Effects of snake venom from Saudi cobras and vipers on hormonal levels in peripheral blood

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ABSTRACT

Objective: Knowledge about the effects of snake venoms on endocrine glands in the Kingdom of Saudi Arabia (KSA) is meager. The aim of the present study is to investigate the acute and chronic envenomation from 4 snakes out of 8 species of Saudi Cobras and Vipers on the tissues of endocrine glands and peripheral hormonal levels in male rats.

Methods: The peripheral blood levels of 4 hormones mainly testosterone, cortisol, insulin and thyroxin were investigated in male Wistar rats following acute and chronic treatment of the rats with poisonous snake venoms at the Department of Physiology, Faculty of Medicine, King Abdul-Aziz University, Jeddah, Kingdom of Saudi Arabia between September 2000 to May 2001.

Results: Using radio immunoassay for hormonal analysis, a rise in testosterone levels in peripheral blood was obtained following acute treatment, which is due to the effect of the venoms on vascular permeability and increased blood flow. In contrast, the chronic treatment with venoms resulted in a delayed effect on vascular permeability and testicular degeneration resulting in a decreased blood flow and a significant drop in testosterone concentration. Cortisol levels were no different from the controls during acute treatment but it demonstrates gradual rise following chronic treatment to withstand the stress imposed on the animals. Similar results were obtained for insulin, which showed normal values with acute treatment but decreased levels of chronic treatment suggesting insulin insufficiently. Likewise, the thyroxin levels were decreased with chronic treatment suggesting a toxic effect of the poison on the rich blood supply of the thyroid follicles with a subsequent decrease in blood flow to the tissues and therefore, decreased thyroid hormone levels.

Conclusion: The effects of venom toxicity on testosterone levels were either normal or stimulatory with acute treatment or inhibitory with chronic treatment depending on the vascular blood flow and testicular degeneration. Cortisol levels were normal at acute treatment but showed a gradual rise reflecting the stress imposed on the animals. The rise in cortisol levels was visualized to potentiate the cardiovascular and metabolic changes. The effects on insulin and thyroxin were similar to those of testosterone level showing normal or stimulatory effect with acute treatment followed by decreased levels of hormones with chronic treatment.

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Snake venoms with heterogenous composition were reported to have various neurotoxic and hematological effects in both humans and animals.[1,2] The hematological effects include mainly hemorrhage, coagulation disturbances, hemolysis and general cardiovascular disturbances.[3] Other complicated symptoms related to physiological and metabolic changes were also reported.[4] The severity of the envenomation maybe both systematic and localized.[5] Depending on the species of the snake involved and the amount of venom injected. Localized tissue damage is found to occur in many internal organs including the brain, lungs, heart, kidney, liver and endocrine glands.[6,7] On the other hand, cobra venoms were reported to contain many active ingredients that beside neurotoxicity it can

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results in multiple pharmacological and metabolic effects.[5,8-10] Injection of sublethal doses of Echis choloratus venom causes a significant rise in liver enzymes in serum of rats accompanied with disturbances in hepatic and renal functions of envenomated animals resulting in hepatocellular injuries and necrosis of hepatocytes and kidney tubules.[11-14] The tissue damage produced by lethal and sublethal doses of Naja haje (N.haje) was not easily reversed even with a polyvalent serum.[15] Some other authors[16,17] stated that injection of lethal and sublethal doses of N.haje snake venom produced stress reaction resulted in a significant increase in cortisol, catecholamine and serum albumin.

However, the effects of these snake venoms on serum hormonal levels have just recently been reported.[10,18,19] We, therefore, conducted 2 experiments to investigate the acute and chronic treatment of snake venoms on some endocrine glands in the male rats with emphasis on the testicular tissue and testosterone concentration. Eight species of snakes out of 50 were described as poisonous snakes in Saudi Arabia. These include 2 cobra species and 6 other vipers.[20] The snake venoms employed in the present study were those of the poisonous types including the 2 cobra species; Naja Haje Arabicus (NH), the black desert cobra Walternnesia Aegyptia (WA) in addition to 2 vipers namely, Cerastes cerastes (CC) and Echis Collarettes (EC).

Methods. Male Wister rats varying in age between 50-60 days and weighing between 300-350 grams, were brought to the Department of Physiology from the Animal House Department at King Abdul-Aziz Research Center (KARC), Jeddah, Kingdom of Saudi Arabia during the years 2000-2001 used in the experiments. The animals were normally kept in a temperature controlled room at 25^{0}C and 45% relative humidity and maintained on a standard pelleted diet with free access to food and water.

Two experiments were conducted to investigate the acute and chronic effects of snake venoms which were milked from Saudi Cobras and Vipers. In the first experiment, 5 groups of animals were employed during the study to investigate the acute effect of snake venoms.

The first group consisted of 8 rats, received subcutaneous injections of normal saline to serve as sham controls. The remaining 4 groups, each group consisted of 6 rats were injected subcutaneously with sublethal doses of snake venoms as treated animals. Four different snake venoms were used namely; NH, CC, EC and WA and each of the 4 groups of the treated rats received subcutaneous injection from one of the above venoms. The injected dose for each rat in the group was calculated previously according to body weight and determined by trial experiments in order to produce acute effects of the venoms on the animals. These were as follows: sub-LD50 (30 µg/kg body weight) for N.Haje, sub-LD50 (4 mg/kg body weight) for CC, sub- LD50 (30 µg/kg body weight) for WA and sub-LD50 (2 mg/kg body weight) for EC. The use of these sublethal doses produced the toxicity required. After a lapse of 24 hours, both the control and treated groups were sacrificed, blood samples were collected and the serum was separated by centrifugation and stored at -20^{0}C until assayed using radioimmunoassay.

Another experiment was then conducted to compare the chronic effects of snake venom with the results obtained in the previous experiment but using 2 snakes venoms namely NH and CC. Seven groups of animals were studied. The first group consisted of 8 rats, which received saline injection subcutaneously as controls. The remaining 6 groups, each consisted of 6 rats, were divided into 2 lots and injected with snake venoms as treated animals. The first lot, which consisted of 3 sub-groups was injected subcutaneously with sublethal doses of NH venom. The first subgroup of rats were treated 24 hours before blood collection, the second sub-group received 2 injections (24 and 48 hours before blood collection) and the third subgroup received 3 injections (24, 48 and 72 hours before blood sampling). The second lots of animals were handled similarly in 3 subgroups but injected with CC snake venom. Doses of venoms were previously calculated according to body weight in trial experiments to produce chronic effects on the animals. Following the prescribed period for each subgroups, the treated animals were sacrificed: blood samples were collected and serum fractions were separated and stored at -20^{0}C for radioimmunoassay.

Hormonal assays. Radioimmunoassays for testosterone, cortisol and insulin in experiment I and for the same hormones in addition to thyroxin in experiment II were carried out on serum aliquots in duplicate using solid phase 125-I radioimmunoassay designed for quantitative measurement for the above hormones (Diagnostic Products Corporation, DPC, Los Angeles, California, USA). The 125-I labeled hormone was left to compete for a fixed time with the hormone in the serum samples for antibody sites. The specific antibodies were bound immobilized to the walls of the polypropylene tubes after thorough incubation of the samples with the radioactive hormone. The tubes were then vigorously decanted, plotted and the antibody-bound fractions of the radio-labeled hormone were read for one minute in a gamma counter. The counts were converted by way of a calibration curve to measure the specific hormone in the serum samples. No extraction for the serum samples or chromatography was needed for the

Table 1 - Blood levels of testosterone, cortisol and insulin in male rats following acute treatment with Walternnesia Aegyptia, Cerastes cerastes, Naja Haje Arabicus, Echis Collarettes snake venoms.

Hormonal findings	**Control N=8**	**Walternnesia Aegyptia N=6**	**Cerastes cerastes N=6**	**Naja Haje Arabicus N=6**	**Echis Collarettes N=6**
Testosterone (nmol/L)	9.58 ± 0.37	17.54 ± 0.26*	18.68 ± 0.56*	10.39 ± 0.84	31.96 ± 1.06†
Cortisol (nmol/L)	6.83 ± 0.21	6.88 ± 0.23	7.41 ± 0.32	8.12 ± 0.30	6.54 ± 0.25
Insulin (pmol/L)	43.8 ± 1.96	39.56 ± 2.03	79.74 ± 2.27	68.25 ± 3.57*	50.71 ± 1.99

*p<0.05, †p<0.01

Table 2 - Blood levels of testosterone, cortisol, insulin and thyroxin following chronic treatment with Naja Haje Arabicus and Cerastes cerastes snake venoms.

Hormonal findings	**Naja Haje Arabicus**			**Cerastes cerastes**		
	At 24 hours	**At 48 hours**	**At 72 hours**	**At 24 hours**	**At 48 hours**	**At 72 hours**
Testosterone (nmol/L)						
Control animals (N=8)	6.81 ± 0.59	8.01 ± 0.79	6.97 ± 0.71	6.14 ± 0.83	6.79 ± 0.42	7.39 ± 0.96
Treated animals (N=6)	2.91 ± 0.51*	0.68 ± 0.11†	0.56 ± 0.21†	11.3 ± 1.01*	6.52 ± 0.81	2.60 ± 0.52*
Cortisol (nmol/L)						
Control animals (N=8)	6.82 ± 0.85	5.87 ± 0.75	6.01 ± 0.85	6.89 ± 0.88	6.91 ± 0.72	5.69 ± 0.99
Treated animals (N=6)	8.95 ± 0.73	11.83 ± 1.03*	10.79 ± 0.93*	7.91 ± 0.85	12.03 ± 1.23*	10.52 ± 0.68*
Insulin (pmol/L)						
Control animals (N=8)	41.71 ± 2.33	37.92 ± 299	38.75 ± 2.51	36.17 ± 3.22	25.29 ± 2.91	40.11 ± 2.65
Treated animals (N=6)	38.76 ± 2.79	26.3 ± 2.04*	34.78 ± 2.88	33.79 ± 2.53	32.98 ± 2.45	23.78 ± 1.33*
Thyroxin (nmol/L)						
Control animals (N=8)	69.52 ± 3.76	67.31 ± 3.01	66.21 ± 3.01	60.93 ± 3.61	58.11 ± 3.29	61.79 ± 4.69
Treated animals (N=6)	62.92 ± 3.64	43.79 ± 2.51*	42.82 ± 1.71	63.82 ± 1.99	66.29 ± 2.58	132.19 ± 8.80†

*p<0.05, †p<0.01

assays. The antisera were highly specific for the hormones with less than 5-8% cross-reactivity with the naturally occurring hormones. Accuracy and precision of the assays were determined within assays using quality control measurements and the procedural losses of the assay were measured by percent recovery. The inter-assay and intra-assay coefficient of variation were very low for the hormones tested (DPC, California, USA). The mean and standard error of the mean were calculated. The results were analyzed according to Mann-Whitney U-tests[21] using SPSS to test the significant differences between the treated groups and the control. Figures were drawn using Sigma Plot (scientific graphing software, version 2.01).

Results. ***a) Hormonal levels following acute treatment.*** Results from the first experiment were shown in **Table 1,** which showed a significant rise in the levels of testosterone concentration in the peripheral blood of the treated rats with WA, CC and EC venoms. However, the level of testosterone in animals treated with NH did not vary greatly from that of the control. The levels of cortisol hormone, on the other hand, were not significantly different in the peripheral blood between the control and the treated rats with the snake venoms; whereas a significant rise in insulin level was obtained in the peripheral blood for rats injected with NH venom but the effects for WA, CC and EC poisons did not vary greatly from that of the controls (**Table 1**).

b) Hormonal levels following chronic treatment. In contrast with the results observed in the above experiment, a highly significant decrease in testosterone concentration in the peripheral blood of the treated rats was observed at 24, 48 and 72 hours following NH injection. Whereas with CC treatment there was a significant rise in testosterone levels at 24 hours followed by an initial decrease at 48 and 72 hours after treatment (**Table 2**). Similarly in comparison with the acute treatment, a gradual rise in cortisol levels were observed in the blood of the treated rats in this experiment at 24 hours following injection with NH and CC venoms (**Table 2**). However, the levels of cortisol hormone tended to increase significantly at 48 and 72 hours following treatment with the NH and CC compared with that of the controls.

At 24 hours following NH and CC treatment, the levels of insulin showed no significant differences between the controls and treated rats but significantly dropped at 48 hours following NH treatment and 72 hours following CC treatment (**Table 2**). The thyroxin level showed a significant decrease at 48 and 72 hours following NH treatment and a gradual rise at 48 hours, which was significant at 72 hours following treatment with CC venom (**Table 2**).

Discussion. The rise in testosterone concentration in the peripheral blood of rats observed following the acute treatment with snake venoms could be explained by the acute effects of the toxins on the vascular permeability in the interstitial tissue of the rats testis. This was associated with an earlier rise in testicular blood flow, which was also potentiated by the release of the pharmacologically active substance in response to the toxins. Consequently, the blood testis barrier might also be affected. Thus, the resultant increase in testicular blood flow would tend to increase the level of testosterone concentration in the peripheral blood of the treated rats. This is in close agreement with earlier reports in the literature using toxins of cadium salts,[22] irradiation[23] or heating the testis.[24]

No such data have been reported using snake venom treatment in male rats. However, Waites and Setchell[22] studied the toxicity of cadmium salts to the testis, obtained increased blood flow and increased permeability of the blood-testis barrier.

In contrast to the above findings the chronic treatment of rats produced a significant decrease in the level of testosterone concentration in the peripheral blood. This could be explained by the continual increase in vascular permeability of the testis and the degenerative changes that would occur in testicular tissues in response to the prolonged effect of the toxins that would consequently result in a significant drop in testicular blood flow and testosterone concentration.[24]

Reduction in testis weights and testosterone concentration were reported following chronic treatment with Aflatoxins fed to male chicken,[25] while testicular atrophy with morphological and histological changes were also observed in chicken fed with triorthocresyl phosphate neurotoxin.[26] Several reports of chronic pituitary failures that resulted in low serum testosterone concentration were documented in human subjects following bites by Russel's Vipers in Burma 2 weeks after envenomation.[27-30] Moreover, the inhibition of human chorionic gonadotropin-stimulated steroidogenesis in cultured Leydig tumor cells was reported[31] using biphenyl-A and octylphenols as environmental chemicals.

In case of CC venom, the earlier rise in testosterone concentration coincides favorably with the results obtained with the acute treatment. It demonstrated clearly that the earlier toxic effect of the venom on testicular tissue was an increase in vascular permeability and the permeability of the blood-testis barrier. This consequently resulted in an increase for testicular blood flow and testosterone concentration in the peripheral blood. However, with continual injection of the venom to produce chronic effects, the testicular blood flow was gradually decreased resulting in a significant drop in testosterone concentration, which was mainly due to a sustained increase in vascular permeability of the testis and an earlier degeneration of testicular tissue. Many factors were known to affect testicular blood flow[32-34] and a significant correlation was obtained between the testicular blood flow and testosterone concentration.[35-38] Positive correlation of testicular blood flow and testosterone concentration in the peripheral blood was obtained by infusion of prostaglandin in rats,[39] in rabbits[40] by irradiation of testis of rats[23] and by a short exposure of the testis to high temperature.[41] On the other hand, we have no definite explanation for the normal level of cortisol in blood of acutely treated rats. It was most probably due to the short exposure of the rats to the venoms, which was not enough to trigger the anti-inflammatory effects for cortisol and its response to withstand stress. In contrast to the above finding, the chronic effects of the venoms showed a gradual rise of cortisol, which demonstrated clearly the noxious effects of the snake venom and the stress imposed on the animals. This is in agreement with that of the reported literature[42-45] and that human and animals subjected to severe stress and noxious agents showed an increase release of glucocorticoids mainly cortisol to resist the effects of imposed stress.

In recent studies, the effects of neurotoxin fraction from NH venom have been shown to increase the plasma levels of cortisol, insulin and cause hyperglycemia, which were observed to be associated with a depletion of glycogen content of

the liver and kidney.[18] It was concluded that the neurotoxin stimulated a release of glucocorticoids that had modulated both insulin and glucose turn-over to maintain the hyperglycemia during the stress period. It was also reported that using the neurotoxic fraction of snake venom from Crotalus durissus in different in vivo and in vitro animal models stimulated the hypothalamo-pituitary-adrenal axis with a rise in adrenocorticotropic hormone, glucocorticoids and glucose levels inducing symptoms common to those of inflammatory stress.[19] Glucocorticoids have a powerful anti-inflammatory action and vital role in metabolism, cardiovascular function, skeletal muscle, central nervous system, lymphoid and connective tissue.[42-45] A rise of ACTH precedes glucocorticoids increase and the stressful stimuli that increase ACTH and cortisol release also activate the sympathoadrenal medullary system to maintain vascular reactivity to catecholamines in an attempt to control the cardiovascular system and the hemodynamics of the blood, which is the major causes in the lethality of the snake venoms.[44,46] More recently the effects of snake venoms of different families on the adrenomedullary cells were investigated.[17] It was found that all snake venoms induced marked catecholamine efflux, which was mainly due to cytolysis.

Besides, the potentiation of the vasoconstrictor effects of catecholamine, glucocorticoids also augment the action of other hormones such as glucagon, growth hormone and exert anti-insulin effect on peripheral tissues.[44,47-48] On the other hand, although the acute treatment showed normal or slightly elevated levels of insulin, the chronic effects produced a significant decrease in insulin level in peripheral blood of the treated animals after a prolonged exposure. It was most probably that the acute effects produced a stimulatory effect on the ß-cells of the pancreas due to the short exposure, accompanied with an interaction with other hormones affecting the carbohydrate and lipid metabolism mainly cortisol, glucagon, catecholamines, growth hormones and thyroxin.

The chronic treatment demonstrated clearly an insulin insufficiency suggesting the toxic effect of the poison and probably the destruction of ß-cells in the islets of Langerhans. Alloxan and other chemicals were known to destroy the B-cells.[47-48] Moreover, the anti-insulin effect produced by the higher level of cortisol in this study and the interaction between cortisol hormone and glucagon, growth hormone and catecholamines as well as the effects of cortisol on the intermediary metabolism for glycogenesis, gluconeogenesis, lipolysis and proteins catabolism should not be ignored.

Finally, the decreased thyroxin levels observed during the chronic treatment with the toxins, could be explained by the toxic effects of the NH and CC venoms on the rich blood supply of the thyroid follicles. These effects would eventually result in increased vascular permeability of the thyroid tissue leading to decreased blood flow and a drop in thyroxin level in the peripheral blood of the treated rats. Reports of decreased serum thyroxin level in human subjects several days following snake-bites were reported.[27-30] No explanation could be offered for the rise in thyroxin level during the last day following CC treatment; however, the levels were initially decreased before starting to rise remarkably on the third day of treatment. The disturbances in metabolism discussed for cortisol, insulin and probably growth hormone, glucagon and catecholamines and the interaction with thyroxin, as major metabolic hormones should be considered.

In conclusion, testosterone and insulin concentration were raised following acute treatment of snake venoms while the concentration levels of both hormones plus thyroxin were decreased following the chronic treatment. On contrast, the concentration of cortisol levels was not different from that of the control following acute treatment of the snake venoms, but showed a significant rise following chronic treatment.

The rise of hormones following acute treatment was thought to be due to the increased vascular permeability and increased blood flow while the decreased level of hormones after chronic treatment might be due to the degeneration of secretory tissues. The significant rise in cortisol levels could be visualized as a response to stress imposed by the treatment with toxic venoms. Our future plan is to study the short and long-term effects of snake venoms on the histology of different endocrine glands.

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