STUDIES ON

THE EFFECT OF CORTICOSTEROIDS, NEOSTIGMINE AND CALCIUM IN COBRA VENOM (*Naja naja*) INTOXICATION

By

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INTRODUCTION

INTRODUCTION

Snake venom, the greatest proportion of which consists of non-cellular proteins, is elaborated and stored in specialised glands which are analogous to parotid salivary glands of mammals. The protein component constitutes 90 to 92 per cent of its dry weight and contains more than one toxic principle. The more minor components are organophosphorus and non-protein organic materials. It also contains a number of metallic and non-metallic ions. According to their mode of action, snake venoms can be broadly classified as neurotoxic, haemotoxic and myotoxic.

The amount of ejected venom in liquid form varies from 4 to 5 drops to 1 ml in a single strike, depending upon the age, size, species of reptile and various other environmental factors. Fresh venom is colourless and transparent or sometimes straw coloured. Its relative viscosity varies from 1.5 to 2.5. Venom can be dried in vacuum without loss of its potency or toxicity, depending on the species. In the dry stage, venom appears light to orange yellow in colour. Dry venom can be refrigerated for years without appreciable destruction of toxicity. On the dry weight basis, the amount of venom injected in a single bite has been put down as - Indian cobra (Naja naja) 211.3 mg; Common krait (Bungarus caeruleus) 64 mg; Banded krait (Bungarus fasciatus) 42.9 mg;

Indian Daboia (Vipera russelli) 72 mg; Phoorsa (Echis carinatus) 12.5 mg and the calculated fatal dose for man would be:- Cobra: 15 mg; Common krait: 1 mg; Banded krait: 10 mg; Viper: 42 mg; Phoorsa: 5 mg (Chaudhuri et al., 1971). Dried venoms are not completely soluble in distilled water. Elapid venom is more soluble than the Viperine venom. Solubility increases in physiological saline. Naja naja venom can dissolve upto 99.5 per cent in physiological saline. The pH of the aqueous solution of Naja naja venom is 6.6 to 7.0.

In addition to proteins, there are also non-protein nitrogen components in snake venom that might exhibit biological activities. Among the metallic ions, zinc is more predominant and occurs at a concentration of 5 mg per g in dried cobra venom. It is not associated with the phospholipase-A or the neurotoxin of the venom. Possibly, by inhibiting the phosphatases and other enzymes, it protects the venom gland from damages by its own secretions. Gitter et al. (1963) observed the presence of copper, bound with protein at a concentration of 4 mg per g in Naja naja venom. Zeller (1948) estimated the riboflavin content of snake venom responsible for yellow colour as 140 to 240 mcg per gram of dried venom. Devi and Srivastava (1963) analysed snake venom for the organic constituents and indicate that

venom contains not only toxic proteins, enzyme proteins and peptides but also contains nucleotides, free amino acids, sugars, phosphorylated sugars and lipids.

The protein components of snake venom can be classified into three groups (1) Protein with toxic properties, (2) Proteins with enzymatic activities and (3) Protein with no known biological activities. It was found that toxicity of snake venom as a whole is the combined effect of various protein components. The non-protein constituents in the venom are also toxic, but they are not lethal as is whole venom. Because of the great number of enzyme systems that were found in venom, it was only natural to associate the toxicity of snake venom with these enzyme system. More recent work has concluded, however, that there is no absolute correlation between those fractions having enzymatic activity and those containing lethal factors. Even as early as 1883, Mitchell pointed out that small molecular weight compounds were responsible for the lethality of snake venom. These observations were also confirmed by a number of investigators. Attempts to track down the precise nature of the toxic substances in snake venom involved isolation, identification and purification of the toxic principle and determination of its chemical structure and clarification of the

relationship between toxicity and structure. The development of more sophisticated techniques as electrophoresis and chromatography, chemical methods and salt precipitatic techniques gave new dimensions and broader perspective to the problem of venom chemistry.

Electrophoretic separation of different components from Naja naja venom

Ghosh and De (1938), Mohammed (1949) and Kazuki et al. (1954) separated the neurotoxin, a major component of elapidae venom from other components of cobra venom by employing different electrophoretic procedures and studied their behavioural pattern in the electrical field. Ghosh et al. (1961) purified the neurotoxin from Naja naja venom free from cholinesterase activity. Hector and Rao (1961), by employing starch gel electrophoresis, obtained nine different components from Cobra venom (Naja naja) like cholinesterase, phospholipase-A, L-amino acid oxidase, protease, phosphodiesterase, 5' nucleotidase, phosphomonoesterase and neurotoxin. Of the several components of Cobra venom that migrated towards the cathode, the one that exhibited the toxic effect of the venom was not associated with any enzyme activity. The fractions on the anode side when tested separately were non toxic; when they were

combined and tested, no toxicity could be detected. The authors also separated the fraction neurotoxin from the same venom which was free from hemolysin.

Hemolysin, a fraction of cobra venom was first separated electrophoretically by Ghosh and De (1938). Iwanaga et al. (1958) also separated hemolysin from cobra venom free from other enzymatic principles using column chromatography and this fraction was found to be stable for a long time without loss of its activity.

A fraction observed in cobra venom namely cardiotoxin which has got direct action on the heart was isolated and purified from Naja naja venom (Sarkar, 1947, 1948 Slotta and Vick (1969) found that the most basic polypeptide isolated from Naja naja venom by chromatography on CM Sephadex column showed a strong cardiotoxic activity.

Lecithinase-A or phospholipase-A, a component of Cobra venom has also been identified and studied by various investigators. Ciasca Benderia and Bovet Nitti (1960) measured lecithinase-A acitivity in Naja naja venom employing paper chromatography and found that Naja naja venom was highly active even at 1 mcg concentration. Condrea et al. (1964) isolated and purified

phospholipase-A directly from Naja naja venom. Phospholipase-A from cobra venom was crystallised and its amino acid composition described (Currie et al., 1968). Slotta et al. (1971) prepared phospholipase-A from Naja naja venom by column chromatography and tested its enzymatic activities on lecithin, red cells, red cell membranes, phospholipids of serum, egg yolk and fresh brain powder and also observed the formation of lysophospholipids by the action of phospholipase-A on phospholipids and, at faster rate, from phospholipoproteins.

An enzyme which is peculiar to the elapid venom and absent in viperine venom is the cholinesterase. This enzyme abundant in Cobra venom has been identified and separated. Chaudhuri (1942) obtained this enzyme from Cobra venom in a purified state and found it was 20 times more active as crude venom. Subsequently Master and Rao (1959) employed electrophoretic procedure fractioned cholinesterase from cobra and banded krait venom. Massaro et al. (1971) using starch gel electrophoresis obtained ten components among which the cholinesterase was found to be higher in this venom.

L-amino acid oxidase, an enzyme present in snake venom which is capable of hydrolysing the L-amino acids

was separated and purified from Cobra venom (Zeller, 1944 and Meister, 1956).

By using spectrophotometric method Amma and Sarkar (1963) investigated the activity of DNase and RNase in different species of snake venoms and observed that the Indian Cobra venom exhibits a very high RNase activity.

Ghosh and Bakacharya (1952) and Johnson et al. (1953) detected an inorganic pyrophosphatase and dephosphorylating enzymes (ATPase and ADPase) in Cobra venom by incubating inorganic pyrophosphate with Cobra venom at 37°C for 6 days. Sulkowski et al.(1963) purified the non-specific phosphatase from venom while attempts to obtain 5' nucleotidase.

A cell respiratory enzyme, cytochrome oxidase has been isolated from Cobra venom by fractionate precipitation with ammonium sulphate at low temperature (Chatterjee, 1949). Meldrum (1963) separated from Naja naja venom by means of starch gel electrophoresis, two fractions, able to irreversibly depolarise skeletal muscle designated as "skeletal muscle depolarizing factor". Brahmbhatt and Patel (1965) had shown that the lethal

factor in Naja naja venom was a glycoprotein. Brahganca et al.(1967) isolated cytotoxin from Cobra venom showing selective distruction to Yoshida sarcoma cell.

A direct hemolytic factor from Cobra venom has been identified, and it was characteristically a basic protein, and it was designated as "direct lytic factor" (DLF) in view of its lytic effect on washed red blood cells. Larsen and Wolff (1969) purified from Naja naja venom by means of chromatography, gel filtration and ammonium sulphate precipitation two basic proteins named cobramines A and B. Various terms like cardiotoxin, skeletal muscle depolarizing factor, cobramine and direct lytic factor (DLF) designate one and the same protein responsible for all the above effects (Condrea et al., 1964 and Aloof Hersch et al., 1968). Kabara (1971) using silica gel thin layer chromatography was able to fractioned Naja naja venom into atleast 4 major chemical classification - large molecular weight protein, peptides of molecular weight less than 10,000, glycosides and lipids.

From the electrophoretic analysis of Cobra venom it is clear that each of the components fractioned from this venom can exert its physiological and biochemical

activity on the different biochemical and physiological systems of the body. Most of the enzyme fractions isolated from the venom do not seems to show inherent toxicity but they will contribute their part to the total toxicity of the main toxic constituents in the venom thereby causing the death of the victim.

Enzymes in Cobra venom and their actions:

From the different fractionation procedures, it is observed that snake venom contains various enzymatic and non-enzymatic principles in different concentrations with various activities in the system. The more important enzymes observed in Cobra venom are phosphatases, nucleases, proteases and esterases, cholinesterase, hyaluronidase, phospholipase etc.

Phosphatases:

Snake venom contains several phosphatases involved in the hydrolysis of phosphate bonds in nucleotides. These are phosphomonoesterase, phosphodiesterase, 5' nucleotidase, ATPase, DPNase and endonuclease. Phosphomonoesterase has properties of an orthophosphoric monoester phosphohydrolase. Nothing is known about the pharmacological properties of venom phosphomonoesterase. It has been suggested that the

enzyme is responsible for the ATPase and DNase activities of venom. Williams et al. (1963) found that phosphodiesterase given intravenously in cats produced profound fall in systemic arterial blood pressure. Phosphodiesterase activity in crude venom can be destroyed by heating at 60°C for 30 minutes in presence of ammonium sulphate. Snake venom phosphodiesterase also can hydrolase cyclic nucleotidases. It attacks the linkage of 3' and 5' of 2'5' cyclic adenylic acid. Phosphodiesterase, biologically & biochemically differentiated from ATPase, has disproportional distribution among venoms (Pereira et al., 1971).

5' nucleotidase:

5' nucleotidase is a common constituent of snake venoms and in most instances, it is the [illegible] phosphatase in the venom. It is a 5' ribonucleotide phosphohydrolase which catalizes the hydrolysis of 5' mononucleotides, yielding ribonucleoside and orthophosphate. 5' nucleotidase, which hydrolysis the terminal phosphate from adenylic acid, is widely distributed in nature. EDTA can inhibit the action of this enzyme and the enzyme loses all its activity within 5 hours whereas in the absence of EDTA it is stable for 8 days even at 37°C (Sulkowski et al., 1963).

ATPase, ADPase and Pyrophosphatase:

Ghosh and Battacharya (1951) have investigated adinosine triphosphatase (ATPase) and ADPase in snake venoms, which are capable of splitting one phosphate bond from the pyrophosphate linkage of ATP, producing ADP and phosphoric acid. Cobra venom also possesses ADPase activity and in addition to these dephosphorylating enzymes, Cobra venom also contains an inorganic pyrophosphatase (Johnson et al., 1955 and Kaye, 1960). The venom attacks in contrast to other tissue ATPase, hydrolyzis alpha and beta pyrophosphate bond of ATP.

DPN - Pyrophosphatase:

Ghosh and Battacharya (1951) observed strong DPN pyrophosphatase activity in Cobra venom, it can hydrolyse DPN, and it can be completely inactivated by heating at 70°C for 30 minutes. The DPN splitting enzyme cannot hydrolyse ATP or thiamine pyrophosphate. When DPN - pyrophosphatase acts on DPN in the presence of Zn^{++} adenylic acid is liberated without any production of phosphoric acid; in the absence of Zn^{++} adenosine and two molecules of phosphoric acid are produced presumably due to the action of 5' nucleotidase present in venoms, on adenylic acid which is formed by the action of DPN-pyrophosphatase on DPN.

Nucleases:

Nucleases in snake venom are capable of hydrolysing phosphodiesterase bonds of DNA and RNA (Georgatsos and Laskowski, 1962 and Nikolskaya et al., 1962). They exhibit both exonuclease and endonuclease activities.

Non-specific phosphatases:

The occurrence of a non-specific monoesterase in snake venom capable of hydrolysing monophenyl phosphate was demonstrated by Gulland and Jackson (1938).

Proteases and Esterases:

The endopeptidases of snake venoms are trypsin like and the venom of elapids are poor in proteases (Zeller, 1951). Rapid disintegration and deep necrosis extending down to the bone with subsequent mummification are mainly due to the action of protease present in venome. Inherent qualities of snake venoms appears to have a definite correlation with protein and phospholipase-A content. The action of purified venom protease is completely inhibited by sodium thioglycolate, cystine and such chelating agents and KCN. Injection of purified protease intravenously into guinea pigs causes significant

myolysis with haemorrhage. Snake venoms are obviously devoided of chymotrypsin like enzymes (Devi and Sarkar, 1956).

Rao and Rao (1956) while investigating the hydrolysis of hemoglobin by crude cobra venom (Naja naja) at different hydrogen ion concentration found very little degradation of substrate in 50 minutes, but noticed appreciable proteolysis when the reaction is continued for 24 hours and also observed there is more than one proteolytic enzyme present in Cobra venom. In general, proteolytic enzymes and hemorrhagic factors are associated in the same fraction of venom.

L-amino acid oxidase:

The existance of a powerful enzyme in snake venoms, capable of oxidising L-amino acids was first reported by Zeller (1944) and designated as Ophio-L-amino acid oxidase. The L-amino acid oxidase present in snake venoms is most active of all the known L-amino acid oxidases occurring in the liver and kidney of animals. The activity of this enzyme varies from venom to venom. It was found that venom L-amino acid oxidase can activate proteases and peptidases (Zeller, 1947). Bragance and

Quastel (1952) found that Cobra venom and other venoms α-amino acid oxidase, can act as anaerobic dehydrogenase and transfer hydrogen to ferricyanide in anaerobic condition in absence of added carrier. The activity of α-amino acid oxidase of any venom can be measured manometrically in Warburg apparatus showing the rate of oxidation of leucine in the presence of an excess of catalase at pH 7.6 in phosphate buffer at 37°C. This enzyme is fairly stable and retains its activity for many hours even in dilute solution at room temperature. The crystallized enzyme in its reduced form is more resistant to heat than its oxidized form. The activity of this enzyme is strongly inhibited by aliphatic and aromatic carboxylic acid, sulfonic acid and sulfonilamides and they act as competitive inhibitors. A close parallelism between flavin content and amino acid oxidase activity of different species of snakes has also been recorded.

Cholinesterase:

The presence of Acetyl cholinesterase and non cholinesterase in venom of snakes has been reported by several investigators. Purified cholinesterase from Cobra venom does not show the toxic properties of crude venom. This fact definitely identifies the Ach enzyme

as a protein other than neurotoxin. Elapid venom contains this enzyme capable of hydrolysing acetylcholine. The occurrance of the enzyme in the venoms of all species belonging to the elapidae was simultaneously reported by Iyengar et al. (1958); Ghosh et al. (1959) and Rao and Rao (1959), while this enzyme is absent in viperine venoms. However, Ghosh et al. (1961) noticed that purified neurotoxin separated from Cobra venom did not exhibit any esterase activity. The cholinesterase of venoms can hydrolyse both methyl acetyl choline but not benzoyl choline. Its activity is inhibited by caffeine, physostigmine and high concentration of substrate (Keller, 1951). Cholinesterase of cobra venom appears to possess wide specificity, since it can hydrolyse acetylcholin-ester, non-cholinester, organion phosphester etc. Devi and Sarkar (1956) noticed that eserine can inhibit the hydrolysis of acetylcholine by Cobra venom cholinesterase. They suggested that cholinesterase of venoms should be regarded as mixture of several isoenzymes. Cobra venom cholinesterase is stable for 70 days at 0°C. but an aqueous solution loses 50% of its activity at room temperature in 3 days and in 1 hour at 25°C. The esterase activity is directly proportional to its concentration in the venom and is inhibited reversibly by physostigmine and prostigmine (Augustinsson, 1951). The cholinesterase

activity can also be blocked by cysteine, citrate, [illegible], gelatin and albumin. It has curare-like action and can block the transmission of nerve impulses by [illegible] the neuromuscular junction (arkar and Maitra, 1951), but does not appear to be responsible for the toxicity of venom as the cholinesterase activity of the venom is completely destroyed without reducing toxicity when it is heated at 55°C for 10 minutes. Moreover, the toxicity of elapid venoms is not parallel to the ChE activity of venom. All the evidence so far obtained suggests that toxicity of elapid venoms cannot be correlated to the activity of this enzyme.

Hyaluronidase:

Duran and Duthie (1940) identified the enzyme hyaluronidase in cobra venom; its role obviously is to hydrolyse the hyaluronic acid barrier, particularly in connective tissue allowing other fractions of the venom to penetrate the tissue, contributing to the edema or swelling caused by snake venom. It thus helps to spread the venom components in the tissue and potentiates the action of toxins.

Phospholipase-A:

The toxicity of snake [illegible] erythrocytes was observed as early as [illegible]

and Myers and in 1902 by Flexner and Noguchi. Naja Naja venom lecithinase-A does not require lecithin or any other phospholipids for its activity (Bolden, 1939 and De, 1944). Lecithinase- in the venom will act on lecithin to form lysolecithin which is a true hemolytic agent. This enzyme is designated as hemolysin, phospholipase-A and lecithinase-A. Phospholipase-A characteristically hydrolyses on one of the fatty acyl ester linkage in diacylphosphatin The phospholipase-A found in mammalian pancreas closely resembles snake venom phospholipase-A in substrate specificity, "activation" requirement and heat stability etc. Bragance and Bastel (1953) thought lecithinase-A of the Cobra venom was responsible for the venom's neurotoxic action. Cobra venom hemolysin or lecithinase-A was also active in dilute solution than in a concentrated one (Lee and Tarver, 1956). Phospholipase-A strongly inhibits respiration as well as oxidative phosphorylation due to its action on mitochondrial membranes. Most of the effects observed with venom lecithinases are second in nature and can be considered as originating from the primary action on lecithin. Although a considerable amount of hemolysis of red cells occurs in vivo, when Cobra venom is injected intravenously into a rabbit, the

action cannot be regarded as the cause of death of the animal, since the venom does not lose its toxicity on destruction of lecithinase-A activity. Purified phospholipase-A isolated directly from Naja naja venom is unable to split phospholipids in washed erythrocytes and to produce hemolysis (Condrea et al., 1964). The purified venom phospholipase-A is found to be non toxic, but very highly active in its action on the substrate. Condrea et al. (1964) using electrophoresis of Naja naja venom, fractionated a direct lytic factor (DLF) among the most electropositive fraction and also phospholipase-A free from direct lytic activity. The DLF closely resembles phospholipase-A in its heat stability. Naja naja venom phospholipase-A can attack osmotic hemolysates of red cells without an activator.

Red cells of different animals present considerable variations in their susceptibility to hemolysis by those of cobra and other venoms (Fellows and Williams, 1933). Some resistant cells such as those of the ox, sheep and goats have a low lecithan content (Ramer et al., 1950).

The role of phospholipase-A, alterin, in nerve, muscle or neuromuscular junctio[n] production and possible central nervous system activit[y] an aspect t[o]

has elicited much controversy. Certainly, its ability to destroy or alter certain phospholipids in nerve tissue essential to electron transport could make it neurotoxic and as has been claimed by some workers this enzyme is probably the neurotoxic component of snake venoms. But it can be hardly responsible for the more severe and profound pharmacological changes provoked by the crude venom on many other tissues. Slotta et al. (1971) observed that venom phospholipase-A can attack cell walls of nervous system and brain. They also noticed that phospholipase can change blood pressure, heart beat, respiration and cortical electrical activity.

Basic polypeptides in Cobra venom

Cardiotoxin:

Earlier study with elapine venom on cardiac muscle (Lapsein, 1939) and the marked electrographic changes in the rhythm and conduction of the heart under experimental condition had suggested that the above effect were produced by the direct action of venom on the myocardium. A factor isolated and purified from Naja naja venom by Barker (1947, 1948) and Lee et al. (1960) named "cardiotoxin". The same polypeptide was later separated and studied by different investigators and

designated as "cobramine-B" (Larsen and Wolff, 1968), "direct lytic factor (DLF)" (Aloof-Hirsch et al., 1968), "toxin gama" (Izard et al., 1969) and "cobra venom cytotoxin" (Braganca et al., 1967). It is still doubtful whether these above said polypeptides separated from Cobra venom are all identical or at least some of them are different substances. In view of the similarity in both their biological and chemical properties they must be very closely related, if not entirely identical; the slight differences in the aminoacid composition among these polypeptides appear to be due to different species of the venom origin. Cardiotoxin is heat labile, loses 50% activity at 65°C and is completely inactivated at 100°C.

Sarkar (1951) demonstrated a direct action of Cobra venom on muscles and Devi and Sarkar (1954) also observed this direct action on frog sartorius muscle preparation. This effect of crude venom on muscles has not been shown by any of the known toxins other than cardiotoxin in the venom. The direct action of cardiotoxin on muscles could be counteracted or reversed by K ions and vice versa (Devi and Sarkar, 1954). The cardiac arrest by Cobra venom or cardiotoxin appears to be primarily due

to direct action on the cardiac muscle and on the cell protoplasm without much involvement of the cellmembrane (Devi et al., 1954).

Braganca et al. (1967), Larsen and Wolff (1967, 1968), Izard et al. (1969) and Slotta and Vick (1969) have found that the most basic polypeptide isolated from Naja naja venom by chromatography on CM-sephadex column comprises the total, rather low, direct lytic activity and also the total, very strong, cardiotoxic activity of Cobra venom. They suggested, therefore that this polypeptide should be named "cardiotoxin" rather than DLF. The cobramine-B and toxin gama and Cobra venom cytotoxin have been claimed to be devoid of any lytic effect on human erythrocytes even in presence of phospholipase-A.

The direct lytic action of cardiotoxin is potentiated by phospholipase-A confirming the findings of various workers (Condrea et al., 1964; Chang and Lee, 1966 and Slotta and Vick, 1969) that DLF acts synergestically with phospholipase-A. The direct lytic action of cardiotoxin is also inhibited by heparin, RNA and gangliosides. Cardiotoxin produces a marked contracture of the chick biventer cervicis muscle (Lee et al., 1968). This action of cardiotoxin is also prevented by pre-treatment with

heparin, RNA or gangliosides. Cardiotoxin consists of 60 aminoacids in a single cross-linked by four disulphide bridge with amino-terminal luciene and carboxy-terminal asparagine (Narita and Lee, 1970). The cobramine isolated by Larsen and Wolff (1963) consists of 52 amino acid residues and also identical with those of cardiotoxin except for some minor differences. The amino acid composition of DLP in the venom is also very similar to that of cardiotoxin (Aloof-Hirsch et al., 1968).

Cardiotoxin, cobramine-B and DLP are all the most basic polypeptides isolated from Cobra venom of the same or different species. They not only share cardiotoxic direct hemolytic and many other biological activities, but they are also similar in their amino-acid composition. Therefore they should be regarded as "isotoxins" if not entirely identical compounds.

From the above, it is clear that the main basic polypeptide cardiotoxin is capable of affecting various kinds of cells causing irreversible depolarization of cellmembrane, contracture of skeletal muscles, systolic arrest of the heart, contraction of smooth muscle, axonal conduction block, local irritation, cytopathic

effect on stable tumour cell cultures etc. However, hence amino acid composition and the molecular weight of ..F are somewhat different from those of cardiotoxin, it is possible that BMF from different venoms may vary slightly in its amino acid composition.

Neurotoxin:

Cobra neurotoxins are proteic substances of small molecular weight, generally high isoelectric point and high pharmacological activity not associated with enzymatic activities of their own. In many cases more than one neurotoxin has been isolated from the cobra venom and individual variations have also been reported (Larsen and Wolff, 1964; Miranda et al., 1970; Tu et al., 1971 and Carlsson et al., 1971). The neurotoxin is heat stable and it is dializable (Tebs, 1969). The neurotoxin is also devoid of sugar moety. Cobra neurotoxin is composed of 61 or 62 residues of 18 or 19 amino-acids in a single peptide chain, cross-linked by four disulphide bridges (Botes and Strydom, 1969 and Strydom and Botes, 1971).

It has been repeatedly demonstrated that the integrity of the disulphide bonds in the neurotoxin molecule is essential for their toxicity (Yang, 1967).

Reduction breaks the disulphide bonds and results in complete loss of toxicity and reoxidation will not restore the full toxicity. Studies on chemical modification of these neurotoxins with group reagents have revealed that basic amino acids, especially lysine residues are essential for their biological activity (Lee et al., 1960 and Chang et al., 1970).

Neurotoxin has been isolated and characterized from other components of Cobra venom using electrophoretic methods by different workers. Recently Nayer and Rao (1961) separated neurotoxin and noticed that neurotoxin has its distinctive action on respiratory center. Earlier investigators has observed that most of the Elapidae secrete a paralysing substance, the action of which may be compared to Indian curare (curare-like action). Further experiments have confirmed this observation (Brown and Harris, 1950; Chang, 1960; Schmidt et al., 196 and Vick et al., 1965). Certain cobroid venoms seem to act as true or 'pachy curares' which exert a specific action on the Ach receptors distributed on the "Plaque motrice". Some others, on the other hand might be responsible for a decrease in the quantity of Ach discharged at the end of the nerve or conversely increase the sensitivity of the involved muscle.

Bellaway (1954) ascribed to the neurotoxin of the Elapidae a special affinity for the cells of the respiratory center of the brain. It has been claimed that the characteristic paralysis produced by neurotoxin is the consequence of either a peripheral curare-like action on the neuromuscular junction (Pradhan, 1954 and Christensen, 1955) or a central action on the brain stem (Schmaper, 1962); it is probable that the effect is produced on the synapses at both sites, as they are physiologically similar. Waldrum (1965) observed two types of effects at the neuromuscular junction, a curare like block produced by polypeptide toxin, but not relieved by eserine or by washing out, and the other type of effect is the impairment of Ach release from the presynaptic terminals. Cobra venom is very active in paralyzing the neuromuscular junction of isolated nerve-muscle preparation as reported by Barlow and Whittaker (1950). Bevi et al. (1964) suggested that the neuromuscular block produced by Cobra venom is of different type. It may also assumed that the neurotoxin does not ordinarily cause organic damage in nervous system, hence all specific symptoms disappear after recovery. Rosenfeld (1971) suggested that neurotoxin probably acts by destroying some chemical mediation substance

that are necessary for the effectiveness of nerve cell activity. Lester, (1970) and Miledi et al., (1971) found that the response to micro-iontophoretically applied Ach or Carbachol in the isolated frog sartorious is blocked by cobra neurotoxin. Cobra venom neurotoxin do not modify the release of Ach by nerve impulses. Its neuromuscular blocking action is entirely at post junctional end plate (Jimenez Porras, 1968 and Lee, 1971). Neurotoxin will not change the resting potentials elicited after complete blockage by direct stimulation of muscle fibres. It progressively diminishes the amplitude of the miniature end plate potentials, which disappear before completion of neuromuscular block. Neostigmine increases the height and duration of the end plate potentials. The neuromuscular blockade produced by cobra neurotoxin is of antidepolarizing type. Such effects are also elicited by d-tubocurarine (Chang and Lee, 1966; Su et al., 1967 and Lester, 1970).

Autoradiographic studies have shown that cobra neurotoxin labelled with ^{131}I accumulates on the motor end plate zone of the mouse and rat diaphragm (Kato et al., 1970). Cobra neurotoxin is also capable of binding specifically to the cholinergic receptors

(Lester, 1971). Naja naja neurotoxin also produces an irreversible blockade at the neuromuscular junction. The neurotoxin does not affect the cardiac and smooth muscles, indicating that they have no affinity to muscarinic receptors (Wei and Lee, 1970). Cobra neurotoxin exerts no appreciable effect on the ganglionic transmission (Chou and Lee, 1969). Neither the crude venom nor the purified neurotoxin can penetrate into the brain in sufficient amount to account for critical effects, which are most probably of a secondary nature (Tseng et al., 1968). Application of neurotoxin fraction prepared from Naja naja venom to the exposed cerebral cortex of the rat produces a long-lasting convulsant effect and whether this effect is caused by the neurotoxin itself or by phospholipase-A is doubtful (Bhargava et al., 1970).

The neuromuscular blocking action caused by several elapidae venoms is very similar. The following characteristics are common to the action of all these venoms on the myoneural junction.

(1) Onset after a more or less prolonged latent period, depending upon the dose employed and slow progress of the block.

(2) Absence of potentiation of the maximal twitch and in the vivo experiments, of fasciculations or contracture.

(3) Partial and transitory antagonism by neostigmine and other anticholinesterase drugs (Chang, 1960 and Su, 1960).

(4) No influence of Ca^{++} or of choline on the block (Chang, 1960).

(5) Synergism with competitive neuromuscular blocking drugs either in causing neuromuscular block (Schmidt et al., 1964) or in the inhibition of end plate depolarization by Ach (Peng, 1960).

(6) Inhibition of the effect caused by Ach and by other cholinomimetic substances in straited muscles, such as contracture of the frog rectus abdominis (Su, 1960) or rat isolated and denervated hemidiaphragm, depolarization of the end plate in the frog sartorius muscle (Peng, 1960) and responses of the cat anterior tibial muscle to intra arterial injection of neurotoxin.

(7) Irreversibility or extremely slow and difficult reversibility of the neuromuscular junction blockade (Vital-Brazil, 1972).

Respiratory paralysis usually is the primary cause of death in envenomation caused by small or moderate doses of elapid venom. Larger doses can produce severe

cardio vascular disturbance, and even death by circulatory collapse. The paralysis is exclusively of peripheral origin.

Bradykinin releasing factor in snake venom

Certain snake venom enzymes (proteolytic enzymes) are capable of releasing bradykinin (a nonapeptide) and calidin from plasma. The main pharmacological action of bradykinin are smooth muscle stimulation, vasodilatation, increase capillary permeability, accumulation and migration of leukocytes and production of pain. This bradykinin releasing factor has been observed in Cobra venom though in low concentration only. Increasing the capillary permeability with a resultant local vasodilatation is probably important for the rapid absorption of lethal toxin. The ability of venom to release bradykinin from plasma can be inhibited by DFP.

Non-protein constituents of venom

Feldberg and Kellaway (1938) reported that injection of Indian Cobra venom into the pulmonary artery of a guinea pig caused the appearance of histamine and coagulable protein from the lung in the perfusate. This has been supported by further experimentation on Cobra

venom by different investigators. Study of the mechanism by which histamine is liberated from tissue when snake venom is introduced, has led to the discovery of lysolecithin and slow reacting substances as the intermediary products of histamine release. The contraction of smooth muscle by venoms is also accounted for in part by the liberation of histamine and by lysolecithin and SRS, which are all apparently produced when venoms act on muscle (Trethewie, 1956). Subsequently, it was shown that an adenyl compound and a deaminating enzyme are released when the heart is perfused with Cobra venom. The shock produced by perfusion of an animal with Cobra venom is also thought to be caused by production of histamine and adenyl compound.

Devi and Sarkar (1954) noticed a factor in the Cobra venom which has got strong physiological action on the perfused frog heart and concluded that this factor may be a non-protein constituent. Based on this idea, Devi and Sarkar (1954) began to study the physiological effect of heated Cobra venom on the anaesthetised cat especially blood pressure and respiration. Heating of Cobra venom at 100°C for 30 minutes will destroy all the enzymes and other constituents of the venom, but such heat denatured venom still showed the physiological

action on the heart hinting at the existence of more thermostable constituents which possessed physiological function.

No definite role had been assigned to the non-protein fraction of the venoms except to suggest that they may function as part of the toxic and enzyme proteins. A direct paper chromatographic analysis of the venom TCA filtrate indicates the presence of the glucose, galactose and mannose in Naja naja venom. The cysteine sulphuric acid reaction for hexose establishes the fact that glucose, galactose and mannose exist as free sugars. No function of these sugars has been elucidated.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Antisnake venom serum or antivenin, which is the only specific therapeutic agent for the treatment of snake poison in man and animals by its very nature of being a foreign protein derived from equines, can induce severe anaphylatic or serum reaction in few instances. The efficacy and therapeutic value of such agents as corticosteroids, neostigmine and calcium employed as supportive medication have been extensively evaluated with these drugs given alone or in conjunction with antivenin both in experimental and clinical cases. Hoback and Green (1953) were among the earliest to report the beneficial effect of cortisone and corticotropin in three cases of snake bite (presumably by copper head moccasin - North American pit viper) with the remarkable ability of this drug to inhibit local reactivity to foreign protein and its symptomatic effect on pain and fever. According to Zimmer (1954) cortisone can be given to patients bitten by snake in 20 to 30 per cent increased dosage and corticosteroids tablets given immediately after snake bite may delay if not prevent death. It was also valuable to reduce the hazards of anaphylatic reaction associated with antivenin therapy.

Wood et al.(1955) reported 11 copper head snake bites, one timber rattle snake bite and 3 bites of unidentified snakes and concluded that in grade I venomation neither cortisone or ACTH accelerated the recovery of the patients whereas in the case of grade II venomation, cortisone and ACTH helped to control severe angioneurotic edema and urticaria in one patient and in another where the patient was extremely sensitive to horse serum, ACTH, cortisone, oxygen, blood transfussion, external heat, vitamin-K and anti-histaminics were credited for saving patient's life. In another series of 52 snake bite cases, mostly of Malayan pit viper and treated with 'Cortigen' orally over 48 to 72 hours in varying doses and at varying intervals, the gross swelling and mucosal bleeding were arrested and none of the 52 patients died or exhibited toxic effects (Ariff, 1955). Gujaral and Dhawan (1956) tested eight drugs like cortisone, ACTH, hyaluronidase, rutin, adrenaline hydrochloride, calcium chloride, sodium salicylate and 'synopen' for their ability to save rats against death caused by experimental poisoning with cobra venom and these drugs were given half an hour before the injection of venom and a second dose given half an hour later. From this study, it was observed that six of these drugs caused a significant

increase in survival period of experimental rats and the maximum degree of protection was caused by calcium chloride and cortisone. The effectiveness of cortisone acetate in experimental poisoning with Agkistrodon piscivorous has been investigated in dogs, and its beneficial effect assessed in the experimental group when compared with untreated control animals. Cortisone treated dogs evidenced very little symptoms of envenomation and all of them recovered from the toxic effect of the venom (Knapp and Flowers,1956). The effect of hydrocortisone was tried in mice that were given one LD50 of viper venom and the effect compared with the antivenin group to show that hydrocortisone was as effective or more so, than antivenin (Ganatra et al., 1957). In a series of experiments on dogs, Diechmann et al. (1958) found that hydrocortisone protected the animals from intramuscular injection of approximately lethal dose of Crotalus adamanteus venom when steroid was given intravenously immediately two/four hours after the venom. All dogs that received hydrocortisone immediately afterwards survived; 68 per cent of those that had the drug two hour after venom survived, 75 per cent of those that received this drug four hour later survived, whereas all control dogs died approximately in 18 hours. In another 60

selected cases of viper bite, treatment was done with cortisone preparation either alone or in combination with antivenin. In some patients of the combined-treatment group that initially received polyvalent antivenin the general condition got worse, whereas the symptoms subsided rapidly after cortisone was given; and hence cortisone was recommended even for primary treatment in other cases with successful results. Cortisone either given alone or combined with antivenin was as effective as antivenin in both early as well as later stages (Denyajati et al., 1960). Wig and Vaish (1960) treated in a year 15 cases of Echis carinatus bite with antivenin and corticosteroids. Although, some of them showed definite evidence of marked envenomation, all survived. The antivenin used for these patients did not contain specific antitoxin against Echis carinatus venom. This showed that corticosteroids alone could probably be life saving. The therapeutic value of hydrocortisone in experimental envenomation with cobra venom (Naja naja) in dogs has been investigated by Morales et al. (1961) who observed that hydrocortisone given intravenously before the injection of venom had no protective action, but repeated doses after envenomation prolonged the survival period of these animals. It was also recorded that

large doses of hydrocortisone were necessary to reduce the lethality of cobra venom in dogs. Benyajati et al. (1961) experimented with cobra venom in a group of seven dogs which were given prednisolone succinate intravenously immediately after the venom and subsequently every four hours until toxic signs had subsided or death supervened. After a period of mild neurotoxicity, five of the dogs recovered. Another group of six dogs given the first dose of prednisolone two hours after the venom and five among the six survived. In a further group consisting of five dogs, four hours elapsed before prednisolone was given; by this time neurotoxic signs were established but nevertheless four out of the five animals recovered. After these encouraging results, six patients with cobra bite were treated with glucocorticoids and all of them showed significant improvement. The author suggested that the glucocorticoids were especially beneficial to overcome the serum reaction but suggested that a large dose of corticosteroid was necessary to overcome the ill effects of cobra bite. Arora et al.(1962) have reported the beneficial effect of hydrocortisone and hydrocortisone-antivenin combination against the Echis carinatus envenomation. In that study the authors confirmed the efficacy of corticosteroids alone against

Echis carinatus poisoning and also showed that corticosteroid itself was as effective as that of antivenin for saving the life in envenomation. Tallquist and Osterlund (1962) noticed among 132 patients with poisonous snake bites, beneficial effects in 31 cases treated with cortisone and/or antivenin. It was surmised that Echis carinatus venom exerts its effect through release of 5HT and histamine, thus increasing capillary permeability and such increased capillary permeability can be controlled by administration of hydrocortisone (Tomani and Arora, 1962). Longaire (1965) agreed with the use of cortisone alone for counteracting the envenomation itself particularly in viperine poisoning. With data on 60 cases of poisonous snakes bite, Mahmood (1968) reports that among 38 patients who had antivenin in addition to corticosteroids two died while none of the remaining 22 patients who had steroid alone survived. In another series, 32 dogs were used to assess the therapeutic value of corticosteroids in experimental envenomation with water moccasin and eastern diamond rattle snake venom. 16 dogs were given varying doses of 'Meticortitone' immediately after at different interval after injection of venom. The survival period averaged 27½ hour. The drug did prolong survival period of these experimental dogs but was not

by itself life saving (Snyder et al., 1971). The effectiveness of hydrocortisone and hydrocortisone-antivenin combination, on plasma fibrinogen level in Russell viper poisoning has been investigated by Seth et al. (1972) and the authors speculated that corticosteroids may control bleeding in envenomation by Russel viper, both by affecting capillary permeability and preventing decrease in the plasma fibrinogen. In their experimental study on rats to evaluate the beneficial effect of hydrocortisone and hydrocortisone-antivenin combination in Russell viper envenomation, it was observed that hydrocortisone alone (10 mg/kg body weight) was quite effective in saving the animals against LD95 of Russell viper venom. When hydrocortisone and antivenin (2.5 mg/kg and 0.5 ml/kg body weight respectively) combination was used against LD50 of venom, a fair degree of protection was observed. Using the same LD50 venom, a high degree of potentiation of protective action was observed when large doses of hydrocortisone (5 mg/kg body weight) was employed in conjunction with same antivenin (0.5 ml/kg body weight). But it was emphasised that higher doses of corticosteroids should be given than what was usually given in routine practice and the drug should be continued for some time and then gradually tapered off, particularly to keep the bleeding

tendencies under control (Seth, 1974). Investigators like Gowdy (1954); Gupta et al.(1960)and Mullins and Naylor (1960) had also reported that combination of corticosteroid and antivenin was more beneficial in snake bite envenomation than antivenin alone. Highly valuable effect of corticosteroids in overcoming the antivenin induced anaphylatic reaction have also been investigated by various workers (Krements and Laville, 1961; Mason, 1963; Russell and Coventry, 1963; Gennaro, 1965; Reid, 1964; Furneaux, 1967 and Ganthavorn, 1971).

On the other hand there are adverse reports also on the use of corticosteroids in the treatment of snake bite in clinical as well as experimental conditions (Schottler, 1954; Allam et al., 1956; Reid, 1956, 1957; Knyvett and Malphy, 1959; Stimson and Engelhardt, 1960; Russell and Emery, 1961; Reid, 1961 and Chatterjee and Dass, 1969).

Christensen (1955) tested a number of substances including neostigmine and calcium gluconate against the neurotoxic manifestation caused by crotalus bite. Formosan elapid venom causes respiratory failure and this cannot be antagonised by neostigmine and "Tensilone" (Lee and Peng, 1961). Reid (1961) could not elecit a reversal of neuromuscular block and bring about improvement

in three cases of sea snake bite, given neostigmine intravenously. Kumar and Usgaonkar (1968) in a case with myasthenia gravis like picture resulting from cobra bite presumed that it might be due to excessive cholinesterase activity, since cobra venom contains high quantity of this enzyme; the patient concerned responded dramatically after injection of neostigmine 1 mg intramuscularly. It was emphasised that such a clinical picture should be given a therapeutic trial of anticholinesterase injection. Conversely it has also been reported that the neuromuscular block and paralysis produced by elapid and sea snake venoms cannot be reversed by antagonist substances such as eserine, neostigmine and prostigmine (Boquet, 1968; Michel Barme, 1968 and Rosenfeld, 1971). Banerjee et al. (1972) has reported in clinical cases, the therapeutic value of neostigmine-atropine combination in conjunction with antigenin. Nigam et al. (1973) recorded two cases of snake bite that were treated with usual first aid and routine antivenin-neostigmine-atropine combination administered to counteract the neurotoxicity produced by the venom. Seth (1974) recommended that in all cases of snake bite, intensive treatment with antivenin and hydrocortisone may be given; however in cases of cobra bite along with artificial respiration neostigmine can also be given as a therapeutic supportive measure to enhance the chances of survival.

McGee (1953) treated a rattle snake bite in dog with calcium gluconate alone intravenously and the animal survived without any complication. Cook (1956) treated four dogs suspected with snake bite employing the usual first aid treatment followed by administration of calcium gluconate solution intravenously and 1 ml 'Pyrohistine' subcutaneously; all the four cases recovered completely from the envenomation. The beneficial effect of calcium gluconate solution intravenously to counteract venom induced hemolysis in animals has been reported by Parrish (1958). Reid (1959) has reported that the renal excretion of venom and myoglobin in sea snake bite may be facilitated in the early stages by intravenous infusion of glucose and calcium gluconate solution. The unpleasant muscular twitch resulting from diamond black rattle snake bite could be modified by introduction of calcium gluconate 10 per cent solution intravenously as observed in a case report by McCleary and Wurzel (1959). Strover (1961) had recommended the administration of calcium gluconate intravenously along with antivenin infusion especially in viper poisoning. Chatterjee (1965) suggested calcium gluconate to counteract haemorrhage produced by snake venom.

PRESENT STUDY

PRESENT STUDY

Apart from the routine antivenin therapy, it had been observed from the literature, that corticosteroids, neostigmine and calcium-alone or in combination-have got high therapeutic and clinical value in the treatment of snake bite in man and animals. It is generally concluded that these agents can reduce the venom toxicity by different mechanisms of action thereby prolonging the survival period or even causing complete recovery.

The present study is undertaken to evaluate the degree and extent of protection afford by corticosteroids, neostigmine and calcium used as ancillary agents against the experimental envenomation with cobra venom (*Naja naja*) in rats. These drugs are given in different dose levels and at varying intervals intraperitoneally in rats challenged with one LD50 cobra venom intramuscularly. The data obtained was analysed to assess the efficacy of these supportive measures.

MATERIALS AND METHODS

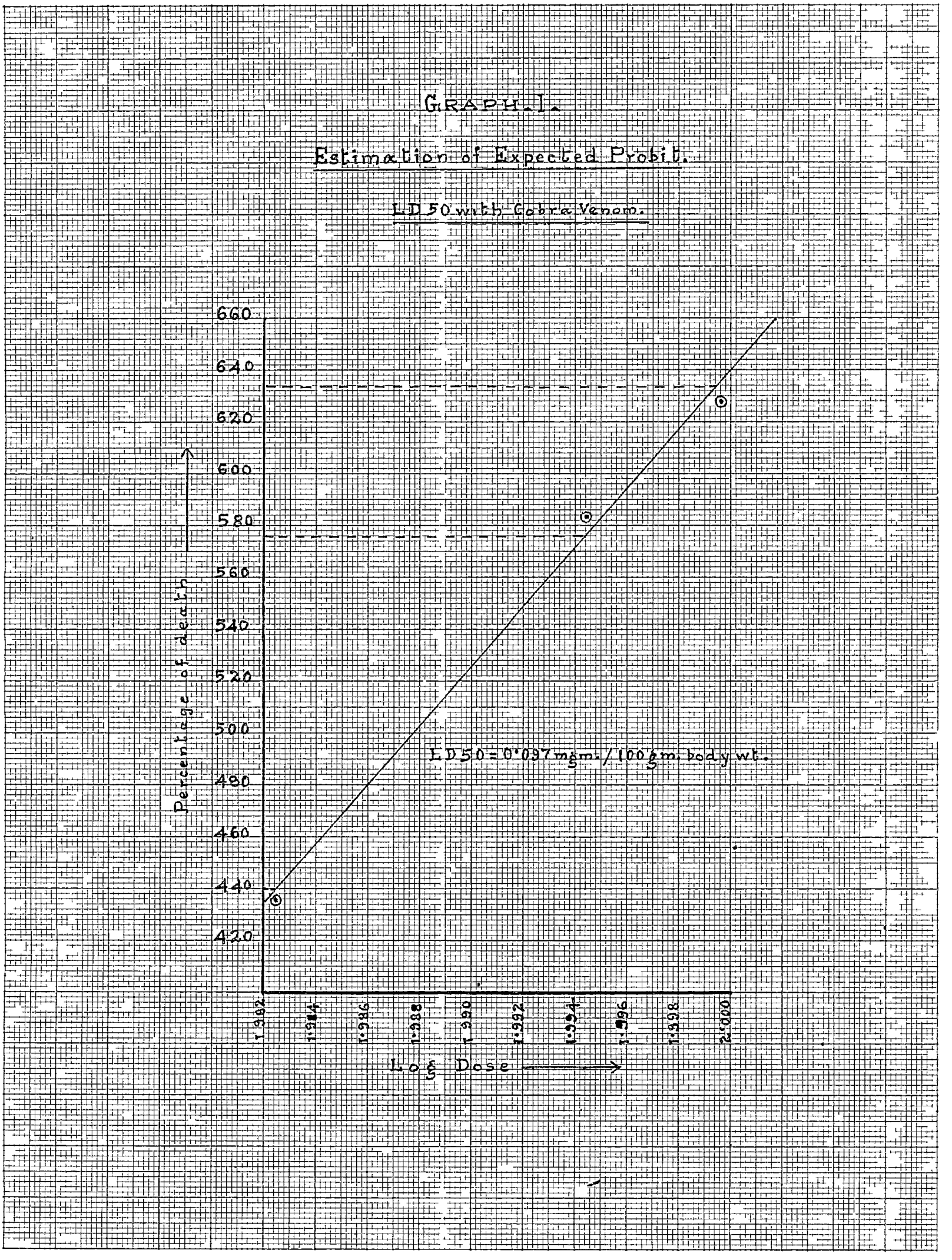
MATERIALS AND METHODS

The experimental animals for the study consisted of healthy and adult Norwegian strain of albino rats of both sexes weighing 100 to 200 g, obtained from the Small Animal Breeding Station, Mannuthy. A total of 400 rats were utilised for the trials. 'Betnesol' (Glaxo brand of betamethazone), neostigmine bromide (E Merck) and calcium gluconate (10% - Sandoz) were tried in rats after injection of cobra venom. Lyophilized cobra venom (Naja naja) was obtained from Haffkine Institute, Bombay as one gram sealed ampules. To determine the LD50, the venom was reconstituted in physiological saline, and a dose range of 0.06 mg to 0.1 mg of cobra venom per 100 g body weight of rats was selected on the basis of available literature. The venom was given intramuscularly to separate batches of rats and by trial and error method, a dosage range of 0.095 to 0.1 mg per 100 g body weight of rats were arrived at for further determination of LD50 of cobra venom. This dose range of cobra venom was employed in batches varied from 4 to 10 animals. A first batch of 4 animals, second batch of five animals and third batch of ten animals were made use of to obtain LD50, the venom being given intramuscularly at a dose of 0.095 mg, 0.098 mg and 0.1 mg per 100 g body weight. The occurrence of mortality within a

GRAPH-I.

Estimation of Expected Probit.

LD 50 with Cobra Venom.



period of 8 hours after injection of venom was observed. To determine the LD50 of cobra venom, the percentage of kill was converted into probit. Measuring this along with vertical axis and logaritham of dose along the horizontal axis, the points were plotted. The best fitting line to the eye for these points were drawn and this was used to obtain the expected probit. These expected probits were used to fit the best probit regression, with the help of which the LD50 was determined (Finney, 1964). Thereby the intramuscular LD50 of cobra venom was found to be 0.097 mg per 100 g body weight of rats.

The study was conducted to assess the beneficial effect of betamethazone, neostigmine bromide and calcium gluconate individually, at differing dose levels and at varying intervals to observe the prolongation of survival period of the experimental rats given one LD50 of cobra venom. The experimental animals were divided into four groups, each group comprising 6 animals. The fresh cobra venom stock solution was prepared in physiological saline every time in such a way that 0.5 ml of the stock solution would contain one LD50 of cobra venom (0.097 mg/100 g body weight). The venom was injected in a dose of 0.097 mg per 100 g body weight intramuscularly to these experimental

group of rats. The drug under trial was also diluted in physiological saline and given intraperitoneally after the injection of cobra venom. The dosage of the three different drugs used in the study were as follows:-

1. Betamethasone - 0.6 mg, 0.8 mg and 1 mg per 100 g body weight.
2. Neostigmine bromide - 30 mcg per 100 g body weight.
3. Calcium gluconate - 8 mg, 10 mg and 12 mg per 100 g body weight.

The stock solution of these drugs were prepared freshly in such a way that 1 ml of the stock solution would contain the required quantity of the drug for 100 g body weight of rats. Each dose of drug was given at fixed intervals ie., immediately afterwards, one hour, two hour and three hour after injection of LD50 cobra venom. The extension of survival period in the treated rats were observed. If the animals survived for 12 hours, it was deemed as protection because the usual survival period of one LD50 venom was reckoned to be approximately eight hours. The prolongation of survival period with these individual drugs at different dose levels and at varying intervals was recorded and tabulated. Food and water were withheld for a

period of 12 hours after injection of venom. The animals which survived for 12 hours were given food and water and the recovered animals were kept for a total period of 48 hours under observation and then sacrificed.

RESULT.

R E S U L T S

The intramuscular LD50 of cobra venom (death within 8 hours) as determined by the help of probit analysis and plotted on a graph (attached) was found to be 0.097 mg per 100 g body weight of rats. However, 100% death was observed within a period of 12 hours after injection of one LD50 cobra venom intramuscularly. It was also observed that both sexes of rats were equally susceptable to the venom toxicity.

During injection of venom solution, the animals showed pain and immediately afterwards they showed excitement. There was tendency to move arround with affected limb lifted up. After a few minutes, the respiration became rapid and the rats became quiet and dull with tendency to lie in a corner of the cage. There was local edema at the sight of injection of venom. Approximately one hour afterwards they showed salivation and the respiration became weak, slow and distressing. Some of the rats which survived more than 10 hours showed slight bloody discharge from the eyes which was similar to the condition known as 'chromodacryorrhea', a peculiar symptom in anticholinesterase poisoning in rats. This syndrome was found to be predominant in animals which survived after neostigmine injection. It was absent in rats which

received Betnesol and calcium. Respiration was the first to fail and there were involuntary movements. Heart continued to beat even up to 10 minutes after respiratory failure. Some recovered rats were kept for 24 days under observation, when they were found to develop extensive necrosis and gangrene of the affected limb extending down to the bone.

Betnesol at 0.6 mg per 100 g body weight when given by intraperitoneal route immediately after one LD50 cobra venom gave 83 per cent prolongation of survival period, 67 per cent prolongation when the drug was given one hour after venom, and it was 17 per cent when the drug was given at either two hours or three hours after venom (vide table I). Betnesol at both dose rate of 0.8 mg and 1 mg per 100 g body weight when given immediately after venom gave 100 per cent protection, when administered one hour after venom it was 83 per cent protection. The survival period was on the other hand decreased by 33 per cent, when the same dose levels were employed after 2 hour interval. When the same 2 dosages viz., 0.8 mg and 1 mg per 100 g body weight were used 3 hours after venom, survival period was decreased by 33 per cent and 17 per cent respectively (vide tables II and III).

Neostigmine bromide at a dose level of 30 mcg per 100 g body weight by intraperitoneal route, adversely affected the survival period of the experimental rats. Neostigmine given immediately after cobra venom resulted in 100 per cent mortality within 8 hours. The same effect was observed when neostigmine was given one hour after cobra venom injection. However, a very small increase of 17 per cent in the degree of protection could be obtained if neostigmine was administered two or three hours after venom (table IV).

Calcium gluconate at dose levels of 8 mg, 10 mg and 12 mg per 100 g body weight when given by intraperitoneal route immediately, one hour and three hour after cobra venom injection did not prolong the survival period of rats whereas all the above three dose levels given two hours after venom prolonged the survival period of 67 per cent, 83 per cent and 67 per cent respectively (vide tables V,VI and VII).

From the above results, it was surmised that Betnesol at dose of 0.8 to 1 mg per 100 g body weight in rats when given intraperitoneally, immediately after experimental envenomation with one LD50 cobra venom intramuscularly would definitely give 100 per cent protection by the prolongation of survival period of the experimental rats.

D I S C U S S I O N

DISCUSSION

Snake venom containing a multitude of components is capable of affecting the normal biochemical and physiological activities of cells in different systems of the body. For example enzymes like protease, lecithinase-A, 5' nucleotidase and hyaluronidase are capable of materially altering the structure and function of cells especially at the site of the entry of the venom. The factor capable of releasing bradykinin from the plasma has properties of smooth muscle stimulation, vasodilatation, accumulation and migration of leukocytes, increasing the capillary permeability and initiation of pain. Snake venom may induce release of many autopharmacological agents or autocoids to contribute to toxicity, local tissue damage and changes in capillary permeability augmenting the absorption of the toxic constituents of the venom.

Treatment after envenomation is directed firstly to prevent absorption of poison and secondly to neutralise as far as possible the effects of absorbed toxin. If local absorption can be successfully arrested for a significant period, suitable therapeutic measures can then be deployed to counteract the effect of toxin already absorbed. The body defence mechanism may succeed in destroying the toxic agents locally if they get sufficient time. Otherwise,

vascular permeability is the salient factor governing the rate of absorption of venom from the site of injection. Therefore, drugs retarding absorption of the venom should serve as useful adjuncts to the established regimen for snake bite.

Corticosteroids have the ability to decrease capillary permeability while maintaining the integrity of capillaries and tone of blood vessel. Of the different varieties of corticosteroids, betamethasone, a potent synthetic glucocorticoid possesses less sodium retension property with high anti-inflamatory activity. The protection in respect of the prolongation of survival period of rats against on LD50 cobra venom obtained with betamethasone during the course of these trails is in concurrence with the observations of Gujral and Dhawan (1956); Mig and Vaish (1960); Lomani and Arora (1962) and Seth (1974). Benyajati et al. (1961) had also reported that prednisolone prevents or reduces the toxic effect of the venom on nerve tissues or at least can antagonize the effect of spreading factor and improve the metabolism of the poisoned cells. The usefulness of corticosteroids in protecting animals and man from shock irrespective of etiological factor has long been recognised and shock is an integral part of snake venom intoxication.

The doses of betamethazone ('Betnesol') used in the study are admittedly quite large when compared with the normal therapeutic dose level for rats. The most serious drawback in making clinical anology between the results obtained in these experiments and conditions of actual practice would be the matter of delay between the time of injection of venom and institution of therapy. One hundred per cent protection obtained with Betnesol when given immediately after venom injection may not be elicited in actual practice on account of time lag. With such delayed administration of Betnesol there could only be a decreased percentage of protection and extension of the survival period of rats, because the venom components have already established and started their deleterious influence on different tissues in the body. There is difficulty to neutralize toxin fixed already in the tissues and it is only the circulating toxin that can effectively be neutralized by anti serum. However, it is safe to postulate that corticosteroids given as early as possible after the bite may be lifesaving or atleast be helpful in prolonging the survival period and overcome the delay in antivenin therapy.

The neuromuscular block produced by cobra neurotoxin is post junctional and of anti-depolarizing type, which is similar to that of d-tubocurare though they are

structurally and pharmacologically different compounds. The neuromuscular block produced by the latter compound could be antagonized by administration of anticholinesterase agents like neostigmine. But it is doubtful whether the neuromuscular block and other toxic effects produced by cobra venom could be adequately antagonized with neostigmine or other anticholinesterase agents. In the present study, the fact that neostigmine given immediately or one hour after cobra venom caused 100 per cent mortality could be explained only by adverse reactions (excess cholinomimetic activity) of neostigmine which was very predominant. Neostigmine appeared to exert its influence on the various system of the body even before the actual toxic manifestation of venom started, thereby causing early death in the experimental animals. On the other hand, a small degree of protection given by neostigmine when it was administered 2 hour and 3 hour after venom might be due to its transient ability to antagonize the neuromuscular block to a certain degree assuming that such a neuromuscular blockade is established only after a time lag - probably after a period of one hour.

The role of calcium in excitation, conduction, coupling of striated muscle and nerve impulse transmission has been well established. The amplitude of the end plate

potentials is also affected by calcium and there is an approximately linear relation between the amplitude of the end plate potentials and the concentration of calcium ion. In the absence of sufficient amount of calcium, neuromuscular transmission fails. Even more numerous are the observations on the effect of calcium in spontaneous activity at the motor end plate. Calcium appears to be a necessary synaptic constituent for all neuromuscular transmissions it augments the amplitude of the end plate potentials and further increases the rate of occurrence of spontaneous miniature potentials in mammals. Calcium is also involved in the release of acetylcholine at the nerve endings and will also facilitate the depolarization of the end plate region (Simpson, 1968). Calcium is also a constituent of cell membrane essential for cell adhesion and intercellular junctional membrane permeability. It can also decrease the capillary permeability.

McGee (1953), Cook (1956) and Chatterjee (1965) have reported on the beneficial effect of calcium gluconate to overcome the toxicity produced by snake venom especially with viper venom. The results of the present investigation shows that calcium administered at different doses immediately afterwards, one hour or 3 hour after venom did not

afford protection, but very strongly the same dose at 2nd hour interval afforded some degree of protection by prolongation of survival period of rats against one LD50 cobra venom. The dose of cholesterol administered was also very small. The quality of cholesterol as supportive measure, thus appears to require further scrutiny and experimental study.

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S U M M A R Y

S U M M A R Y

The following observations were made during the course of investigation on the effect of corticosteroids, neostigmine and calcium in cobra venom (Naja naja) intoxication in experimental rats:-

(1) The intramuscular LD50 of cobra venom (Naja naja) causing death within 8 hours was found to be 0.097 mg per 100 g body weight of rats.

(2) 'Betnesol' (Glaxo brand of Betamethasone) at dose of 0.8 to 1 mg per 100 g body weight of rats when given by intraperitoneal route immediately after experimental envenomation intramuscularly with one LD50 cobra venom gave 100 per cent protection by prolongation of survival period of rats. The degree of protection given by the drug decreases when there is a time lag for administration of Betnesol.

(3) Neostigmine bromide (E Merck) in a dose of 30 mcg per 100 g body weight of rats was not found to be of much value to protect rats from the neurotoxicity produced by the cobra venom.

(4) No definite conclusion could be drawn by the trial employing calcium gluconate as a supportive measure in cobra venom intoxication in rats.

(5) Some of the rats which survived for more than 10 hours after injection of venom showed a peculiar syndrome similar to that of 'chromodacryorrhea' which is a specific symptom in anticholinesterase poisoning in rats. This feature was more predominant in rats which were given neostigmine whereas it was absent in rats treated with Betnesol and calcium gluconate.

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T A B L E S

TABLE I

Survival period of rats with 'Betnesol'

0.6 mg/100 g body weight intraperitoneally after experimental envenomation

Sl. No.	Body weight of rats in gram	Cobra venom in milligram	Interval for Betnesol injection	Betnesol in milligram intraperitoneally	Survival period in hours
1	120	0.1164	Immediately after venom injection	0.720	10.42
2	135	0.1309	,,	0.810	20.00
3	145	0.1406	,,	0.870	15.00
4	140	0.1358	,,	0.840	48.00
5	150	0.1455	,,	0.900	48.00
6	200	0.1940	,,	1.200	10.40
7	150	0.1455	One hour after venom injection	0.900	7.00
8	140	0.1358	,,	0.840	14.00
9	160	0.1552	,,	0.960	30.00
10	130	0.1261	,,	0.780	20.00
11	145	0.1406	,,	0.870	8.40
12	130	0.1261	,,	0.780	15.20
13	170	0.1649	Two hour after venom injection	1.020	10.30
14	140	0.1358	,,	0.840	7.20
15	155	0.1503	,,	0.930	20.00
16	130	0.1261	,,	0.780	5.00
17	135	0.1309	,,	0.810	9.30
18	160	0.1552	,,	0.960	8.00
19	145	0.1406	Three hour after venom injection	0.870	5.20
20	160	0.1552	,,	0.960	9.25
21	130	0.1261	,,	0.780	10.00
22	155	0.1503	,,	0.930	7.00
23	125	0.1212	,,	0.750	6.30
24	145	0.1406	,,	0.870	22.00

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TABLE II

Survival period of rats with 'Betnesol'

0.8 mg/100 g body weight intraperitoneally after experimental envenomation

Sl. No.	Body weight of rats in gram	Cobra venom in milligram	Interval for Betnesol injection	Betnesol in milligram intraperitoneally	Survival period in hours
1	135	0.1309	Immediately after venom injection	1.080	48.00
2	140	0.1358	"	1.120	48.00
3	105	0.1018	"	0.840	48.00
4	175	0.1697	"	1.400	12.35
5	110	0.1067	"	0.880	30.00
6	150	0.1455	"	1.200	14.30
7	120	0.1164	One hour after venom injection	0.960	48.00
8	165	0.1600	"	1.320	14.00
9	120	0.1164	"	0.960	12.00
10	120	0.1164	"	0.960	14.20
11	125	0.1212	"	1.000	32.00
12	115	0.1115	"	0.920	7.30
13	140	0.1358	Two hour after venom injection	1.120	7.00
14	140	0.1358	"	1.120	48.00
15	145	0.1406	"	1.160	13.00
16	150	0.1455	"	1.200	10.00
17	145	0.1406	"	1.160	5.30
18	130	0.1261	"	1.040	8.00
19	140	0.1358	Three hour after venom injection	1.120	13.30
20	145	0.1406	"	1.160	30.00
21	135	0.1309	"	1.080	8.40
22	115	0.1115	"	0.920	9.20
23	110	0.1067	"	0.880	5.15
24	125	0.1212	"	1.000	6.00

TABLE III

Survival period of rats with 'Betnesol'

1 mg/100 g body weight intraperitoneally after experimental envenomation

Sl. No.	Body weight of rats in gram	Cobra venom in milligram	Interval for Betnesol injection	Betnesol in milligram intraperitoneally	Survival period in hours
1	135	0.1309	Immediately after venom injection	1.35	15.00
2	155	0.1503	,,	1.55	42.00
3	120	0.1164	,,	1.20	42.00
4	175	0.1697	,,	1.75	42.00
5	130	0.1261	,,	1.30	20.00
6	120	0.1164	,,	1.20	12.44
7	165	0.1600	One hour after venom injection	1.65	30.00
8	130	0.1261	,,	1.30	14.00
9	155	0.1503	,,	1.55	12.20
10	185	0.1794	,,	1.85	32.00
11	120	0.1164	,,	1.20	6.20
12	145	0.1406	,,	1.45	10.40
13	145	0.1406	Two hour after venom injection	1.45	10.40
14	140	0.1358	,,	1.40	5.00
15	175	0.1697	,,	1.75	24.00
16	120	0.1164	,,	1.20	8.00
17	175	0.1697	,,	1.75	14.00
18	160	0.1552	,,	1.60	7.20
19	130	0.1261	Three hour after venom injection	1.30	6.50
20	165	0.1600	,,	1.65	8.00
21	135	0.1309	,,	1.35	11.00
22	145	0.1406	,,	1.45	24.00
23	165	0.1600	,,	1.65	5.00
24	170	0.1649	,,	1.70	9.40

TABLE IV

Survival period of rats with Neostigmine bromide 30 mcg/100 g body weight intraperitoneally after experimental envenomation

Sl. No.	Body weight of rats in gram	Cobra venom in milligram	Interval for Neostigmine injection	Neostigmine in microgram intraperitoneally	Survival period in hours
1	170	0.1649	Immediately after venom injection	51.00	3.25
2	160	0.1552	,,	48.00	3.10
3	165	0.1600	,,	49.50	3.15
4	160	0.1552	,,	48.00	3.25
5	130	0.1261	,,	39.00	5.37
6	190	0.1843	,,	57.00	4.45
7	125	0.1212	One hour after venom injection	37.50	4.18
8	130	0.1261	,,	39.00	3.00
9	140	0.1358	,,	42.00	3.00
10	125	0.1212	,,	37.50	3.15
11	120	0.1164	,,	36.00	3.40
12	130	0.1261	,,	39.00	4.20
13	150	0.1455	Two hour after venom injection	45.00	3.30
14	150	0.1455	,,	45.00	3.00
15	150	0.1455	,,	45.00	3.30
16	120	0.1164	,,	36.00	6.14
17	130	0.1261	,,	39.00	10.08
18	120	0.1164	,,	36.00	24.00
19	165	0.1600	Three hour after venom injection	49.00	5.21
20	150	0.1455	,,	45.00	5.20
21	150	0.1455	,,	45.00	48.00
22	130	0.1261	,,	39.00	4.30
23	120	0.1164	,,	36.00	4.42
24	110	0.1067	,,	33.00	5.20

TABLE V

Survival period of rats with Calcium gluconate 8 mg/100 g body weight intraperitoneally after experimental envenomation

Sl. No.	Body weight of rats in gram	Cobra venom in milligram	Interval for Calcium gluconate injection	Calcium gluconate in milligram intraperitoneally	Survival period in hours
1	165	0.1600	Immediately after venom injection	13.20	10.45
2	140	0.1358	,,	11.20	8.00
3	110	0.1067	,,	8.80	10.00
4	115	0.1115	,,	9.20	7.30
5	165	0.1600	,,	13.20	11.00
6	110	0.1067	,,	8.80	9.25
7	130	0.1261	One hour after venom injection	10.40	6.20
8	195	0.1891	,,	15.60	12.25
9	110	0.1067	,,	8.80	10.30
10	165	0.1600	,,	13.20	12.50
11	100	0.0970	,,	8.00	12.20
12	170	0.1649	,,	13.60	10.00
13	105	0.1018	Two hour after venom injection	8.40	3.00
14	120	0.1164	,,	9.60	13.00
15	105	0.1018	,,	8.40	12.30
16	170	0.1649	,,	13.60	12.20
17	165	0.1600	,,	13.20	5.15
18	110	0.1067	,,	8.80	32.00
19	160	0.1552	Three hour after venom injection	12.80	8.40
20	105	0.1018	,,	8.40	7.30
21	110	0.1067	,,	8.80	11.40
22	160	0.1552	,,	12.80	3.00
23	110	0.1067	,,	8.80	12.30
24	165	0.1600	,,	13.20	12.48

TABLE VI

Survival period of rats with Calcium gluconate 10 mg/100 g body weight intraperitoneally after experimental envenomation

Sl. No.	Body weight of rats in gram	Cobra venom in milligram	Interval for Calcium gluconate injection	Calcium gluconate in milligram intraperitoneally	Survival period in hours
1	165	0.1600	Immediately after venom injection	16.50	10.00
2	120	0.1164	,,	12.00	15.30
3	115	0.1115	,,	11.50	6.30
4	155	0.1503	,,	15.50	12.10
5	140	0.1358	,,	14.00	48.00
6	170	0.1649	,,	17.00	9.20
7	110	0.1067	One hour after venom injection	11.00	12.45
8	120	0.1164	,,	12.00	10.55
9	140	0.1358	,,	14.00	15.30
10	155	0.1503	,,	15.50	7.42
11	140	0.1358	,,	14.00	24.00
12	120	0.1164	,,	12.00	9.35
13	165	0.1600	Two hour after venom injection	16.50	12.40
14	135	0.1309	,,	13.50	24.00
15	110	0.1067	,,	11.00	10.30
16	120	0.1164	,,	12.00	14.00
17	170	0.1649	,,	17.00	14.10
18	110	0.1067	,,	11.00	24.00
19	135	0.1309	Three hour after venom injection	13.50	6.00
20	110	0.1067	,,	11.00	48.00
21	120	0.1164	,,	12.00	11.45
22	165	0.1600	,,	16.50	10.37
23	155	0.1503	,,	15.50	11.10
24	120	0.1164	,,	12.00	10.00

TABLE VII

Survival period of rats with Calcium gluconate 12 mg/100 g body weight intraperitoneally after experimental envenomation

Sl. No.	Body weight of rats in gram	Cobra venom in milligram	Interval for Calcium gluconate injection	Calcium gluconate in milligram intraperitoneally	Survival period in hours
1	165	0.1600	Immediately after venom injection	19.80	11.15
2	170	0.1649	,,	20.40	9.25
3	155	0.1503	,,	18.60	12.25
4	165	0.1600	,,	19.80	24.00
5	160	0.1552	,,	19.20	4.35
6	155	0.1503	,,	18.60	11.15
7	110	0.1067	One hour after venom injection	13.20	24.00
8	135	0.1309	,,	16.20	24.00
9	190	0.1843	,,	22.80	11.30
10	170	0.1649	,,	20.40	5.00
11	160	0.1552	,,	19.20	4.00
12	110	0.1067	,,	13.20	4.15
13	180	0.1746	Two hour after venom injection	21.60	5.30
14	155	0.1503	,,	18.60	5.55
15	175	0.1697	,,	21.00	13.25
16	115	0.1115	,,	13.80	12.20
17	120	0.1164	,,	14.40	12.35
18	140	0.1358	,,	16.80	30.00
19	115	0.1115	Three hour after venom injection	13.80	11.20
20	150	0.1435	,,	18.00	9.05
21	120	0.1164	,,	14.40	12.55
22	105	0.1018	,,	12.60	10.00
23	120	0.1164	,,	14.40	24.00
24	115	0.1115	,,	13.80	7.30

A B S T R A C T

Abstract of "STUDIES ON THE EFFECT OF CORTICOSTEROIDS, NEOSTIGMINE AND CALCIUM IN COBRA VENOM (*Naja naja*) INTOXICATION"

Administration of specific antiserum as early as possible after snake bite is of utmost importance for better chances of survival. Supportive measures are definitely capable of reducing the venom toxicity and prolonging survival period eg., if the absorption of venom from the site of bite could be delayed or retarded it will be highly beneficial to the patient. It has been shown that certain drugs are capable of decreasing capillary permeability and altering the rate of absorption of venom from the site while other agents may alleviate neurotoxicity or reduce haemotoxicity of venoms on the system.

The present study was undertaken to evaluate the beneficial effect of (1) 'Betnesol' (Glaxo brand of betamethazone), (2) Neostigmine bromide (E Merck) and (3) Calcium gluconate - 10% (Sandoz) in experimental envenomation with cobra venom (*Naja naja*) in adult rats of both sexes. One LD50 cobra venom (0.097 mg per 100 g body weight - death within 8 hours) was given intramuscularly to these experimental rats and the trial drugs viz., 'Betnesol' (0.6, 0.8 and 1 mg per 100 g body weight), neostigmine bromide (30 mcg per 100 g body weight) and

calcium gluconate (8, 10 and 12 mg per 100 g body weight) were given intraperitoneally at varying intervals to evaluate the efficacy of these drugs in respect of their ability to prolong the survival period of experimentally envenomated rats.

It is observed that 'Betnesol' at dose rate of 0.8 to 1 mg per 100 g body weight of rats when given immediately after injection of one LD50 cobra venom gave 100 per cent prolongation of survival period and the degree of protection decreases when the time lag for the administration of this drug. Neostigmine bromide was found to be of not much value to counteract the neurotoxicity produced by cobra venom. Results of calcium administration were of a varying nature and require further investigation.