# TOXOIDING OF SNAKE VENOM AND EVALUATION OF IMMUNOGENICITY OF THE TOXOIDS

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## Abstract

The venom of Pakistan species of snakes (Naja naja, Vipera russelli and Echis carinatus) and lethal fractions of Cobra (Naja naja) venom were converted to toxoid with formalin. Detoxification of the crude venoms and toxic fraction was attained by addition of formalin in several steps and incubation at 37°C maintaining the pH 7.0. Relationship of formalin concentration and incubation period was studied. The detoxified product retained immunogenicity as revealed by titrating the circulating anti-toxin and response of the animals immunized with the crude venom toxoids when challenged with homologous venoms. Vipera russelli and Echis carinatus anti sera neutralized 10 and 15 M.H.D. respectively and on challenging immunized animals with homologous venoms of Naja naja, vipera russelli and Echis carinatus survived 5-10 MLD of the respective venoms. The lethal fraction toxoid of cobra showed lesser immunogenicity (JPMA 30: 9, 1980).

## Introduction

It is estimated that in Pakistan snake bite deaths occur in the range of 10,000 to 12,000 annually (Minton 1968). They are mostly due to Cobra (Naja naja), Russel's Viper (Vipera russelli) saw scaled viper (Echis carinatus) and Kraits (Bangarus cerealis and B. candid us). The rural population of the country and the army in strategic movements are much exposed to the bites of these snakes. There are no protective measures against the venomous effects of the bites of these snakes except post-exposure therapy with hyperimmune serum and other supportive therapy. Sero-therapy has its own limitations. It is effective only when applied soon after the snake bite before manifestation of tissue damage. In most of the cases it is too late when the anti-venom is available to the victim of the snake bite. Moreover the reactions occurring in certain cases have their own hazards. People prone to the hazards of the snakes bites could be protected by active immunization if immunogenic toxoids of snakes venoms could be prepared and used.

The Biological Production Division of the National Health Laboratories, Islamabad, manufactured monovalent and polyvalent hyperimmune sera of the above named Pakistan species of snake venoms for post exposure therapy. Preparation of immunogenic toxoid (s) of snake venom (s) for active immunization was undertaken as a research project by the Division.

Preparation of a toxoid of snake venom involves complete detoxification of the venom and at the same time retention of immunogenicity of the product at its maximum. As reported by Kondo et al (1971) attempts of toxoid-ing the snake venoms by treatment with formalin or other chemicals, by irradiation with x-rays and/or by photo-oxidation by a number of workers had only partial success as detoxification was attained but the immunogenicity was impaired. Attempts at preparation of highly immunogenic toxoids of three kinds of venoms by graded treatment with increasing concentrations of formalin following the methods of Sadahiro et al (1970) and Kondo et al (1971) were made and briefly reported by Khan et al (1977).

## Material and Methods

**Snake Venoms:** The venoms used were pools of lypholized venom batches of Cobra Naja naja, Russell's Viper (Vipera russelli)and Echis carinatus. The lethal fraction of Cobra venom (fraction pool III) was obtained by column chromatography on CM. Cellulose as reported earlier (Khan et al., 1974).

**Determination of Toxicity:** The lethal toxicity of all the three venoms and the lethal fraction III of cobra venom were determined (separately) by intervenous injections into mice of 16-18 gms body weight and expressed in terms of LD5o whereas the hemorrhagic activity was determined by intracutaenous injections into depleted skins of rabbits and expressed in terms of MHD. (Minimum Hemorrhagic Dose) following the methods of Kondo et al (1960) and Khan et al (1973, 1974).

**DetoRification of crude venom:** Each of the crude venoms of Cobra, Russell Viper and Echis carinatus was dissolved in 30 M phosphate buffer saline (pH 7.0) to make 1% solution. Detoxification of the venom solutions was carried out following the methods of Sadahiro et al (1970) and Kondo et al (1971). Aliquots of venom solutions were treated with formalin 0.2%, 0.4%, 0.6% and 0-8%. The final formalin concentrations were attained by increasing concentration by 0.2% on every alternate day. Similarly lethal fraction pool III of cobra venom isolated earlier (Khan et al., 1974) was treated with formalin by increasing concentration of 0.2% finally reaching the maximum level of 0.8%. The formalinized venom solutions were incubated at 37°C for a maximum period of 21 days maintaining the pH at 7.0 using N/ I NaOH solution. The course of detoxification was studied on 3, 5, 7, 10, 14 and 21 days after the addition of formalin. For the experimental purpose a part of the forma-linising solution (crude venom toxoid) and also that of Cobra lethal fraction III toxoid was dialized in cellophane tubing at 2-4°C in buffered saline (pH 7.0) to remove excess of formalin. The toxicities of both dialized and undialized toxoids were determined at intervals by intravenous injections into mice of 16-18 gms body weight, as shown in Table I.

**Immunization with the toxoids:** Guinea pigs weighing 350-450 gms and rabbits weighing 1500-1600 gms body weight were immunized with each toxoid preparation per immunization schedule (Table II). After complete cycle of immunization the animals were bled and circulating anti toxin titres and ED5o of immunized sera were determined following the methods of Kondo et al (1965, 1971).

Immunogenicity tests of the toxoids: Standard monovalent antivenom that is a hyperimmune horse serum, prepared by the Biological Production Division of the National Health Laboratories, Islamabad against Cobra, Russell's viper and Echis carinatus venoms were used as standard antivenin in the experiments for titration of circulating antitoxins of the sera obtained from the animals immunized with the toxoids. Units of anti-sera were determined by the method introduced by Ipsen (1942)- As such the units of monovalent anti serum of cobra, Russell's viper and Echis carinatus were established and kept at 200 units/ml after carrying out the neutralizing effects of the serum concerned on the toxicity of corresponding venom in a series of mice injected by the intravenous route with mixtures in which amounts of the serum were varying but the amount of antigen (venoms) was maintained constant. The number of deaths occurring in the mice within an interval of 24 hrs upto 5 days were utilized in the calculation, since mice receiving injections containing much free toxin (venom) die more rapidly than mice receiving injections containing small amounts of free toxin (venom). Since there are no international standard anti-sera against Russel's viper and Echis carinatus determination of relationship between the units of anti-sera of our laboratory with the international standard was not possible.

Each serum specimen of Russell's viper and Echis carinatus toxoid was titrated for anti-hemorrhagic activity as the Cobra venom is devoid of hemorrhagic activity (Ohsaka et al., 1966; Yang, 1965; Khan et al., 1974).

Gel-diffusion test on microscopic slides and precipitation tests in capillary tubes were also carried out for qualitative determination of the presence of corresponding anti-bodies in the sera of the immunized guinea pigs and rabbits using homologous and heterologous venom solutions. Positive controls were also maintained in all the tests using corresponding monovalent antivenin.

The other groups of immunized animals (guinea pigs and rabbits) were challenged 10' days after the last injection with 2-10 M.L.D. of respective crude venom by intramuscular injections in thigh muscles.

**Results**

The snake venom solutions treated with formalin at concentrations ranging from 0.2 to 0.8 and incubation at 36-37\°C for a maximum period of 21 days resulted in detoxification at a concentration as low as 0.2%. At concentrations 0.6% and 0.8% detoxifications were attained by 14th and 10th day respectively (Fig. 1).

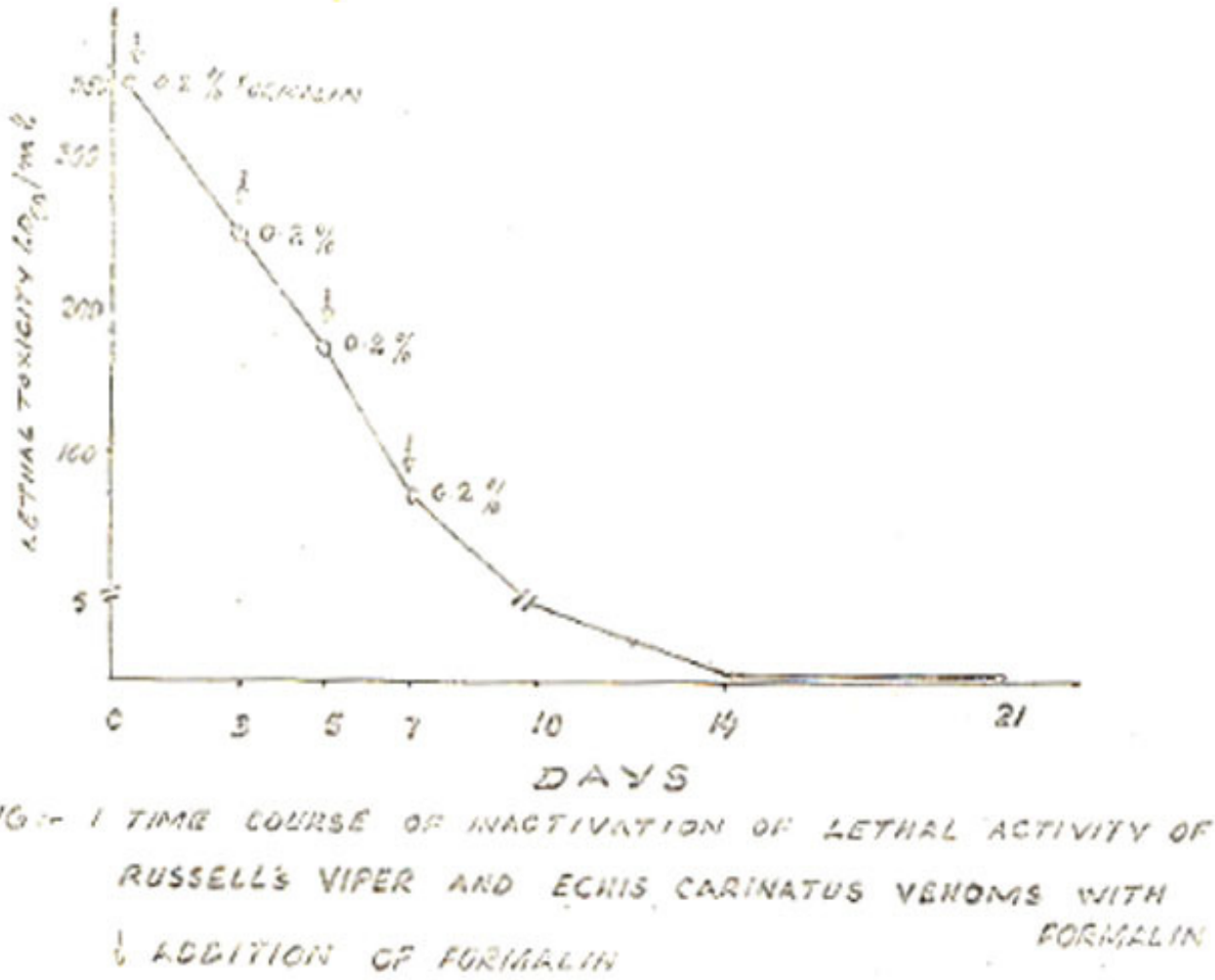


FIG:- 1 TIME COURSE OF INACTIVATION OF LETHAL ACTIVITY OF RUSSELL'S VIPER AND ECHIS CARINATUS VENOMS WITH FORMALIN

↓ ADDITION OF FORMALIN

The course of detoxification collectively is depicted graphically (Fig. I) and detoxification test results at the level of 0.6% and 0.8% formalin treatment are summarized in Table I.

Table I:Detoxification Schedule of Venoms of Cobra,Russell's Viper and Echis Carinatus with Formaline and Toxicity Test Results

| Time interval in days | Formaline added in % | Incubation temp. | Toxicity test in mice of 16-18mgs. CVT 0.6% | CVT 0.8% | RVT 0.6% | RVT 0.8% | EVT 0.6% | EVT 0.8% |
|---|---|---|---|---|---|---|---|---|
| Ist day | 0.2 | | | | | | | |
| 3rd day | 0.2(0.4) | | | | | | | |
| 5th day | 0.2(0.6) | | | | | | | |
| 7th day | 0.2(0.8) | 37°C | | | | | | |
| 7th day | | | (+) | N.T. | (+) | N.T. | (+) | N.T. |
| 10th day | | | (+) | (-) | (+) | (-)0 | (+) | (-) |
| 14th day | | | (-) | (-) | (-) | (-) | (-) | (-) |
| 21st day | | | (-) | (-) | (-) | (-) | (-) | (-) |

Key: 1. CVT=Cobra venom toxoid
RVT=Russell's Venom toxoid
EVT=Echis carinatus venom toxoid
2. Fig. in () indicate final conc.of formalin
3. (+)= Toxic
4. (-)= Free from toxicity
5. N.T.=Not tested.

Immunization schedule of animals (rabbits and guinea pigs) with toxoids has been elaborated in Table II.

Table II:Immunization Schedule to Animals with Toxoids of Cobra,Russell's Viper and Echis Carinatus

| | | Rabbits | Guinea Pigs |
|---|---|---|---|
| Ist Week | Ist day | 0.2 ml. | 0.1 ml. |
| | 3rd day | 0.2 ml. | 0.1 ml. |
| | 5th day | 0.3 ml. | 0.2 ml. |
| 2nd Week | | REST | |
| 3rd Week | Ist day | 0.3 ml. | 0.2 ml. |
| | 3rd day | 0.5 ml. | 0.3 ml. |
| | 5th day | 0.5 ml. | 0.3 ml. |
| 4th Week | | REST | |
| 5th Week | Ist day | 0.5 ml. | 0.3 ml. |
| | 3rd day | 1.0 ml. | 0.5 ml. |
| | 5th day | 1.0 ml. | 0.5 ml. |

8 Rabbits of 1.5-1.6 KG and 8 Guinea Pigs of 350-450 GMS were used for each kind of Toxoid preparation.

The results of circulating anti-toxin titres and ED estimations of immunized sera of rabbits and guinea pigs with formal toxoids of Cobra, Russell's viper and Echis carinatus are summarized in Table III.

Table III: Circulating Antitoxin Titres and $ED_{50}$ of Sera of Rabbits and Guinea Pigs Immunized with Formal Toxoids of Cobra, Russell's Viper and Echis Carinatus

| Toxoids for immunization | Anti toxin titre in u/ml. | | $ED_{50}$ | |
|---|---|---|---|---|
| | Pooled Rabbits sera | Pooled Guinea Pigs sera. | Pooled Rabbits sera. | Pooled Guinea Pigs sera. |
| Cobra | 20 | 10 | 2.15 | 1.5 |
| Russell's viper | 70 | 30 | 9.5 | 4.6 |
| Echis carinatus | 50 | 25 | 6.5 | 3.3 |
| Std.Anti-Sera. | 200 units/ml | | | |

Pooled sera of 4 Immunized Rabbits and 4 Immunized Guinea Pigs was separately titrated with crude venom.

Precipitations in capillary tubes and gel diffusion tests performed with the sera of the immunized animals with the toxoids gave positive results as shown in Table IV.

Table IV: Results of Gel-Diffusion and Precipitation Tests of Immune Sera of Animals with Corresponding Venoms/Toxoids.

| Dilution of Toxoid/Venom | CAV | | RAV | | EAV | |
|---|---|---|---|---|---|---|
| | Rabbit | Guinea Pig | Rabbit | Guinea Pig | Rabbit | Guinea Pig |
| Undilluted | +++ | + | +++ | + | +++ | + |
| 1:10 | ++ | - | ++ | - | ++ | - |
| 1:100 | + | - | + | - | + | - |
| Saline Control | - | - | - | - | - | - |
| Normal Serum Control | - | - | - | - | - | - |

+++ Strong Positive
++ Moderate Positive
+ Weak Positive
- Doubt ful
- Negative

CAV= Cobra Anti-Serum
RAV= Russell's Anti Serum
EAV= Echis Carinatus Anti Serum

The gel diffusion and precipitation tests showed distinct precipitating bands for toxoids of Russell's viper and Echis carinatus using homologous venoms whereas somewhat diffused precipitation bands were observed in case of Cobra toxoid immunized sera. This is probably for the reason that the cobra venom is less antigenic as compared to Russell's viper and Echis carinatus venoms. No cross reactions were observed, in the animals immunized with toxoids.

Table V: Protection From Intramuscular Challenging with the Crude Venom to Rabbits and Guinea-Pigs Immunized with Toxoids of Cobra, Russell's Viper and Echis carinatus.

| | Challenging Dose Corresponding Venom | Rabbits | | | Guinea Pigs | | |
|---|---|---|---|---|---|---|---|
| | | CVT | RVT | EVT | CVT | RVT | EVT |
| Immunized Animals | 10 MLD | + | - | - | + | + | + |
| | 5 MLD | - | - | - | + | - | - |
| | 2 MLD | - | - | - | - | - | - |
| Unimmunized Animals | 10 MLD | + | + | + | + | + | + |
| | 5 MLD | + | + | + | + | + | + |
| | 2 MLD | + | + | + | + | + | + |

CVT= Cobra Venom Toxoids RVT= Russell's Viper Venom Toxoid

EVT= Echis Carinatus Venom Toxoid + = Died - = Alive

Table V shows that immunized animals, rabbits and guinea pigs challenged with the 2-10 MLD doses of corresponding venoms were protected.

Table VI: M.H.D. Neutralization Results of Immunized Sera with Corresponding Venoms.

| Venom | M.H.D. | Immunized Sera | |
|---|---|---|---|
| | | Guinea Pig | Rabbits |
| Russell's Viper | 5 | - | - |
| | 10 | - | - |
| | 15 | + | + |
| | 20 | + | + |
| Echis carinatus | 5 | - | - |
| | 10 | - | - |
| | 15 | - | - |
| | 20 | + | + |

- Neutralized. (less than 8mm haemorrhegic zone).

+ Positive reaction. (over 8mm " " " ).

In table VI it may be noted that Russell's viper serum has the capacity to neutralize 10 MHD homologous venom where Echis carinatus anti-serum neutralized 15 MHD with its homologous venom. MHD of Russell's viper and Echis carinatus venoms was determined as per methods of Ohsaka et al (1966)

The cobra venom lethal fraction pool III toxoid was detoxified as the crude cobra venom but the toxoid elicited poor antigenicity. In diffusion test clear precipitation bands could be observed only upto 1/10 dilution. The immunized rabbits could survive with challenge of 5 MLD only and at higher challenge dose the animal did not survive. The immunogenic response in guinea-pig was not exhibited at this challenge dose.

## Discussion

Detoxification of snake venoms irrespective of snake species were achieved on treatment with low concentration of formalin and incubation at temperature of 37°C and pH 7.0. Formalin concentration and incubation period have inverse relationship as lesser the concentration the longer the incubation period and with increasing concentration the incubation time was shortened. This is in agreement with the finding of Sadahiro et al (1970) and Kondo et al (1971). The crude venoms detoxified with gradually at increasing concentrations by 0.2% and finally reaching at 0.6% and 0.8% on tests have shown that the preparation retained the immunogenicity.
The anti-sera of the toxoids exhibited anti-toxin qualitatively by precipitation and gel diffusion tests. The immunized animals survived 5-10 MLD challenge doses of respective venoms and the anti-sera neutralized 10-15 MHD doses of Russell's viper and Echis carinatus venoms. The toxoids of lethal fraction of cobra venom (Fraction pool III) was less immunogenic as compared with crude venom toxoid.

## References

1. Ipsen, J. (1942) Systematische and Zufallige Fehler-quellen bei Messung Kleiner Antitoxinmengen; teta-nusantitoxin. Ztschr. f. Immunitatsforsch. J. Exper. Therap., 102:347.
2. Khan, Z.H., Jan, Z.A. and Ahmad, M. (1973) Fractionation of Russellis viper venom by chromatography on CM. Cellulose with special reference. Japan J. Med. Sc. Biol., 26:39.
3. Khan, Z.H., Lari, FA. and Ali, Z. (1977) Preparation of toxoids from the venoms of Pakistan species of snake, (Naja naja), Vipera russelli and Echis carinatus. Japan J. Med. Sc. Biol., V. 30:19.
4. Kondo, H., Kondo, S., Ikezawa, H., Murata, R. and Ohsaka, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of Habu snake venom. Japan J. Med. Sc. Biol., 13:43.
5. Khan, Z.H., Jan. Z.A., Zaidi, T.A. and Faruqui, A.Q. (1974) Fractionation of Cobra venom on C.M. Cellulose with special reference to biological activities. Japan J. Med. Sc. Biol., 27:49.
6. Kondo, H., Kondo, S., Sadahiro, S., Yamauchi, K., Ohsaka, A. and Murata, R. (1965) Standardization of antivenin. I. A method for determination of anti-lethal potency of Habu antivenin. Japan J. Med. Sc. Biol., 18:101.
7. Kondo, H., Kondo, S., Sadahiro, S., Yamauchi, K., Ohsaka, A. and Murata, R. (1965) Standardization of antivenin. II. A method for determination of anti-hemorrhagic potency of Habu antivenin in the presence of two hemorrhagic principles and their antibodies. Japan J. Sc. Biol., 18:127.
8. Kondo, S., Sadahiro, S., Yamauchi, K., Kondo, H. and Murata, R. (1971) Preparation and standardization of toxoid from the venom of Trimeresurus flavo-viridis (Habu). Japan J. Med. Sc. Biol., 24:181.
9. Cecil, R.L.F. Cecil Leeb textbook of medicine. 12th ed. edited Paul B. Beeson and Walsh McDermott, Philadelphia, Saunders, 1967, pp. 420-6.
10. Ohsaka, A. (1960) Fractionated of Habu snake venom by chromatography on CM. Cellulose with special reference to biological activities. Japan J. Med. Sc. Biol., 13:193.
11. Ohsaka, A., Omori-Satok, Y., Kondo, H., Kondo, S. and Murata, R. (1966) Biochemical and

pathological aspects of hemorrhagic principle in snake venoms with special reference to Habu (Trimeresurus flavovi-ridis) venom. Mem. Inst. Butantan, 33:193.
12. Reed, L.J. and Muinch, H. (1938) A simple method of estimating fifty per cent and points. Am. J. Hyg., 27:493.
13. Sadahiro, S., Kondo, S., Yamauchi, K., Kondo, H. and Murata, R. (1970) Studies on immunogenicity of toxoid from Habu (Trimeresurus flavoviridis) venom. Japan J. Med. Sc. Biol., 23:285.
14. Yang, C. (1965) Crystallization and properties of cobrotoxin from formosan cobra venom. J. Biol. Chem., 240:1616.