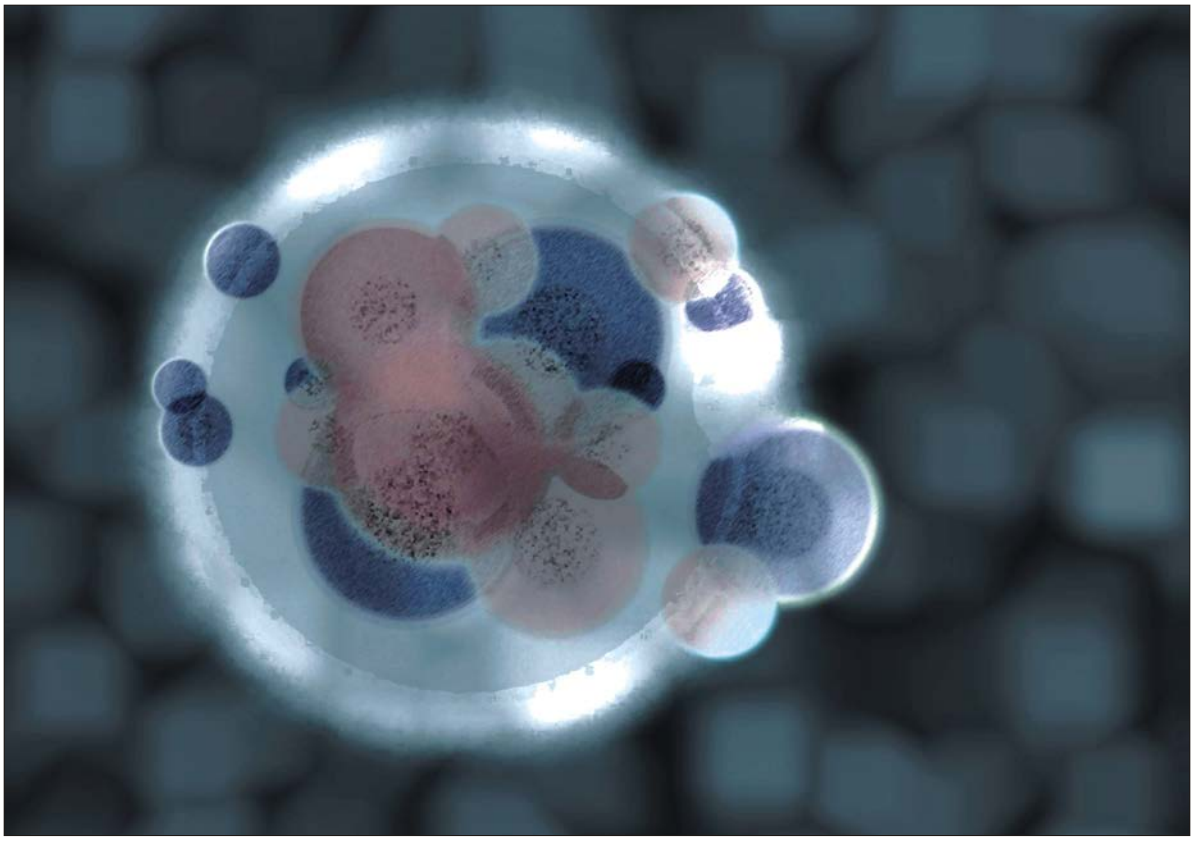

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# Dose-Dependent Effect of Cobra Venom on Testes in Mice

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**Abstract:** In this study, the effect of phospholipases, Cobra Venom Factor (CVF) on the morphology of testis in mice as well as on some mineral contents was investigated. Mineral analysis shows changes in the concentration of many elements. This report however, will only discuss calcium, potassium and phosphorus and their possible roles during testis development. Toxin administration results in severe structural damage to the testis. These toxin-induced abnormalities are associated with a reduction in the intracellular concentrations of calcium, potassium and phosphorus in testis cells. Inhibition of calcium suggests a signaling-mediated role of the CVF-induced effect on the testis of intoxicated mice. Potassium depletion is believed to mediate cellular deformation through hyperpolarization of the plasma membrane. Reduction of the intracellular phosphorus causes many physiological abnormalities within testis cells as a result of the reduced production of internal cellular regulators that are mostly phosphorylated.

**Key words:** Cobra, venom, testes, calcium, phosphorus

## INTRODUCTION

Snake venoms comprise complex mixtures of enzymatic and non-enzymatic proteins and small organic compounds. The pathology of envenomation includes both local and systemic effects such as neurotoxicity (pre/postsynaptic), myotoxicity, cardiotoxicity, coagulant (pro/anti), hemostatic (activating/inhibiting), hemorrhagic, hemolytic and edema forming activities (Girish *et al*., 2004). The pathology involves the degradation of proteins and glycosaminoglycans in the extracellular matrix, connective tissue surrounding blood vessels and capillaries and myonecrosis. Nevertheless, the relative abundance of these enzymes/toxins may vary with the venom source. For example, myotoxic phospholipase $A_2$ and cardiotoxins are rich in cobra venom while hemorrhagic metalloproteases are rich in viper venoms. Very little is known about the envenomation effect on the morphology of the testes and the cellular mechanism that induce that effect.

Fertilization is essential for sexual reproduction and for the generation of a new individual. This fundamental process requires communication between competent male and female gametes so that they may fuse. In internal fertilizers such as reptiles, birds and mammals, sperms develop the potential for motility as they pass through the epididymis (Morisawa and Morisawa, 1994; Shiba *et al*., 2006). The behavior of the sperm after being released from the gonads is a most dramatic example of continuous coupling between the cell machinery and the outer environment. The sperm is not a deterministic device, oblivious to the external medium and tuned only to the chemical signals from the egg outer layer. It must avoid fusing with any other cells but the egg. The concentration of ions, pollutants, pH, temperature and other physicochemical variables influence sperm behavior and metabolism. Importantly, signals from the egg modulate sperm physiology, inducing sperm to undergo series of ordered changes in configuration that enables it to complete fertilization.

Phospholipase activity was first described in pancreatic juice and cobra venom at about the turn of the century. Phospholipase $A_2$s ($PLA_2$s) are those enzymes that hydrolyze the sn-2 fatty acid acyl ester bond of phosphoglycerides to free fatty acid and lysophospholipids. $PLA_2$s have been divided into several groups based on molecular weight, amino acid sequence and homology, calcium dependence and cellular localization. Two groups of; 14-kDa snake venom $PLA_2$s, Group I from cobras and kraits and Group II from rattlesnakes and vipers, are well known.

In situ hybridization of testis tissue sections indicates that the group IIC $PLA_2$ gene is expressed mainly in pachytene spermatocytes, secondary spermatocytes and round spermatids but not in spermatogonia, elongating spermatids, or Sertoli cells (Chen *et al*., 1997; Irino *et al*., 2005). It is not known whether there is compensatory activity of one of the other $PLA_2$ genes in human testis or other tissues.

The goal of this study is to investigate the effect of CVF as a source of group I phospholipase on testicular morphology. The concentration of a selected number of minerals in mice testes will also be assessed using a programmable simultaneous plasma emission spectrometer. The role of some of the examined elements in maintaining the integrity of the testes will also be discussed.

## MATERIALS AND METHODS

**Reagents:** CVF purchased from sigma was used at 0.015 (low dose) and 0.03 (high dose) mg $g^{-1}$ body weight and was intrapretonially injected into mice (Holt *et al*., 2001). Multi-element standard solutions were prepared from Spectrosol Single Element stock solutions (1000 mg $L^{-1}$) purchased from PDH Laboratory Supplies U.K. Calibration standard were made by successive dilutions of the stock solution to obtain a final concentration of 2500 μg $L^{-1}$ (2.5 ppm) for Cu (39.3 μmol $L^{-1}$), Fe (44.8 μmol $L^{-1}$) and Zn (38.2 μmol $L^{-1}$); 500 μmol $L^{-1}$ for Se (6.3 μmol $L^{-1}$); 300 μmol $L^{-1}$ for Al (11.1 μmol $L^{-1}$), B (27.7 μmol $L^{-1}$) and Pb (1.4 μmol $L^{-1}$); 100 μmol $L^{-1}$ for Li (14.4 μmol $L^{-1}$), Ba (0.7 μmol $L^{-1}$), Be (11.1 μmol $L^{-1}$), Co (1.7 μmol $L^{-1}$), Mn (1.8 μ mol $L^{-1}$), Ni (1.7 μ mol $L^{-1}$) and Sr (1.1 μmol $L^{-1}$) and 50 μmol $L^{-1}$ for Cd (0.4 μmol $L^{-1}$) and Cr (1.0 μmol $L^{-1}$). Ultra pure nitric acid and suprapure hydrogen peroxide were purchased from PDH Laboratory Supplies U.K.

**Animals:** Six to eight weeks old male to mice were obtained from the Animal House, UAE University, U.A.E. Mice were grouped (n = 8) and were kept under specified pathogen-free conditions. They had free access to water and were fed standard laboratory chow. Mice were kept in polycarbonate cages with wood chip bedding under a 12 h light/dark cycle and room temperature 22-24°C. Mice were acclimatized to the environment for one-week prior to experimental use. All experiments were performed in accordance with the international guidelines of the Animal Experiments Committee.

**Histochemistry:** Tissues were fixed in Unifix (Klinipath, Duiven, Netherlands), dehydrated and embedded in paraffin. Sections 4 μm thick were stained with Hematoxylin and Eosin using standard protocols.

**Digestion and sample preparation for trace element analysis:** Known dry weights of testis samples were digested each in 10 mL of concentrated nitric acid for 30-45 min at 150°C. After heating, sample solutions were allowed to cool down then 5-10 mm of perchloric acid were slowly added to each sample. Mixtures were then heated up gently to 200°C until digestion was complete.

**Instrumentation:** Mineral concentrations were measured using a programmable simultaneous plasma emission spectrometer (Inductively Coupled Plasma-Optical Emission Spectroscopy; ICP-OES) (Varian vista-MPXCCD Simultaneous ICP-OES) from Varian, Australia with a charged injection device detector and an axial viewing mode. A microcentric nebulizer (Micromist nebulizer) was used. The ICP-OES was equipped with a microcentric nebulizer (Micromist nebulizer) and the analyses were performed under the following conditions: Plasma frequency, 27.12 MHz; RF power, 1200 Waat; Torch gas flow, 560 mL $min^{-1}$; Auxiliary gas flow, 1500 mL $min^{-1}$; Plasma viewing mode, Axial; Nebulizer gas flow, 750 mL $min^{-1}$; Spray champer, Cyclone; Nebulizer uptake, 0.75 mL $min^{-1}$; Sample volume used, PUT LATER; Flush time, 20s; Purge time, 90s; Detector, C. C. Device detector.

## RESULTS

**Testis deformation in intoxicated mice:** In the testes of animals exposed to CVF, the seminiferous tubules underwent significant time-dependent degenerative alterations. There were fewer layers of seminiferous epithelium (Fig. 1B-D). Intercellular spaces were

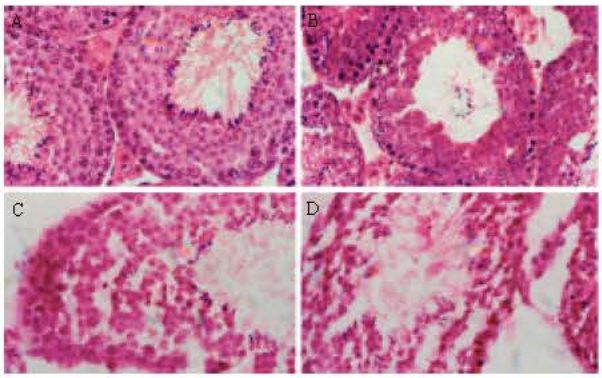


Fig. 1: Effect of intoxication on the integrity of the seminiferous tubules in the testis. Sections of the testis of mice treated with saline (A) or treated for 12, 24 or 48 h with CVF (0.03 mg $g^{-1}$) (B, C and D, respectively) were stained with H and E (original magnification, X 400). Exposure to CVF resulted in a variety of structural damages in the testis, as illustrated by the severe disarray of the developing spermatogonia and the malformation of the mature spermatozoa

Table 1: Effect of intoxication with Low Dose (LD) of CVF (0.015 mg $g^{-1}$ body weight) on the intracellular content of some minerals in testis

| Element | Control | LD 12 | LD 24 |
|---|---|---|---|
| Al | 64.23 | 38.32** | 106.94** |
| B | 148.8 | 165.89 | 65.68** |
| Ba | 4.43 | 1.67** | 5.34* |
| Ca | 512.7 | 228.35** | 646.6* |
| Cd | 1.892 | 1.653 | 0.076** |
| Co | 0.215 | 0.1734* | 0.152* |
| Cr | 6.23 | 6.098 | 49.71** |
| Cu | 13.05 | 11.069 | 3.282** |
| Fe | 146.3 | 585.83** | 81.79** |
| K | 1534.6 | 2047.0** | 1777.4* |
| Mg | 321.6 | 214.21** | 284.7 |
| Mn | 2.28 | 3.439** | 1.2213** |
| Mo | 1.661 | 3.05** | 3.816** |
| Ni | 6.346 | 3.699** | 1.488** |
| P | 1396.9 | 1321.67** | 1678.4** |
| Pb | 5.31 | 1.981** | 2.061** |
| Sr | 4.553 | 1.531** | 1.87** |
| Zn | 45.88 | 31.87** | 38.93 |

*Significant change when compared with the control group; **Highly significant change when compared with the control group

Table 2: Effect of intoxication with the High Dose (HD) of CVF (0.03 mg $g^{-1}$ body weight) on the intracellular content of some minerals in testis

| Elements | Control | HD 12 | HD 24 |
|---|---|---|---|
| Al | 64.23 | 179.75** | 55.39* |
| B | 148.8 | 258.67** | 28.39** |
| Ba | 4.43 | 2.809** | 3.29** |
| Ca | 512.7 | 426.85** | 183.1** |
| Cd | 1.892 | 1.983 | 0.102** |
| Co | 0.215 | 0.206 | 0.102** |
| Cr | 6.23 | 11.19** | 20.78** |
| Cu | 13.05 | 5.247** | 2.81** |
| Fe | 146.3 | 141.03 | 55.9** |
| K | 1534.6 | 1105.7** | 879.7** |
| Mg | 321.6 | 247.93** | 131.2** |
| Mn | 2.28 | 2.479 | 0.26** |
| Mo | 1.661 | 1.818 | 8.23** |
| Ni | 6.346 | 7.231 | 0.843** |
| P | 1396.9 | 1203.3** | 875.7** |
| Pb | 5.31 | 3.14** | 2.53** |
| Sr | 4.553 | 2.148** | 1.48** |
| Zn | 45.88 | 41.28 | 16.89** |

*Significant change when compared with the control group; **Highly significant change when compared with the control group

exaggerated and many of the nuclei appeared pycnotic. Most tubules displayed subluminal nuclei that morphologically could be identified as part of spermatozoa heads; these usually lacked tails, indicating that the treatment interfered with spermiogenesis.

**Minerals analysis:** To examine the effect of intoxication at tissue level, 18 different minerals were measured in both control and treated animals. At the low dose level, most of the examined elements show little changes (Table 1). Potassium (1534.6, 2047.0, 1777.4), phosphorus (1396.9, 1321.67, 1678.4) levels rise dramatically. Calcium concentration (512.7, 228.35) on the other hand, decreases. At a higher dose, levels of phosphorus (1396.9, 1203.3, 875.7) and calcium (512.7, 426.85, 183.1) decrease in a time-dependent manner (Table 2).

**Relation between intoxication and $K^+$ efflux:** $K^+$ efflux from CVF-treated mouse testis in relation to intoxication with the high dose is shown in Fig. 2. $K^+$concentration is dramatically reduced from normal level in untreated testis (1534.6) to (1105.7) 12 h post toxin treatment and to (879.7) 24 h post-toxication. It is worth mentioning here that the histological alterations in the testes of intoxicated mice are more severe when the high toxin dose was used. These results suggest that $K^+$ efflux is an indicator of toxin-mediated membrane perturbation and is associated temporally with intoxication.

**Intoxication reduces calcium levels of testis:** Calcium is crucial for motility in addition of being indispensable second messenger for a wide range of intracellular signaling pathways in mammalian systems. This study

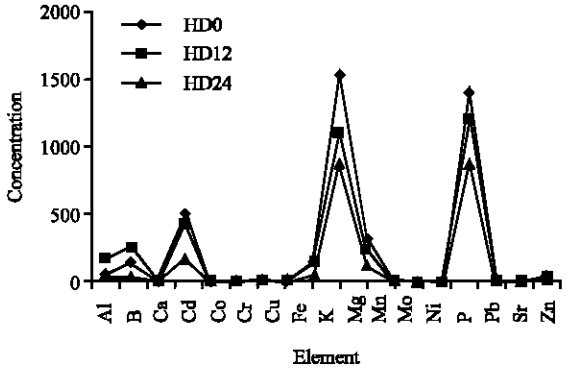


Fig. 2: Effect of high intoxication dose on some mineral concentration in testis. Of the tested elements phosphorus, calcium and potassium concentrations are discussed. Concentrations of phosphorus, potassium and calcium decrease dramatically 24 h after toxin exposure

therefore examines whether intoxication affects the calcium concentration in testis. In response to mild intoxication, calcium concentration drops in 12 h from the control levels (512.7) to (228.35). Twenty four hours following intoxication, $Ca^{2+}$ level is higher than that of the control (Table 1). With the higher dose, the level of calcium decreases from 512.7 to 426.85 and then to 183.1 in a time-dependent manner (Table 2). This decrease in intracellular $Ca^{2+}$ content reflects a toxin-induced hyperpolarization of the studied cells.

## DISCUSSION

The mechanism by which CVF causes its anti-spermatogenic effects is not understood, although it has

been suggested that other toxin-mediated disruption of spermatogenesis in the testis of rats and mice act through Sertoli cells. Several possible factors such as direct cytotoxic effect, a hypo-cholesterolemic effect and androgen deficiency due to Leydig cell damage or increased microsomal enzymes of the liver have been suggested to account for the toxin-induced damage to the testis (Singh and Chakravarty, 2003). In this study, however, it is likely that CVF causes an anti-spermatogenic effect in the mouse testis by acting through Sertoli cells.

The ionic environment encountered by spermatozoa in its journey through the epididymis undergoes significant changes. Potassium for example, rises significantly in the caput and the cauda (Jenkins *et al*., 1980). Capacitation in bovine and mouse sperm is accompanied by $K^+$ permeability increases that hyperpolarize the sperms (Zeng *et al*., 1995). Hyperpolarization of sperms in the sea urchin stimulates adenyle cyclase (AC) (Beltra *et al*., 1996).

The decrease of intracellular potassium levels of target cells has also been described as a marker of pore formation in cell membranes by a variety of toxins (Gary *et al*., 1998) and its demonstration here in response to CVF toxin (Table 2 and Fig. 2) might suggest the involvement of intracellular $K^+$ in the mechanism by which this toxin may function. This study relates the structural deformation of testis to potassium concentration. In agreement with Szabo *et al*. (1994), the results of this study suggest that the insertion of monomeric CVF toxin allows $K^+$ release, which is followed by a time- and concentration-dependent oligomerization leading to the formation of a larger transmembrane pore required for cellular deformation.

During sperm maturation, $Ca^{2+}$ progressively rises in some species (Baldi *et al*., 1991), leading to hyperactivated motility and spontaneous acrosomal reaction. Seminal plasma factors and other factors present in the female fluids *in vivo* or added to the capacitating media (Yanagimachi, 1994) can regulate $Ca^{2+}$ and sperm capacitation (Okamura *et al*., 1990). The role of internal $Ca^{2+}$ stores in capacitation is not yet understood. Calreticulin, a $Ca^{2+}$-binding protein (Nakamura *et al*., 1993) has been detected in the acrosome of several mammalian species, indicating that $Ca^{2+}$ may be stored and released from this organelle. Compounds that favor $Ca^{2+}$ release from internal stores, like thapsigargin, appear to accelerate this process (Mendoza and Tesarik, 1993). It is possible to assume that the toxin-induced testis deformation (Fig. 1), at least in part, is the result of the CVF-mediated decrease in calcium concentration (Tables 1, 2 and Fig. 2). It has been reported that heparin, which is required for bovine sperm *in vitro* capacitation, regulates $Ca^{2+}$ by modulating voltage-dependent $Ca^{2+}$ channels, possibly through binding to specific plasma membrane receptors (Parrish *et al*., 1989). The gene encoding a receptor for $PLA_2$ has been cloned from cow (Ishizaki *et al*., 1994), rabbit (Lambeau *et al*., 1994) and human (Ancian *et al*., 1995). The $PLA_2$ receptor has also been implicated in the induction of group IIA $PLA_2$ mRNA transcription by the group IB pancreatic enzyme in rat mesangial cells (Kishino *et al*., 1994). Perhaps, the $PLA_2$ receptor also mediates cross talk between additional; 14-kDa $PLA_2$s in a cell type-or species-specific manner (Darszon *et al*., 1999).

The lipid content of membranes may modulate their fluidity and ion channel activity (Lundbaek *et al*., 1996). Bovine serum albumin-scavenging of cholesterol during capacitation is thought to change the membrane fluidity and the permeability to $Ca^{2+}$. Protein phosphorylation during mouse (Visconti *et al*., 1995), bovine (Galantino-Hommer *et al*., 1997) and human sperm capacitation (Baldi *et al*., 1996) are modulated by $Ca^{2+}$. Hyperactivation and phosphorylation of several proteins by tyrosine kinase follow the increase in cAMP (Boatman and Robbins, 1991). The increase in cAMP activates a protein kinase (A), which in turn stimulates tyrosine kinases, which finally phosphorylate a set of proteins important for capacitation (Visconti and Kopf, 1998). The reduced level of phosphorus associated with CVF administration, shown in this study is possibly one of the factors that mediate the toxin-induced malformation in testis. This effect is accomplished through the depletion of phosphorylated intracellular regulators particularly cAMP, a major intracellular second messenger for many physiological adjustments.

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