Evaluation of Approximate LD50 and pharmacological effects of Echis carinatus (Saw-scaled viper) venom from Central Punjab, Pakistan

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Citation

Abstract
The assessment of toxic and pharmacological properties of snake venoms and their components is based predominantly on the animal experiments. Several modifications using fewer animals than the classical LD\textsubscript{50} assay have been published. Using these methods an Approximate LD\textsubscript{50} can be determined, the precision of and reproducibility of which is sufficient for most purposes of lethality testing. The pharmacological studies of the saw-scaled viper help us to locate and work on the components in the venom responsible for bringing these changes in the victims. So far in Pakistan the toxicity of snake venoms has been evaluated only through the classical LD\textsubscript{50}. The present work comprises the estimation of the toxicity of crude venom of Echis carinatus (saw-scaled viper) by evaluation of its Approximate LD\textsubscript{50} in mice. This study has been taken as a model for the venom bioassays of snakes from other proximate species. The subsequent portion comprises the characterization of pharmacological effects of the crude venom of the said snake in mice.

INTRODUCTION
The total toxicity of snake venom encompasses the entire effects of all the toxic constituents. This toxicity of the venom and its other pharmacological effects on the victim are tested and studied through various animal experiments. The venom amount that kills 50\% of the test animals is defined as the LD\textsubscript{50}, where LD abbreviates for Lethal Dose. Researchers have usually carried out LD\textsubscript{50} calculations according to Reed and Muench (1958) Litchfield and Wilcoxon (1949) method. The LD\textsubscript{50} test introduced by Trevan (1927) has gained acceptance as a measure of acute toxicity. However the LD\textsubscript{50} figure is by no means constant with a given substance and can be affected by wide range of different factors. Nevertheless, the information on the lethality of snake venoms is required although in many cases it may result in unnecessary waste of experimental animals (Zbinden and Flurry, 1981, Brown, 1985).

Several modifications using fewer animals than the classical LD\textsubscript{50} assay have been published. Using these methods an approximate LD\textsubscript{50} can be determined, the precision and reproducibility of which is sufficient for most purposes of lethality testing. Meier and Theakston (1986) calculated the approximate LD\textsubscript{50} values of some snake venoms by using the method of Baccari (1949) as modified by Molinengo (1979). This technique is based on the results obtained by comparison of doses injected with observed survival times of experimental animals. Several researchers have studied the biochemical and pharmacological effects of venoms from different species of snakes from different localities (Al-Asmari, 2005). Meier and Theakston (1986) applied this technique and got consistent results. Some workers suggested that although statistical precision and reproducibility of an LD\textsubscript{50} test could probably be improved by sacrificing large number of animals, its outcome is influenced by a considerable number of factors. In Pakistan some work has been done regarding the determination of toxicity of cobra venom through the LD\textsubscript{50} (Alam and Ali, 1998), though in this work, the conventional classical LD\textsubscript{50} has been performed.

Saw-scaled viper, Echis carinatus is considered to be one of the most dangerous snakes in the world because of its venom toxicity and high population densities in rural agriculture areas. However, little information is available on the pharmacological effects of venom from this snake (Zahra et al., 2005). The principal segment of the resent study comprises the estimation of the toxicity of the Echis carinatus venom by calculating its approximate LD\textsubscript{50} in mice. This study has been taken as a model for the venom
bioassays of snakes from other proximate species. Calculating the approximate LD$_{50}$ for the Echis carinatus will lead us towards the calculation of similar lethal doses and estimations of toxicity of other snake venoms using the same method. The subsequent portion elucidates the characterization of the diverse pharmacological effects and changes inflicted by the crude saw-scaled viper venom in mice.

MATERIAL AND METHODS

SNAKES AND VENOM

Fifteen Saw-scaled vipers, Echis carinatus were captured from different regions of Central Punjab of Pakistan. The snakes were kept in captivity for two weeks before their milking was performed. All snakes selected for milking were adult. Milking was performed without anesthetics. A specialized team was responsible for scientific classification, milking of specimens and storage of the venom. Venoms of all snake specimens were pooled and dissolved in saline (final concentration: 10mg/ml). All the venom samples were stored at 4°C until used to avoid any disruption of their natural toxic properties.

TOXICOLOGICAL STUDIES

Fifty adult albino white mice belonging to both sexes were purchased from Manawa Research Institute (MRI) Lahore, Punjab. The pooled venom was injected intraperitoneally; separately into groups of 10 mice with doses ranging from 1 to 10-mg/kg-body wt. As all the snakes used in the experiment belonged to the same species, pooled venom was suggested and preferred to the individual venom in order to have a cumulative effect of the toxins present in a particular snake species. Survival times (time between injection and death) of each animal for 24 hours were recorded. The LD$_{50}$ of each snake species was determined according to the mathematical scheme adopted by Meier and Theakston (1986) some modification was however made in their method where by the maximum three-hour survival time was increased up to 24 hours for bringing convenience and authenticity to results.

No variables related to age, geographical origin, sex, and diet of snakes were controlled although variation in venom composition, even in natural conditions, may be associated with these variables. The effects of these variables on the toxicity of the venom in mice, although certainly present, were therefore not observed.

RESULTS AND DISCUSSION

CALCULATION OF APPROXIMATE LD:

Survival times observed after intraperitoneal (i.p.) injection of different doses of Echis carinatus venom in mice are given in table 1. The scheme for the calculation of Approximate LD$_{50}$ is given table 2.

Figure 1

Table 1: Survival times observed after i.p. Injection of different doses of venom in mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Weight of mouse (grams)</th>
<th>Application volume (10 ml/kg)</th>
<th>Dose (mg/kg)</th>
<th>Actual (mg) venom entered mouse (ml)</th>
<th>Survival time (min)</th>
<th>Dose (mg/kg)</th>
<th>Survival time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>1.5</td>
<td>10</td>
<td>0.05</td>
<td>18</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1.5</td>
<td>20</td>
<td>0.015</td>
<td>16.33</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>10</td>
<td>5</td>
<td>0.37</td>
<td>9</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>10</td>
<td>7</td>
<td>0.33</td>
<td>2.42</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>2.5</td>
<td>8</td>
<td>0.10</td>
<td>3.5</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>5</td>
<td>9</td>
<td>0.16</td>
<td>1.33</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>32</td>
<td>7.5</td>
<td>10</td>
<td>0.23</td>
<td>0.33</td>
<td>13.0</td>
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<tr>
<td>8</td>
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<td>9</td>
<td>11</td>
<td>0.31</td>
<td>0.3</td>
<td>14.0</td>
<td></td>
</tr>
</tbody>
</table>
The pharmacological effects of the crude venom were characterized on a number of mice, however some significant effects observed in various models with visible symptoms are described.

**INCREASED HYPERSENSITIVITY WITH NON-LETHAL DOSE**

When a mouse was injected intraperitoneally with a non-lethal dose 1 mg/kg of viper venom, it grew surprisingly energetic and hypersensitive. It ate more feed than the normal and, during one of the countless attempts of escape from the finely wired jar; it sometimes excreted the fecal matter that was larger, almost double the normal fecal matter size. The mouse remained alive and active and did not by any means seem to suffer badly from the effects of the non-lethal dose. It showed no symptoms of pain or agitation.

**SLOUGHING OF SKIN WITH SUB-LETHAL DOSE**

When a sub lethal dose (2 mg/kg) was injected to a mouse, there was a little sloughing of the dead tissue around the injection site after a day or so. The being grew weaker, ate poorly, and appeared generally miserable for about two days before it recovered its health.

As the venom of the viper Echis carinatus literally dissolves and liquefies some tissues that it contacts, one mouse with a sub lethal dose bled to death not due to the lethal effect of the venom but more because of the large wound at the site of the injection. The wound grew so black and large that the entire viscera of the mouse literally began to pop out of the wound and the mouse died almost 12 hours after the injection. The survival time of this mouse was not included in the data.

**PARALYSIS WITH PARTIALLY LETHAL DOSE**

When a partially lethal (4.5 mg/kg) dose was injected to a mouse, the weakness and difficulty in breathing developed and lasted for a few hours. The tissues at the site of the injection were dissolved. The animal would be obviously uncomfortable from the start; it scratched, rubbed at the injection site and was generally irritable and hyperactive and hypersensitive. Next came a quiet period during which the animal would sit huddled up but alert. The injection site was dark. There would be a short period of almost normal activity followed by another quiet stage that eventually terminated in collapse and death. The skin around the injection site grew gangrenous. The mouse became flat, immobile with its hind legs paralyzed. The survival time of the mouse was about 18 hours (table 1).

**RESPIRATORY FAILURE WITH LETHAL DOSE**

When a mouse was injected intraperitoneally with a lethal, but not overwhelming, dose of viper venom (5 mg/kg), it showed little immediate evidence of pain or discomfort. For a short time it behaved normally but soon it started showing signs of weakness. Its breathing got labored, and its flanks became peculiar, punched in appearance. Soon it could no longer get to its feet with the breathing becoming ever more difficult. As respiration ceased, the heart continued to beat for a few minutes. The survival time of the mouse was noted to be about 16 hours and 20 minutes (table 1).

**CONVULSIVE SEIZURES AND NECROSIS AT HIGHER DOSES**
At the next lethal doses (6 to 12 mg/kg) the sequence of events was not altogether different. The duration of the each event was brief and it started quicker in time and closer to each other. The mice would sometimes lick the place where the venom was inoculated. Within a few minutes the breathing became irregular and abdominal respiration began which was also spasmodic. Soon the mice became prone, motionless with their hind legs paralyzed. In some mice powerful convulsive spasms rarely passed from the tail through the whole body. The bodies of the mice became stiff near death. The place of the skin where the venom was inoculated became darker in color and black spot appeared at the site of injection within half an hour or so. The area of the skin around the site of injection became hairless and acute tissue digestion began at the site. This showed the obvious necrotic effect of the venom. The survival time of the mouse having the highest dose (12 mg/kg) of venom was recorded to be about 20 minutes only.

Pharmacological studies clarify the clinical and pathological features of human fertility. Furthermore these help to understand specific resistance and susceptibility of various warm blooded and cold-blooded species (Perez et al. 1978, 1979). This could lead to gauging the extent of physiological tolerance to natural snakebites and thereby enable preparation of better antisera to neutralize the venom activity.

Two type of toxic effects were most easily observable in the all the mice, i.e. the change in color and dissolution of the skin at the site of the injection and the mice becoming prone, motionless with their hind legs paralyzed. The dissolution of the skin is an evident consequence of the myotoxic effect of the venom. Paralysis of hind legs making the mouse flat motionless with their hind legs paralyzed. In some mice which was also spasmodic. Soon the mice became prone, breathing became irregular and abdominal respiration began near death. The place of the skin where the venom was inoculated became darker in color and black spot appeared at the site of injection within half an hour or so. The area of the skin around the site of injection became hairless and acute tissue digestion began at the site. This showed the obvious necrotic effect of the venom. The survival time of the mouse having the highest dose (12 mg/kg) of venom was recorded to be about 20 minutes only.

The intraperitoneal Toxicity of Echis carinatus determined through Approximate LD50 in our study was 6.23 (Table 2). This value is authenticated by the intravenous toxicity of E. carinatus determined through Approximate LD50, which was 2.98. The difference between the two figures is mainly due to two factors. Firstly the two experiments were performed under different conditions and circumstances Secondly there is always substantial difference between an intraperitoneal and an intravenous dose of the same drug (in this case the venom).

As classical LD50 experiments, owing to their high level of variability, are far from satisfactory, the approximately method of LD50 suggested for scientific, economic and ethical reasons was tried and found relatively unproblematic, suitable and effective. Approximate method has thus been found a satisfactory alternative to classical LD50 determinations for animal venoms, owing to its exactitude and reproducibility for most purposes of lethality testing in animal models.

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