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Research Paper on Observation of Toxicity and Hemolytic Properties of Indian Common Krait Along With Effect of Chemicals, Ph, Light and Radiations

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***Abstract*- Nearly 20,000 people die every year from snakebite in India alone. More than 285 species of snakes are known to exist in India itself. Among these, nearly 58 are known to cause fatal bites. The venom of B. caeruleus is highly lethal: LD50 is 10-12 µg for 70-75 Kg man. Here the specific characters of Indian common krait venom like toxicity and haemolytic activity are studied. Effect of light, pH, chemicals and radiations has also been studied. Hydrogen peroxide reduced the toxicity to 99% but immunogenicity is also lost up to 29%. The effect of electromagnetic spectrum on the venom of Bungarus caeruleus has also been observed. It was found that the venom turns totally non-toxic at 22°C and 37 °C when stored for 28 days, but immunogenicity of the venom is reduced too. After irradiation, there is a change in a number of physical and chemical properties of the proteins. It may further have produced rearrangement of the protein. We can observe the loss of immunogenic components when exposed to UV, infra-red rays and to direct sunlight. The venom is subjected to various temperatures and the effect on reduction of toxicity and immunogenicity has been observed.**

***Key Words*: Bungarus caeruleus, light, ph, temperature, radiations, toxicity, immunogenicity and haemolytic activity.**

I. INTRODUCTION

Snakes are found all over the earth with an exception of Newzeland, where they are never known to exist. These creatures are limbless and like heat, which means life and vitality to them. Therefore, the snakes are more prevalent in tropical and semi-tropical areas of the globe. In warm temperature, they can be seen at any hour of the day, although Bungarus caeruleus is nocturnal in habitat. Nearly 20,000 people die every year from snake bite in India alone. More than 285 species of snakes are known to exist in India itself. Among these, nearly 58 are known to cause fatal bites. The venom of B. caeruleus is highly lethal: LD50 is 10-12 µg for 70-75 Kg man. The snakes may take a heavy toll of life every year in nature. They keep the growth of fast breeding rodents at check. They feed upon insects, rodents and other small creatures that are a nuisance for everyone around. The snakes are a valuable source of venom that is used in various research fields and medicines. The systematic study of venom began in 17th century when Francesco Redi investigated that route of inoculation was important for venom to be effective1. Later, scientists described the effects of haemolysis and toxic effects of various snake venoms2. In 1938, a crystallisable protein from venom of Crotalus durissus terificcus was prepared3. With the advancement in science, newer methods for isolation and purification were introduced. Techniques like ion exchange, polyacrylamide gel electrophoresis or sieve chromatographies are very fast and they retain biological properties of the venom under experiment. In the present study, an effort has been made to observe toxicity and haemolytic activity of venom of B. caeruleus. The effects of physical and chemical agents have also been observed so as to characterise this venom. Here sun light, day light, darkness, UV rays and infrared rays that are parts of electromagnetic spectrum and temperature like 0, 4, 8, 22 and 37 degree Celsius are taken as different parameters to expose the venom and observe the effects on toxicity and immunogenicity on it. To see effects of chemicals on the venom, it has also been exposed to different pH range from 5, 6, 7, 8 to 9; and hydrogen peroxide, formaldehyde, glutaraldehyde and iodine separately to see the effect on venom.

II. MATERIALS AND METHODS

Material

1) Venom: The common krait (B. caeruleus) venom used in this study was obtained from Central Research Institute Kasauli. The venom was dissolved in sterile normal saline. It was centrifuged at 1500 rpm for 10 minutes and then passed through membrane filter of pore size 0.22 micron. The sterile solution was then used in the experiments.

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2) The buffers of different pH (acetate buffer of pH 5.0, Phosphate buffer of pH 6.0, 7.0, 8.0, Barbitone buffer of pH 9.0) were prepared4. The pH of all the buffers was checked accurately with pH meter.
3) Chemicals used in the experiments were prepared in lab, like formaldehyde, glutaraldehyde, iodine and hydrogen peroxide.
4) Laboratory mice of 18-20 gm were taken as experimental animal. They were obtained from random breeding in a closed colony.
5) The normal horse serum was obtained by drawing blood from jugular vein of the normal horse with sterile syringe and collected in a sterile glass tube and incubated at 37oC for half an hour in slanting position. The tube was kept at 4oC overnight. The clot formed in the tube was then broken with the help of a sterile glass rod and centrifuged for 10 minutes at 1,500 rpm. The clear serum was stored at 4oC for use in experiments.

III. METHODOLOGY

Experimental Method was followed.
Toxicity test: It was done by inoculating venom in mice by intravenous route and LD50 was calculated5.
Haemolytic Activity6: A series of ten, two fold dilutions from 5 mg/ml of stock venom solution were prepared in duplicate to observe the direct and indirect haemolysis. The percent transmission of lysed cells in the supernatant was observed with Specord at 540 nm and this was compared with standard.
Standard: 10 ml of 1% RBCs under experiment were taken in a test tube and centrifuged at 1,500 rpm for 10 minutes. The supernatant was discarded and to the remaining cells 10 ml of distilled water was added and shaken to lyse the RBC. Now two fold dilutions of these lysed RBCs were made and percentage transmission was observed at 540 nm.
Graph between log (venom dose) and probit absorption was drawn after making transformation of venom dose to log (venom dose). Percentage extinction due to haemolysis was transformed to probit extinction. Then graph was plotted by taking log (dose) on X- axis, and the probit extinction on Y-axis. 50% haemolysis was observed by drawing a straight line from a point 'p' (probit 5.0 on Y- axis) parallel to the X- axis which crossed the line of haemolysis at point 'q' and a perpendicular was drawn from q to X- axis at point T.

Now OQ = OT log (HD50)

Hence HD50 was calculated by finding out the antilog (PQ).
To observe the effect of temperature and electromagnetic spectrum on venom, different sterile vials having equal quantity of venom in solution form, were kept at 0°C, 4°C, 8°C, 22°C and 37°C for 28 days, and the temperature was monitored from time to time.

Immunodiffusion tests: The immunological components were observed by immunodiffusion in gel by method. For this gel diffusion was done7and 8 followed by immunoelectrophoresis 9. The results were noted by observing precipitation lines.
To see the effect of hydrogen ions and chemicals the venom of Bungarus caeruleus was mixed with buffers of pH 5, 6, 7, 8, 9, in 1:1 proportion separately and kept at +4oc for 28 days. The aliquots of solution were taken after every seven days interval and checked for toxicity in vivo in albino mice by intravenous route of inoculation. Same was done in case of chemicals.
The immunological components were observed by immune diffusion in gel.
Results
a. Toxicity test: The toxicity of Indian Common Krait venom was observed in mice through different routes of inoculations and the results are being presented below in table 1. Toxicity test of Common Krait venom through each route of inoculation has been repeated six times and the comparative results have been presented below in fig.1:

Table 1: Route of inoculation & dose of venom

Route of Inoculation	Dose of venom
S/C	0.5
I/M	0.5
I/P	0.5
I/V	0.2
I/C	0.03

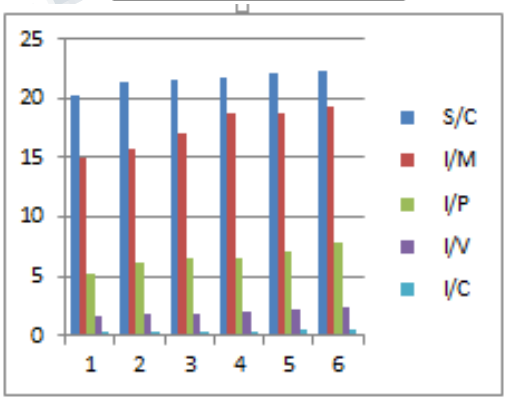

Figure 1: Comparative results of toxicity through different routes of inoculations.[Abbreviations: s/c: subcutaneous; i/m: intramuscular; i/p: intraperitoneal; i/v: intravenous; i/c: intracerebral]

The bar diagram in figure 1, shows the comparative results of all five routes of inoculation. Very high dose of venom is required in s/c route to be effective. The common krait

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venom was found most toxic through intracerebral route and least toxic through subcutaneous route.

b) Haemolytic activity: The common krait venom contains haemolytic component known as haemolysin and it has been analysed here using erythrocytes from nine species of animals (depending on availability). It can be seen from the Table 2, that the erythrocytes from guinea pigs in presence of normal horse serum are most sensitive to the action of krait venom and erythrocytes from fowl are least sensitive. Moreover erythrocytes from none of these species of animals showed haemolysis without the addition of normal horse serum. This clearly indicates that the common krait venom is indirectly haemolytic and it doesn't contain the direct lyric factor like the venom of Naja naja.

Table 2: Haemolytic activity of Common Krait Venom using erythrocytes from different species of animals

Species of animal	HD_{50} in µg/ml
Guinea pig	2.138
Mouse	4.571
Horse	39.114
Sheep	56.230
Mule	138.000
Human	177.800
Rabbit	239.900
Rat	257.000
Fowl	500.000

From Table 2, it is evident that the erythrocytes from guinea pig and mice are more sensitive to Common Krait venom. But the haemolytic activity was observed using horse erythrocytes because of large source of red blood cells from a single animal.

c) Effect of temperature: To observe the effect of temperature on venom, different sterile vials having equal quantity of venom in solution form, were kept at 0°C , 4°C, 8°C, 22°C and 37°C for 28 days, and the temperature was monitored from time to time.

Table 3. Results of toxicity & immunogenicity after exposing to different temperatures

Property		Venom Exposed to				
		0°	4°	8°	22°	37°
Toxicity %	R	100	89.36	89.36	49.97	43.48
	L	0	10.64	10.64	50.03	56.52
Immunogenicity %	R	100	100	100	85.71	85.71
	L	0	0	0	14.29	14.29

The venom turns turbid when stored at a temperature of 22°C and 37° C. Toxicity was retained up to 49.97% at 22°C and up to 43.48% at 37° C. The temperatures 40 C and 80 C, showed similar results and 89.36% toxicity was retained in both cases. Immunological components were retained at 0o C, 4o C and 8o C. No antigenic component was lost at 0o C, 4o C and 8o C after storing the venom for 28 days.

d) Effect of light: The results of venom kept for 7 days did not produce any visible change in the venom kept in darkness, day light or sunlight. After 14 days some turbidity appeared in the vial kept in sunlight. After 28 days the turbidity appeared in all the venom vials kept in dark, day light and sunlight.

Table 4. Effect of light on toxicity and immunogenicity on venom of Bungarus caeruleus

Property		Venom Exposed to					
		Unexposed	UV Rays	Infra-red Rays	Complete darkness	Day light	Sunlight
Toxicity %	R	100	0.90	0.94	100	66.67	66.67
	L	0	99.10	99.06	0	33.33	33.33
Immunogenicity %	R	100	57.14	57.14	100	44.87	1.47
	L	0	42.86	42.86	0	55.13	98.53

Abbreviations: R – Retained, L - Lost

The toxicity showed fall in the case of day light and sun light. The venom became non-toxic when kept in sun light. Daylight reduced the lethality of venom when kept for 28 days. The venom lost one immunogenic component in complete darkness and 2 components were lost in day light and sunlight condition after 28 days.

The venom exposure to infra-red rays and UV rays showed that the toxicity was lost up to 99.10% in infra-red while it was lost up to 99.06% in venom solution exposed to ultra-violet. Loss in toxicity was 33.33% in day light as well as sun light, but immunogenicity was lost up to 55.13% in day light and 98.53% in case of sun light.

e) Effect of pH: The toxicity and immunogenicity of venom was tested after mixing and keeping the venom with buffers of different pH.

Table 5. Results of toxicity and immunogenicity of venom after exposure to different pH

Property of venom		pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
Toxicity%	Retained	50.42	88.52	100	70.82	50.42
	Lost	49.58	11.58	0	29.18	49.58
Immunogenicity%	Retained	85.71	85.71	100	100	100
	Lost	14.29	14.29	0	0	0

It can be seen from the Table 5 that the common krait venom is most stable at pH 7.0. There was negligible change in the toxicity of venom at pH 7.0 and +4oc of temperature even after storing for 28 days whereas toxicity of venom is affected on acidic and alkaline sides. There was not much change in immunogenicity also as can be observed in Table 5.

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f) Effect of chemicals: Venom was exposed to different chemicals and toxicity as well as immunogenicity of venom was tested.

Table 6. Effect of chemical agents on toxicity and immunogenicity of Indian common krait venom

Property		Venom Exposed to				
		Normal venom control	Formaldehyde	Hydrogen peroxide	Iodine	Glutaraldehyde
Toxicity %	R	100	1.03	0.98	0.91	0.18
	L	0	98.97	99.02	99.08	0.18
Immunogenicity %	R	100	85.71	71.42	28.57	0
	L	0	14.29	28.58	71.43	100

Abbreviations: R- retained, L- lost

When the venom was exposed to various chemical agents like formaldehyde, hydrogen peroxide, glutaraldehyde and iodine, reduction in its toxicity was observed. Formaldehyde showed least reduction on immunogenic components (Table 6).

IV. DISCUSSION

Toxicity: The venom was found toxic through intracerebral route which indicates that venom is neurotoxic in nature10.

Haemolytic activity: The erythrocytes from guinea pigs in presence of horse normal horse serum are most sensitive to the action of common krait venom and erythrocytes from fowl are least sensitive. Moreover, erythrocytes from none of these species of animals showed haemolysis without the addition of normal horse serum11. This clearly indicates that the common krait venom is indirectly haemolytic and it doesn't contain the direct lyric factor like the venom of Naja naja. The indirect lytic factor or PLA present in the common krait venom is active only in presence of phospholipids (lecithin) present in normal horse serum. Phospholipase A converts the phospholipids into lysophospholipids which is a strong haemolytic agent12. Effect of temperature: Venom is mainly composed of proteins and enzymes (made of proteins), are needed for the proteins to be toxic. The action of enzymes gets activated when we increase the temperature of a reaction. When venom is subjected to temperature variation for a long period of time, like in this present study, for 28 days, the effect is rather different and the toxicity as well as the immunogenicity of the venom are altered. There may be structural changes in the enzymes which lead to reduced toxicity and loss of immunogenic components. The lower temperature favours the retention of toxicity and immunogenicity of the venom13. Further research is needed.

Effect of light: Ionizing radiations like UV rays and non-ionizing radiations like infra-red rays have been used to observe the effect on Indian common krait venom along with visible part of the electromagnetic spectrum. The basic difference between these two types is the amount of energy they possess. Ionizing radiations have higher energy and have an ability to break chemical bonds, causing ionization of atoms and production of free radicals that can result in biological damage of a protein. Non-ionizing radiation doesn't have enough energy to cause ionization but disperses energy through heat and increased molecular movement14 & 15. Venom is almost denatured by the action of day light, sunlight, UV rays and infra-red rays. The venom kept in darkness showed slight fall in toxicity with retention of 6 immunogenic components. Almost half of immunogenic principles are retained after exposing the venom to infrared and UV rays16. Effect of pH: There was negligible change in the toxicity of venom at pH 7.0 even after storing for 28 days. The venom can be stored at pH 7.0 as neither toxicity nor immunogenicity is lost at this pH. On alkaline side of pH scale the venom is losing its toxicity. Same effect can be observed on the acidic side17. Effect of chemical reagent: Formaldehyde has turned venom into toxoid/ venoid and toxicity is reduced by 98.97% yet retaining 85.71% immunogenicity. Hydrogen peroxide reduced the toxicity to 99% but immunogenicity is also lost by 28.58%. Iodine has reduced toxicity up to 99.08% but loss of immunogenicity is 71.43%. Glutaraldehyde is not good for this purpose as it practically denatured the venom. The toxicity as well as immunogenicity, both are destroyed18. Aldehydes are good reducing agents so may be used for converting venom into toxoid/venoid under some specified norms.

V. CONCLUSION

Author has tried to characterise venom of Indian common krait or B. caeruleus as not much literature is available on this. This particular snake is small, timid but very poisonous and neurotoxic in nature. The antivenom serum is raised in animals and is the only remedy when a snake bite case comes around. For this, the venom is injected in to the body of animal and in this event, local necrotic reactions occur. These inflammatory reactions bring discomfort to the animal. In order to reduce the discomfort to the animal and to know some of the basic properties of venom from B. caeruleus, the present study was done. If further studies are continued on this venom, it may be possible to turn venom into venoid with the action of physical or chemical agents. This will remove the need of using an animal source for raising antivenom sera. The present study indicates the neurotoxic behaviour of common krait venom. As LD50 is being estimated in mice so it cannot give us the exact measure in case of humans. Therefore LD50 in mice obviously does not give us the clear picture. But it is a baseline comparison and has merits, although standards may

differ from one to another laboratory [17]. Venom is indirectly haemolytic and lysis of RBCs occurs only in the presence of normal horse serum which contains lysophospholipids. The lysophospholipid acts on the cell membrane of the erythrocyte producing leakage of haemoglobin into the suspending medium. Again, the venom can be stored at 4o C and 8o C, as there is retention of immunological components but reduction in toxicity of venom. This could be helpful in reducing toxicity of venom before injecting in horses. This can prevent the local reaction at the site of inoculation in the animal. There is potential for further research in this area. The lower temperature could be used for prolonged storage when the venom is in solution form.

Among chemical compounds, hydrogen peroxide is found to modify the protein structures. This reduces toxicity of venom. Iodine is an oxidising agent as well as electrophilic agent. It is not selective in its action. It oxidises the peptide chain in the venom and reduces the antigenicity when exposed to prolonged treatment. Glutaraldehyde is not good for this purpose as it has practically denatured the venom and toxicity as well as immunogenicity is destroyed. Formaldehyde turned venom into toxoid as it reduced toxicity but retained immunogenicity. The enzymatic components of the venom are affected by changes in pH because each enzyme requires an optimum pH. The shape of enzyme as well as properties of the enzyme change, so that either the substrate cannot bind to the active site or it cannot undergo catalysis. This may be due to the blockage of active sites that help in a reaction.
The venom is affected by the action of chemicals. Hydrogen peroxide is found to modify the protein structures. This reduces toxicity of venom. Iodine is an oxidising agent as well as electrophilic agent. It is not selective in its action. It oxidises the peptide chain in the venom. This reduces the antigenicity when exposed to prolonged treatment. In present study the venom has been used in solution form in order to carry out experiment on venom by exposing to chosen electromagnetic rays. Irradiation of aqueous solutions induces chemical and structural alterations in proteins and peptides related to an attenuation or abolishment of biological activity and interferences in immunological properties. When the venom was subjected to visible and invisible spectrum of light it showed decrease in toxicity and immunogenicity. Whether the wave length of light was small as in UV light, large as in infra-red case or the middle spectrum that is direct sun light, it denatured the proteins and the immunogenicity was lost along with toxicity. The study also served to characterise the venom of Bungarus caeruleus but further research is needed.

REFERENCES

1. https://embryo.asu.edu/pages/francesco-redi-1626-1698

2. Flexner, S. & Noguchi, H. 1902: Snake venom in relation to haemolysis, bacteriolysis and toxicity. Jour. Exp. Med.vol. 6, pp. 277.

3. Slotta, K. & Frankel Conrat, 1938. Cited by Lee in 1977 in Handbook of Experimental Pharmacol, vol.52, pp.1.

4. Cruickshank, R. et al, 1975. The practice of medical microbiology. Med. Microbiol, vol. 2, 12th edition. Churchill, Livingstone, New York, London.

5. Reed, L.J. and Muench, H.C 1938. A simple method of estimating 50% and proteins. American J. Hyg. Vol. 27, pp. 493.

6. Tu, A. T. 1977. Phospholipase A2 venoms. Chem & Mol. Biol. Johan Willey & Sons. New York, London, Sydney, Toronto.

7. Weir, D.M. 1967. Method for the preparation of water in oil& multiple emulsions for use as antigen. Blackwell Scientific Publications. Pp. 207, Oxford & Edinburgh.

8. https://www.amazon.com /...Rickwood-Hames /s?...27 %3AB %20D %20Rickwood %5Cc...

9. Anju Dhir, Test of the venom of Bungarus caeruleus; an attempt to observe lethal dose. IJAPBR, 2016; 1(2) http://goo.gl/Z7FUeH April 20, 2016.

10. Anju Dhir, Haemolytic activity of Indian Common Krait Venom.http://ijsae.in/ijsaeems/index.php/ijsae/article/view/1203 11 April 2016

11. Devi A. (1968): Protein and non-protein constituents of snake venoms. Venomous animals and their venoms (1), 128. [Eds. W. Bucherl, E.E. Buckley, V. Deulefeu].

12. Effect of temperature on venom of B. caeruleus (Indian common krait). ijsae/article/view/1203. http://dx.doi.org/10.21474/IJAR01/2016

13. Zaider and Rossi, 1985. Dual radiation action and the initial slope of survival curves. Radiat. Res. Suppl., 8, pp. 568 - 576.

14. Hall, E.J., (2000): A radiation biological looks to the future. Int. J. Radiat. Oncol., Biol. Phys., 46, 1 - 2.

15. Anju Dhir, Effect of light on venom of Bungarus caeruleus (Indian Common Krait). http://dx.doi.org/10.21474/IJAR01/2979

16. Anju Dhir, (2016). Effect of pH on venom of Bungarus caeruleus. IJSDR, vol.1, issue 10, pp. 97-99. http://www.ijsdr.org/papers/IJSDR1610016.pdf.

17. Anju Dhir, Effect of Chemical Agents on Venom of Bungarus caeruleus/ Indian Common Krait, International Journal of Science & Engineering Development Research (www.ijsdr.org), ISSN:2455-2631, Vol.2, Issue 1, page no.103 - 106, January-2017,

18. R. Turner, "Acute toxicity: The determination of LD50", Screening Methods in Pharmacology, Academic Press New York, 1965, pp. 300