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IN VIVO STABILIZATION STUDIES ON INDIAN COBRA (*NAJA NAJA*) VENOM FOR ITS TOXICOLOGICAL ACTIVITY (LD_{50}) IN MICE

Samir Nimbark*[1] and Rosaiah Gorrepati [2]

Department of Biotechnology [1], Department of Botany and Microbiology [2], Acharya Nagarjuna University, Nagarjunanagar - 522510, Andhra Pradesh, India

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Correspondence to Author:

Samir Nimbark

307, Shubh Kalp C.H.S., Survey no. 80/5, Nr. Oxygen valley, Manjri (BK) – 412 307, Dist. Pune, Maharashtra , India

E-mail: samirnimbark@yahoo.co.in

ABSTRACT: Biological/Toxicological activity of Cobra venom (CV) is usually estimated in terms of LD_{50} which varies at each time of test, when stored in liquid condition at non-native environment, as the CV contains most unstable complex proteins in it. Prepared & stored working solution of cobra venom at 5±3ºC in liquid condition for experimentation without any stabilizing agent, it becomes unstable and show huge variations in LD50 test result (performed on Swiss albino mice, weight: 17-22g). Such variations in biological activity of CV have adverse impact on venom dependent potency test of anti-snake venom sera (ASVS) which has direct commercial value. In this study, working stock solution of CV was prepared in the three different diluent sets (B, C and D) of solutions commonly used in laboratories and stored at 5±3ºC, which was tested for LD_{50} at pre-defined interval, up to 120 days. Obtain results were statistically calculated for variation from mean value in terms of Standard deviation (SD). The study result indicates that if the working stock solution of CV is prepared, stored at 5±3ºC using solution of SET C and repeatedly used up to 120 days, shows considerably less variations in its toxicological activity (LD_{50}) which is beneficial in potency test of ASVS.

INTRODUCTION: Indian Cobra (*Naja naja*) also known as **Nag** or **Spectacled Cobra** is a species of the genus Naja found in the Indian subcontinent and a member of the "Big Four", the four medically important snake species which inflict the most snakebites in India [1, 2]. This snake is revered in Indian mythology and culture, and is often seen with snake charmers. It is now protected in India under the Indian Wildlife Protection Act (1972).

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The Indian cobra is native to the Indian subcontinent which includes present day Nepal, Pakistan, India, Bangladesh and Sri Lanka. It can be found in plains, jungles, open fields and the regions heavily populated by people [2]. Its distribution ranges up to 1800 meters above sea-level and this species mainly feed on rodents, toads, frogs, birds, lizards and other snakes [3]. Its diet of rats leads it to areas inhabited by humans including farms and outskirts of urban areas.

The Indian cobra's venom generally contains a powerful post-synaptic neurotoxin and cardio toxin. The venom acts on the synaptic gaps of the nerves, thereby paralyzing muscles and in severe bites leading to respiratory failure or cardiac arrest [1].

The venom components include enzymes such as hyaluronidase that cause lysis and increase the spread of the venom. Envenomation symptoms manifest immediate after snake bite and person usually die within 2 to 6 hours following the bite [2]. In India about 46, 000 people are dying every year from snakebite, out of which Indian Cobra is considered as one of the most known and poisonous snake [4].

Cobra venom contains 70 - 80 % protein components of toxic proteins (like; cardiotoxin, myotoxin, neurotoxin, cytotoxin) and nontoxic proteins along with many enzymes (like; phospholipase A2, L-Amino acid oxidase, nucleosidase, ribonuclease) which are responsible for its poisonous activity over the living organisms [5]. Toxic proteins and enzymes belong to extremely heterogeneous class of biological macromolecules and many of them are unstable when not in their native environment.

Snake venom is used in manufacturing and testing of anti-snake venom serum product, which is produced by immunizing the horse and mules with the cobra venom, many times with the mixture of two to four types of venoms to produce polyvalent product [6]. The amount of antibody will rise after repeated inoculations, in gradually increased quantity of venom. Serum / plasma from animals which having antibody against the cobra venom inoculated is collected and isolated from other components of blood [6]. The quality and quantity of collected serum is tested *in vivo* on Guinea pigs and mice. In quality control testing of ASVS, Snake Venom Activity (Lethality, LD_{50}) plays vital role in neutralization of venom specific antibody of ASVS. The capacity of antibody neutralization by venom determine the quality (selective neutralization by venom) and quantity (amount of venom neutralized by antibody) of antibody to be used to treat the snakebite victim.

Cobra venom when diluted to working solution and stored at 5±3ºC, it becomes unstable in biological activity because of non-native environment. Currently this problem has been handled by repeated preparation of working solution, which increases the experimental / testing cost for venom and anti-snake venom serum.

In this context, there was a need for suitable stabilizer in which the working solution of venom can be prepared and preserved for extended period of time without changing its toxicological / venomic activity (LD_{50}) to provide the consistent results in the experiment / test.

MATERIALS AND METHODS:

SET A: Hundred milligram of lyophilized Indian Cobra venom (received from Erula snake farm, Tamilnadu) was reconstituted using 10 ml of 0.85% physiological saline to make the parent stock solution of 10 mg/ml (w/v). The reconstituted solution was mixed gently and stored at 5±3 ºC for overnight.

The working stock solutions of 1mg/ml (w/v) were prepared by adding 1ml of above stock solution (SET A) to the 9 ml of experimental diluents (SET B, SET C and SET D) as given below.

SET B: In this set of working stock solution, Cobra venom of 1mg/ml (w/v) was prepared by mixing of 1 ml of SET A solution with the 9 ml of 0.85 % physiological saline to make total volume of 10 ml, mixed the content gently and stored at 5±3 ºC during study up to 120 days.

SET C: In this set of working stock solution, Cobra venom of 1mg/ml (w/v) was prepared by mixing of 1 ml of SET A solution with the 9 ml of mixture of 0.85 % physiological saline and glycerol (The final concentration of glycerol was 25 % v/v) to make total volume of 10 ml, mixed the content gently and stored at 5±3 ºC during study up to 120 days.

SET D: In this set of working stock solution, Cobra venom of 1mg/ml (w/v) was prepared by mixing of 1 ml of SET A solution with the 9 ml of Phosphate buffer saline (pH 7.0) to make total volume of 10 ml, mixed the content gently and stored at 5±3 ºC during study up to 120 days.

The Above stored working stock solutions (SET B, SET C and SET D) of 1mg/ml (1000 µg/ml, w/v) were taken out from the storage condition of 5±3 ºC at the interval, start from day 0 to 3rd, 7th, 15th, 30th, 45th, 60th, 75th, 90th, 105th and 120th day from day of preparation and tested for its toxicological activity as a LD_{50} in mice.

The Volume of 0.640 ml of working stock solution from all the three sets (SET B, SET C and SET D) was used to perform the LD_{50} test during each time interval. It was further diluted using 0.85 % Physiological Saline to cover 50% lethal dose. For Indian cobra venom, the LD_{50} value ranges between 9 µg/ mice to 15 µg/ mice, hence the serial dilution range of 16 µg/ mice, 12.8 µg/ mice, 10.24 µg/ mice and 8.192 µg/ mice (four dilutions) with dilution factor of 1.25 was used to get desired LD_{50} result within the test range.

Swiss albino mice of same sex group, weighing between 17 – 22 g with the equal weight distribution were selected for the study. The mice were grouped as four mice / dilution; the distributed mice were kept for acclimatization with the surrounding environment for overnight prior to inoculation. The mice were provided with unrestricted diet and water.

The prepared four serial dilutions for all the three set (SET B, SET C and SET D) were inoculated to respective mice group with 0.5 ml dose, at intravenous route, in tail vein using 26 G, ½ inch pre-sterilized, disposable needle. The inoculated animals were observed for 48 hrs (2 days) from the day of inoculation for survival or death and the test results were recorded for each inoculated group. The obtained results were calculated for their LD_{50} values according to Reed and Muench (1938) statistical analysis [7]. The received LD_{50} test results of each sets (SET B, SET C and SET D) tested at different time intervals were further statistically analyzed for average value and Standard Deviations (SD) for each sets, the values are tabulated in **Table 1**.

RESULTS AND DISCUSSION: The experimental test result shows that, there is no significant variation between different set of working stock solutions on the 0^{th} day of testing (Table I), but when the storage period extends from the day 0, the individual test results of all the three sets start showing fluctuation from the initial value obtained on day 0, The variation for individual set and comparison of the variation among experimental sets are detailed as below.

SET B: The lowest LD_{50} value recorded for SET B is 9.87 µg/mice. It is reported for two times in entire study on 7^{th} and 75^{th} day (Table I). The highest LD_{50} value reported for SET B is 12.8 µg/mice, it was also reported for two times in entire study on 15^{th} and 105^{th} day. The average of the entire set was 11.18 µg/mice. The standard deviation (SD) for SET B was 0.99, which is one of the highest among all the three study sets.

SET C: The lowest LD_{50} value observed for SET C was 10.76 µg/mice. It was observed for a single time in the entire set of study i.e. on 60^{th} day whereas the highest LD_{50} value recorded in SET C was 12.11 µg/mice. This was also reported for the single time in the entire study i.e. on 75th day (Table I). The average of the entire set is 11.42 µg/mice. The standard deviation (SD) for SET C is 0.47, which is one of the lowest in the entire study.

SET D: The lowest LD_{50} value reported in SET D was 9.51 µg/mice. It was also the lowest reported value in all the three sets. It was reported for three times in the entire study on 15^{th}, 30^{th} and on 90^{th} day. The highest LD_{50} value reported in SET D was 11.88 µg/mice. This was reported for single event in entire study on 45^{th} day (Table I). The average of the entire set is 10.67 µg/mice. The standard deviation (SD) for SET D is 0.82, which is moderately high in the entire study.

The stability of proteins in aqueous solution is routinely enhanced by co-solvents such as glycerol. Glycerol is known to shift the native protein ensemble to more compact states. Glycerol also inhibits protein aggregation during the refolding of many proteins. However, mechanistic insight into protein stabilization and prevention of protein aggregation by glycerol is still lacking. Glycerol preferentially interacts with large patches of contiguous hydrophobicity where glycerol acts as an amphiphilic interface between the hydrophobic surface and the polar solvent.

Accordingly, Glycerol prevents protein aggregation by inhibiting protein unfolding and by stabilizing aggregation-prone intermediates through preferential interactions with hydrophobic surface regions that favor amphiphilic interface orientations of glycerol, these interactions shift the native protein toward more compact conformations [8].

TABLE 1: VARIATION IN LD_{50} (µg/mice) ACTIVITY OF WORKING STOCK SOLUTIONS AGAINST DAYS

S. No.	Day of Testing	LD_{50} (µg/mice)		
		SET B	**SET C**	**SET D**
1	0	11.03	11.88	11.45
2	3	10.76	11.45	10.78
3	7	09.87	10.78	10.76
4	15	12.80	11.03	09.51
5	30	11.88	11.88	09.51
6	45	11.03	11.45	11.88
7	60	10.76	10.76	11.03
8	75	09.87	12.11	10.76
9	90	10.76	11.88	09.51
10	105	12.80	11.03	10.76
11	120	11.45	11.45	11.45
Average		11.18	11.42	10.67
SD		0.99	0.47	0.82
Level of Variation		**Higher**	**Lower**	**Moderate**

CONCLUSION: Out of three experimental sets (SET B, SET C and SET D), SET C containing 0.85 % physiological saline with Glycerol mixture (25 % v/v) showed lowest level of variation by means of its standard deviation (0.47) when compared with other two experimental sets, SET B (0.99) and SET D (0.82). These results confirm that SET C is more suitable working stock solution for the stabilization of cobra venom *in vivo* rather than SET B and D.

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REFERENCES:

1. Whitaker R and Ashok C: Snakes of India: The field guide, Draco Books, First Edition, 2004.
2. Gopalakrishnakone P and Chou L M: Snakes of medical importance (Asia-pacific region), Venom and Toxin Research Group, National university of Singapore, First Edition, 1990.
3. Daniel J C: The Book of Indian reptiles, Bombay Natural History society, Oxford University Press First Edition, 1983.
4. Mohapatra B, Warrell DA, Suraweera W, Bhatia P, Dhingra N: Snake bite Mortality in India: A Nationally Representative Mortality Survey 2011.
5. Chellapandi P: Purification and Antibacterial Activity of Indian Cobra and Viper Venoms, Electronic Journal of Biology 2008; 4(1): 11-16.
6. WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulin, World Health organization (WHO) – Technical Report Series 2008; 19-28 and 33-73.
7. WHO, Laboratory Procedures, Serological detection of avian influenza A (H7N9) infections by microneutralization assay 2013; 10-11.
8. Vagenende V, Yap MG, Trout BL: Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. Biochemistry 2009; 48(46):11084-11096.

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