CHAPTER II

BACKGROUND INFORMATION

Cobra Venom

The cobra is reptilian in family elapidae. In Thailand, the most common of cobra is Siamese cobra that scientific name is *Naja naja kaouthia*, common name is monocellate cobra. They have monocellate on the back of hood and venom glands located at both side of back of head. Snake venom have a variety of deleterious effects on almost every organ system. In most cases of snake venom poisoning, circulatory disturbance is one of the most frequently encountered events. Since a single snake venom contains a variety of enzymes as well as nonenzymatic components of different biologic activities. The venom enzyme has been designated phospholipase A that important in lipid metabolism. It had been clearly demonstrated that an enzyme in cobra venom formed a hemolytic substance when it acted on horse serum or egg yolk (Lee, 1979).

A venomous snakebite is not always followed by evidence of envenomation and certainly is not always followed by death. Most snakebite are "startled" bites or "defensive" bite and the snake frequently does not inject venom even though several human lethal doses of venom may be present in the venom glands at the time of the bite (Reid, 1957).

Pain In the bitten area is and inconstant feature of elapid envenomation probably more often absent than present. Pain may also, of course, be associated with a nonvenomous bite. It is, however, a constant feature of an effective cobra bite (Rowlands et al., 1969).

Local swelling is a constant feature of cobra envenomation. Swelling may also occur in a nonenvenomated limb if a tourniquet is used, and thus edema may be present after a nonvenomous snakebite or after a suspected bite. Considerable swelling may result from this treatment so much so that local swelling was disregarded as a sign of envenomation (Jutzi et al., 1953). If a tourniquet has not been used, local swelling indicates that envenomation has probably occurred, but its absence does not indicate that envenomation has not occurred (Lee, 1979).

Furthermore, severe local pain, sometimes described as burning in character, is an almost constant feature of an effective cobra bite. The pain commences in the bitten area and frequently radiates up the limb and may last from less than 1 to over 10 days depending or the extent of the local necrosis (Reid, 1964). A dusky discoloration around the bite marks, which occasionally ooze serosanguineous fluid (Hanna and Lamb, 1901; Ahuja and Singh, 1954), extends and taken until after 3-4 days a greyish black area is present, demarcated by an erythematous margin. Sanguineous blisters may develop. Fluctuation later becomes apparent, and necrosis of skin and subcutaneous tissue is usually evident by the 5th day, the area of skin necrosis may vary from a few cm^2 up to 600 cm^2 (Reid, 1964). The extensive skin loss may take several months to heal and may give rise to deformity if inadequately treated (Kuo and Wu, 1972); chronic relapsing ulceration may also occur and lead to amputation at a limb (Warrell et al., 1976).

Action on the Heart of Cobra Venom

Although the primary cause of death in envenomation by elapid snake has been shown to be peripheral respiratory paralysis in many species of animals (Lee, 1972), these venoms also produce cardiovascular changes. For instance, cats poisoned with cobra venom may die of cardiac failure rather than respiratory paralysis (Epstein, 1930; Lee and Peng, 1961) and the venom of spitting cobra (*Naja nigricollis*) invariably causes a profound cardiovascular depression predominating over its paralytic action on envenomated animals (Lee, 1979). One of the earliest studies considered that cobra venom acted on the cerebro-spinal nerve centers, and in large doses it also acted on the

ganglia of the heart, causing arrest of cardiac action, little stress was laid on the role played by circulatory failure. They were unable to come to any definite conclusion as to the exact influence of the venom on the heart, but they thought that the heart's arrest in systole, which they at times observed, was due to same action on cardiac on ganglia (Brunton and Fayrer, 1873). Furthermore, large dosed of cobra venom stopped the frog heart in systole. In the isolated frog heart, the amplitude showed a slight preliminary increase, soon followed by a marked diminution, chiefly the result of a lessened relaxation, and the ventricle finally ceased in systole. An increase in tone of the muscle was produced. Changes in the rate were not marked and irregularities in rhythm were seldom observed (Epstein, 1930). In the mammal strong doses of venom lessened the amount of the heart's movements, while smaller doses increased it. In dog, intravenous injection of the Indian cobra venom (0.2 mg/kg) caused a marked augmentation of the amplitude of ventricular contraction with some extrasystoles (Gautrelet et al., 1934). Cobra venom caused diminution of the contractions, leading to systolic contracture in the isolated frog heart (Gottdenker and Wachstein, 1940). Immediately after the addition of the venom to the perfusion fluid there was occasionally some augmentation of the contractions, followed by a lessened of diastole, and finally the heart passed into a systolic contracture. In the absence of calcium ions in the perfusion fluid, the cardiac muscle became less sensitive to the venom, which produced stoppage of beat without signs of systolic contracture (Gottdenker and Wachstein, 1940).

Electrocardiographic studies on the effect of cobra venom were made and described the following changes in the rabbit and guinea-pig heart after intravenous injection of nonlethal doses of cobra venom : sinus bradycardia, T wave changes, S-T segment depression, atrial flutter, extrasystoles, ventricular bigeminy, slow ventricular rhythm, and A-V dissociation (Lee, 1979). In dogs, intravenous injection of large doses (1.5-2 mg/kg) of cobra venom produced electrocardiographic changed. The first changed to be noted, occurring within a minute or two of the injection, are in the form of the T wave. Next, the P-R interval becomes progressively longer and the ventricular complexes wider. The development of aberrant ventricular complexes may follow,

sometimes preceded by a great diminution in the voltage of the ventricular complexes, or an abrupt on set of heart block occurred. The toxic effects of the venom on the atria were longer delayed than those on the conducting tissues and on the ventricles. In the late stage the P waves were reduced in voltage and sometimes disappear altogether (Feldberg and Kellaway, 1937b). In rabbit heart, they observed the following changes : bradycardia, increased P-R interval, S-T deviation, and terminal heart block (Kellaway and Trethewie, 1940). Furthermore, cobra venom caused are increased of heart rate, inversion of T wave, prominent P wave, extrasystoles, increased P-R interval, aberrant QRS-T complex resembling bundle branch block, and ventricular fibrillation in cats (Kellaway and Trethewie, 1940). Thus, although the changes in the electrocardiograms were somewhat different from one species to another, they all show as the dominant features of the action of the venom.

Hemodynamic Effects

The most prominent and constant hemodynamic change produced by crude venom in general is a fall in systemic arterial pressure, sometimes preceded by an initial transient rise if the venom was administered intravenously. In rabbits, low dose of cobra venom caused a steady rise of blood pressure from the beginning, and sometimes attained very high levels. The conclusion of the high level of blood pressure was due to : (1) the direct action of the circulating venom on the muscular tissue of the arterioles, causing a constriction of these vessels ; (2) the increased force of the myocardium ; and (3) the stimulation of the vasomotor center as a result of the steadily increasing venosity of the blood. The last factor may be responsible for the asphyxial increase in blood pressure before respiratory arrest, but certainly not for the immediate initial rise in blood pressure (Elliot, 1905). After small intravenous doses (e.g., 0.1 mg/kg) of the cape cobra (*Naja nivea*) venom, a slight rise in blood pressure was usually observed in cats and rabbits (Epstein, 1930). In cats that anesthetized with urethrane, Indian cobra venom sometimes caused a fall and sometimes a rise in blood pressure (Chopra and Iswariah, 1931). The rise in blood pressure after injections of sublethal doses of the venom was not due to any stimulant action on the accelerator mechanism of the heart or on the myocardium. They attributed it to the stimulation of the vasomotor center in

the medulla, as it was absent in decerebrated animals. Since weak concentration of venom caused vasoconstriction in the rabbit's ear perfused with physiological salt solution, they were in favor of a direct peripheral vasoconstriction as the cause of the rise in pressure (Gottdenker and Wachstein, 1940).

The primary hypotensive effect of cobra venom is peripheral in nature (Gautrelet et al., 1934; Peng, 1952; Bhanganada and Perry, 1963). In early suggestion, large doses of cobra venom produced falling of blood pressure was due to inhibition of the heart, which was mainly brought about by the direct action of the venom on the vagal centers. The studies those cutting off central vagi impulses from the heart, the hypotensive effect of cobra venom has not prevented (Elliot, 1905; Gautrelet et al., 1934). The initial precipitous fall in arterial pressure produced by cobra venom has been attributed to : (1) vasodilatation in the periphery (Gautrelet et al., 1934; Peng, 1952; Bhanganada and Perry, 1963), combined with constriction of the hepatic veins, especially in dogs (Feldberg and Kellaway, 1937b) ; (2) a pronounced pulmonary vasoconstriction, especially in cats (Yonegawa, 1926; Feldberg and Kellaway, 1937a; Chiu et al., 1968; Lee et al., 1971), or (3) a direct venom action on the heart (Elliot, 1905; Devi and Sarkar, 1966; Phillips, 1972).

The depressor effect is accompanied by loss of fluid from circulation, but whereas in cats, part of the fluid loss is accounted for by edema of the lungs, in dogs, edema of the lungs is absent, and the whole of the fluid loss must be attributed to changes in the permeability of the capillaries of the general circulation (Feldberg and Kellaway, 1937a, 1937b). Despite the differences between the circulatory effects of cobra venom in the cat and dog, there are many points of resemblance. In both animals, the venom has a powerful toxic action upon the heart which may prove fatal after large doses. The capillaries are dilated by the venom, and an increase in their permeability is indicated by the fluid loss from circulation. Although the venous outflow from the cat's limb was decreased and that from the dog's limb was increased after arterial injections of small doses of venom, the capillaries may have been dilated in both, and the difference is accounted for by reactions on the arterial side of the vascular tree, which is constricted in the cat and dilated in the dog. Even in the dog, large doses

of venom decrease the venous outflow from the limb. Difference in the reaction of the arterial side of the vascular tree are therefore in part quantitative rather than qualitative (Feldberg and Kellaway, 1937b). This interpretation of the vascular effects of the venom has much in common with that given for histamine, which constricts the arterioles in the cat and dilates them in the dog, but causes dilatation of the capillaries in both species (Dale and Richards, 1918).

Cardiotoxin

Many snakes, especially Elapidae venoms, contain a variety of chemically similar basic protein which, however, exhibit different types of pharmacological actions including cardiotoxic, cytotoxic and potent neurotoxic activities (Tu, 1977). The most lethal are the α-neurotoxins, which are single-chain proteins of about 60 or 70 residues. These toxins bind to nicotinic acetylcholine receptors, and they death by paralyzing respiratory muscles. Cobra venoms also contain large quantities of other proteins that are similar in size (60 to 62-amino acid residues, 6000-7000 MW.) to the neurotoxins, but do not bind to cholinoceptors (Lee, 1979; Tu, 1991). Their toxicity is primarily associated with direct effects on the heart and, hence, they were named "cardiotoxins" (Sarkar, 1947). Because cardiotoxins (particularly when contaminated with trace amounts of phospholipase A_2) can have lytic effects on a wide range of cells, other names have been proposed for them: cytotoxin (Braganca et al., 1967), cobramine (Condrea, 1974), membrane disruptive polypeptide (Yang, 1974), membrane toxin (Karlsson, 1979), and membranotoxin (Muzskat et al., 1984). The venom of the Indian cobra, *N. naja*., and African Ringhals cobra, *H. haemachatus*. were separated by paper electrophoresis method. The most basic fractions had no detectable phospholypase A activity, but could produce a slow lysis of human red blood cells. The active components were named "direct lytic factors" (Condrea et al., 1964). Most of these compounds act on various membranes increasing their permeability. An electrostatic interaction between the positively charged toxin and the negatively charged surface of the membrane is presumed to be involved in the initial binding. For the further process, two hypotheses have been proposed.

According to the first view, the hydrophobic parts of the toxin penetrate into the hydrophobic layer of the membrane and disrupt its structure (Klibansky et al., 1968). This resulting disorder and leakiness should render the membrane phospholipids accessible to be attacked by phospholipase A. The hydrolysis products, lysophosphatides, are lytic and will further increase the damage.

According to the second hypothesis, the membrane is disrupted by and interchange reaction between disulfides in the toxin and sulfhydryl groups in the membrane (Vogt et al., 1970). Such a mechanism should probably require a particularly reactive disulfide in the toxin, but all the disulfides appear to have the same reactivity. Reduction of a membrane toxin, toxin γ , with an equimolar amount of dithiotreitol reduces all disulfides to about the same extent (Fryklund and Eaker, 1975). Another indirect evidence against the disulfide interchange mechanism is that the lytic action of melittin, a basic polypeptide from bee venom, which is devoid of disulfides, is also potentiated by phospholipase A (Vogt et al., 1970).

From the earliest days of research on snake venoms, cobra venom has been known to affect the cardiovascular system. Respiratory paralysis was main lethal action of Indian cobra venom and cardiac arrest was caused by intravenous injection of venom (Brunton and Fayrer, 1873). Mark contracture and loss of rhythmic activity were direct action of Indian cobra venom in isolated frog heart (Elliot, 1905). These effects were irreversible. Similar effects were found with the venom of the South African Cape cobra, *Naja nivea* (Epstein, 1930).

The effects of *N. naja* venom, an isolated neurotoxin preparation, and an isolated hemolysin preparation were compared. The hemolysin and the crude venom caused irregular beats when applied to toad hearts in situ. The hemolysin was 11 times more potent than crude venom on a weight basis. Large doses of the hemolysin, but not the neurotoxin, could mimic the crude venom in causing cardiac arrest. In anesthetized rabbits or guinea pigs, hemolysin caused cardiac and circulatory failure that was not prevented by artificial respiration (Sarkar et al., 1942). Subsequently, cardiotoxin was isolated from respiratory toxin of Indian cobra venom. The cardiotoxin

fraction had about 15 times the activity of the whole venom, and it was demonstrated to contain basic protein (Sarkar, 1947). Both whole venom and the purified cardiotoxin caused systolic arrest in isolated perfused toad hearts and in anesthetized cats.

Calcium plays an important role in maintaining the functional integrity of biological membrane, and because calcium is the trigger for muscular contraction. Displacement of Ca^{2+} ions from their binding sites on muscular membranes was suggested to be first stage in the action of cardiotoxins (Earl and Excell, 1972; Lin-Shiau et al., 1976), although the nature of the binding site was not specified. Furthermore, cardiotoxin acted directly on muscle membranes. Purified cardiotoxin caused irreversible depolarization of isolated skeletal muscle preparation (Meldrum, 1963, 1965; Lee et al., 1968; Earl and Excell, 1972; Lin-Shiau et al., 1976; Harvey et al., 1982).

The cytotoxic fraction from Indian cobra venom was found to inhibit Na^+-K^+ ATPase activity of membrane fragments of various cells. It was conceivable that lysis of cells may be due to an imbalance of K^+ and Na^+ in the cell which leads to swelling and disintegration of the membrane structure (Zaheer et al., 1975). Controversial, hemolysis of guinea-pig red cells by phospholypase A was not enhanced when the erythrocyte ATPase had been blocked by ouabain (Lankisch et al., 1972). Since, the basic assumption of an action of cardiotoxin on Na^+-K^+ ATPase has been questioned.

Calcium Ion Channels

Electrophysiological studies have shown unequivocally that the plasma membrane of cells that respond to stimuli which changes in membrane potential channels that selectively allow the entry of calcium ions down its electrochemical potential gradient (Reuter, 1983). These channels have two main functions : to allow the participation of calcium ions currents in the rising phase of the action potential ; and to allow the inflow of extracellular calcium ions that leads to the rise in cytoplasmic ions concentration and the consequent cellular response to the stimulus. Calcium ion channels are controlled by voltage-dependent gating, that is, their opening or closing kinetics are a consequence of changes in membrane potential. The channels show both

time and voltage dependent inactivation. Usually they open at more positive membrane potentials than the sodium pump which participate in the rising phase of the action potential. Although calcium ion channels are primarily regulated by the membrane potential, their properties are modulated by neurotransmitters, hormone and drugs. In some cases, modulation seems to imply phosphorylation of the channels or of membrane proteins closely associated with it. Calcium ion channels are detected in sarcoplasmic reticulum and plasma membrane of smooth muscle and all renal tubular segments ; the highest activity are found in distal convoluted tubule (Doucet and Katz, 1982).

Na^+-K^+ ATPase

There is abundant evidence to show that the Na^+-K^+ ATPase, a unique enzyme, spans the basolateral membranes of most renal tubule cells, Na^+-K^+ ATPase catalyzes the movement of sodium and potassium ion across the cell membrane utilizing ATPase the energy source. The enzyme is present in all tissues of higher organism but it is most abundant in the kidney where it is responsible for reabsorbing sodium ions from the glomerular filtrate. Na^+ is transported across the basolateral membrane into the interstitial fluid, creating a gradient for Na^+ from the tubular lumen to be drawn passively into the cell. The accumulation of Na^+ in the interstitial fluid then creates an osmotic force, resulting in water reabsorption from the tubular lumen into the interstitial space. Interstitial Na^+ and water are then taken up the peritubular capillaries. The net result is transport of Na^+ and water from lumen to the capillary.

The Na^+-K^+ ATPase is located on the interstitial site of the cell and, as Na^+ ions are transported out of the epithelial cell, more Na^+ ion are drawn in from the tubular lumen as a result of the reduced into a cellular sodium ion concentration. The enzyme is composed of two subunits, a larger catalytic α subunit and a smaller, glycosylated β subunit. Both are required for function, however the major role of the β subunit may be in maturation of the enzyme and localization to the plasma membrane (Lingrel et al., 1994).

Transcellular active Na^+ reabsorption is directly coupled to enzyme, which is located in the basolateral cell membrane in close association with mitochondria, converts the energy of ATP into electrochemical gradients : Na^+ ions are pump out the cells in exchange with K^+ ions. This generates and maintains a low Na^+ concentration into the cell, thereby providing the main driving force for Na^+ entry across the apical membrane which contains Na^+ transports.

Thus, for a given load of Na^+ in the tubular lumen, the capacity of the tubular cells to reabsorb Na^+ depends on two parameters : The overall Na^+ permeability of the apical membrane and the activity of basolateral pump. The activity of the quantity of enzyme present in each cell and on the intrinsic activity of each Na^+-K^+ ATPase molecule.

It appears clearly that Na^+-K^+ ATPase is closely involved in the mechanism of reabsorption of filtered Na^+.

In order to circumvent the problem of kidney heterogeneity, several attempts have been made to quantitate Na^+-K^+ ATPase at the level of single, will define segments of nephron. The hydrolytic activity of Na^+-K^+ ATPase is usually determined on broken or permeabilized cells (in order to permit free access of exogenous ATP to its intracellular site of action) and under optimal conditions (Doucet, 1992).