hemorrhagic venoms could be visualized after 2-4 hours of incubation at 37°C. For each venom dilution, the concentration required to cause a hemorrhagic corona of 2mm wide was accepted as a standard hemorrhagic dose-SHD (Figure 3).

Estimation of antivenom activity

1ml of the antivenom from the commercial preparation (Vins), was able to neutralize 0.60mg of dried Russell’s viper venom and 0.45 mg of dried saw-scaled viper venom. Dilutions were made from the commercial preparation, so that each of the hemorrhage causing venom dilutions could be exactly neutralized. To estimate the efficacy of the antivenom, the antivenom and venom were mixed accordingly to the specific dilutions and were incubated at 37°C for 30 minutes. The mixture was then applied to a sterile disc and kept on yolk sac membrane over a blood vessel in a partial shell-less egg preparation as previously described and observed for 2 to 4 hours. The minimal amount of antivenom dose required to abolish the hemorrhage was recorded as the Minimum Effective Neutralizing Dose (MEND).

Figure 1: Methodology

Figure 1: In Stage 1 (top row) Fertilized eggs are collected on the morning of being laid and transferred directly to a humid incubator. Once the presence of embryonated yolk is confirmed by candling technique the eggs are transferred to sterile hammocks for the procedure. Stage 2 (middle row) hemorrhagic activity of the venoms is assessed and...
titrated to achieve consistent 2mm corona around the paper disk. Stage 3 (last row) the neutralization efficacy of antivenom with venom was assessed.

A. Top view

B. Side View

Figure 2: Preparation of Clingfilm-Hammock set up: A) View from above with visible air sacs and covered by petri-dishes. B) Side view showing the eggs suspended in the cup by cling-film and elastic band.
Figure 3: Measurement of corona of hemorrhage

The first egg is control (left, with disc removed to reveal underlying, intact vessels) and the second egg with arrow (right) is treated with venom-loaded disc showing corona of hemorrhage. The transparent ruler was used to measure the size. Filter paper was removed from the first egg to show the intact blood vessels of the control egg.

RESULTS

Formation of Hemorrhagic corona

A corona of hemorrhage measuring ~5mm in diameter was observed in the eggs to which venom was applied via filter paper discs confirming the hemorrhagic activity of the venoms. In this study, the coronas of hemorrhage were obtained at the concentration of 100µg/5µl for Russell’s viper and 200µg/10µl for saw-scaled viper venom (Figure 4). This indicates the hemorrhagic activity of these venoms could be reliably studied using the hen’s egg technique.
Egg 1- labeled “Ec” (left), contains venom-loaded disc showing corona of hemorrhage with 200 µg/10 µl for saw-scaled viper venom.

Egg 2- labeled as “Dr” (right), contains venom-loaded disc showing corona of hemorrhage with 100 µg/5 µl for Russell’s viper venom.

*The dilutions 200 µg/10 µl for saw-scaled viper venom and 100 µg/5 µl for Russell’s viper venom were the least venom concentrations that produced hemorrhage in the assay and hence they are inferred as Standard hemorrhagic dose (SHD).

Estimation of Standard Hemorrhagic Dose (SHD)

The SHD was determined by the observations of the corona of hemorrhage developed in the embryonated eggs. The estimation of SHD was measured using a transparent ruler scale. From the results, the measurement of SHD was estimated as:

100µg/5µl for Russell’s viper- *Daboia russellii* (Table 1)
200µg/10µl for saw-scaled viper- *Echis carinatus* (Table 2)

| Table 1: Estimation of Standard Hemorrhagic Dose (SHD) for Russell’s viper venom |
|---|---|---|---|
| S. No | Venom Dilutions | Formation of corona of hemorrhage | Standard Hemorrhagic Dose |
| 1 | 0.5 µg/2 µl | Negative | Negative |
| 2 | 1 µg/2 µl | Negative | Negative |
| 3 | 1.5 µg/2 µl | Negative | Negative |
| 4 | 2 µg/2 µl | Negative | Negative |
| 5 | 3 µg/2 µl | Negative | Negative |
| 6 | 4 µg/2 µl | Negative | Negative |
| 7 | 100 µg/5 µl | Positive | Positive |
| 8 | 200µg/10 µl | Positive | Positive |

| Table 2: Estimation of Standard Hemorrhagic Dose (SHD) for saw-scaled viper venom |
|---|---|---|---|
| S. No | Venom Dilutions | Formation of corona of hemorrhage | Standard Hemorrhagic Dose |
| 1 | 0.5 µg/2 µl | Negative | Negative |
| 2 | 1 µg/2 µl | Negative | Negative |
| 3 | 1.5 µg/2 µl | Negative | Negative |
| 4 | 2 µg/2 µl | Negative | Negative |
| 5 | 3 µg/2 µl | Negative | Negative |
| 6 | 4 µg/2 µl | Negative | Negative |
| 7 | 100 µg/5 µl | Negative | Negative |
| 8 | 200µg/10 µl | Positive | Positive |
Estimation of Minimum Effective Neutralizing Dose (MEND)

The MEND was determined based on the Standard Hemorrhagic Dose (SHD). Antivenom and venom were mixed accordingly to the specific dilutions and incubated as a mixture at 37°C for 30 minutes. Afterwards, the mixture containing one SHD of venom and varying concentrations of antivenom were mixed and applied on a sterile disc. The absence in formation of corona of hemorrhage was noted and the concentration was estimated as Minimum Effective Neutralizing Dose-MEND (Figure 5). Typically the corona of hemorrhage in untreated (control, venom only) eggs show signs of hemorrhage within a minute.

For this experiment the MEND was estimated to be:
167 µl for Russell’s viper- *Daboia russelii* (Table 3)
444 µl for saw-scaled viper-*Echis carinatus* (Table 4)

*Figure 5: Minimum Effective Neutralizing Dose (MEND)*

Egg 1 (Left)- saw-scaled viper venom/antivenom coated disc
Egg 2 (Right)- Russell’s viper venom/antivenom coated disc

*Both the eggs showed minimal hemorrhage which indicates the neutralization of venom by antivenom and hence this dose has been considered as Minimum effective neutralizing dose (MEND).*

<table>
<thead>
<tr>
<th>Standard Hemorrhagic</th>
<th>Antivenom used*</th>
<th>Dilutions of Antivenom</th>
<th>MEND</th>
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<tr>
<td>Dose (SHD)</td>
<td>Antivenom used*</td>
<td>Dilutions of Antivenom (in µl)</td>
<td>MEND</td>
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<td>100 µg/5 µl</td>
<td>Polyvalent Antivenom</td>
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<td>Polyvalent Antivenom</td>
<td>166.6</td>
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*produced form immunized horse by Vins Bioproducts Hyderabad

Table 4: Estimation of Minimum Effective Neutralizing Dose (MEND) of antivenom for saw-scaled viper Venom

DISCUSSION

The Standard hemorrhagic dose (SHD) of 100 µg/5µl for Russell’s viper and 200 µg/10µl for saw-scaled viper were obtained in our study, but every individual batch of venom needs to be assessed independently. The results obtained from our study suggests the efficacy of the antivenoms against hemorrhagic activity of snake venoms can be studied utilizing embryonated hens eggs. Results from Sells et al.[12] illustrates the vast geographical and species differences (2µg/egg for Bothrops jararaca) with respect to venom activity. Shell-less egg cultures have been used in the past for a variety of toxicology studies and we are now extending that experience to study medically important snakes of India.[14,15]

In this study conventional neutralization of hemotoxic venom with antivenoms has been quantified by pre-incubation of various concentration of venom with the MEND. Importantly, the results of our egg study correlate well with rodent studies as animal models though we did not make a direct comparison in the present study.[12] Our results give hope that the current model can be utilized effectively to evaluate the efficacy of antivenoms against the hemorrhagic activity of snake venom and could augment or, eventually, replace conventional animal screening models.

CONCLUSION

In the present study, our choice of embryonated egg model as a potential supplement or alternative to rodent models is based on the fact that avian and mammalian tissue shares many common characteristics. Our results suggest this is the case, as well. Birds, mammals
and other reptiles are prey items of snakes so it is not surprising that many types of venom have promiscuous physiological effects on different animal orders. Previous studies have reported the use of chick biventer cervicis muscles and myoblasts for the investigation of cardiotoxic activity from *Naja siamensis* and *Naja naja kouthia* venom. The notable advantages of embryonated egg assay for venom-induced hemorrhagic activity and neutralization include: Cost effectiveness, simplicity of performance and painless experimentation on the model. This method has the ability to test vast number of venom samples simultaneously and very inexpensively when compared to an expensive animal models. The entire procedure can be accomplished within 5 hours and does not require technically advanced facilities.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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