# PHARMACOLOGICAL CHARACTERIZATION OF RAT PAW EDEMA INDUCED BY *Naja haje arabica* VENOM

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**ABSTRACT:** This investigation was performed in order to assess the inflammatory response induced by *Naja haje arabica* venom (NhaV) in rat hind paw. The inflammatory response was estimated by measuring the edema with a Plethysmometer. The venom (0.625-10µg/paw) produced a dose and time-dependent increase in non-hemorrhagic paw edema. The response to NhaV was maximal within 15 min and disappeared in 24 h. Five µg/paw of NhaV was chosen to test the effect of various drugs on the edema induced by this venom. Quinacrine (QNC), a phospholipase $A_2$ ($PLA_2$) inhibitor, and dipyridamole (DPM), an adenosine transport inhibitor, attenuated venom-induced edema in rat paw (P<0.001). Commercially available antivenom was ineffective when administered intravenously, whereas its local administration with NhaV attenuated the edema formation (P<0.001). In conclusion, NhaV-induced edema in rat paw involves $PLA_2$ and adenosine mechanisms. Additionally, the use of polyspecific antivenom, intravenously, was ineffective in preventing NhaV-induced edema.

**KEY WORDS***: rat paw edema, *Naja haje arabica* venom, inflammatory mediators, inhibitor, antivenom.

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## INTRODUCTION

Snakebites are significant medical problems in many parts of the major continents, especially Africa and Asia. In West Africa, the annual bite incidence is 40-120 bites per 100,000 people (50). Cobra is the common name for some members of the Elapidae family; known for their intimidating looks and deadly bites. Updated information about cobras may be found at the professional website Cobra Information Site (http://www.cobras.org/).

Many venomous snakes of medical importance inhabit the Kingdom of Saudi Arabia and surrounding areas, where several of the most lethal snakes are found (24, 25). *Naja haje arabica* (the Arabian cobra) and *Walterinnesia aegyptia* (the Egyptian black cobra; the desert cobra) are representatives of the Elapidae family. Researchers have reported their habits, habitats, distribution (8-10, 24, 25), clinical manifestations caused by their bites, and their venom characterization (2, 3, 20, 34, 39, 52, 63, 66, 70-72). Highly complex pathophysiological features of local and systemic nature characterize the cases of snake envenoming (70). Local manifestations usually include edema, pain, hemorrhage, and necrosis (6, 35-37, 47, 55, 70, 71). Systemic complications are characterized by hypofibrinogenemia, thrombocytopenia, and a decline in the coagulation factors V and VIII:C (40, 41, 57, 74). Edema is a common feature of the cutaneous inflammatory response and is dependent on a synergism between the mediators of vascular permeability and the blood flow (17, 75, 77, 78). An important consequence of altered capillary permeability in local inflammation is the extravasation of leucocytes (29). The degree of accumulation of these cells at inflammatory sites in the skin is related to local blood flow (18, 31, 32).

Several researchers have studied the biochemical and pharmacological effects of venoms from different species of snakes from different localities, but the inflammatory effect of the Arabian cobra *(Naja haje arabica)* venom has not been studied so far. This work shows dose-related and time-course inflammatory effects of NhaV by using the rat paw edema model. The effects of quinacrine (QNC), dipyridamole (DPM), and commercial antivenom were examined. The objectives of this study were to examine the ability of NhaV in inducing paw edema in rats, and to identify the underlying pharmacological mechanisms of this effect.

## MATERIAL AND METHODS

### Venom and antivenom

*Naja haje arabica* venom was obtained from the National Serpentarium, Riyadh, Saudi Arabia. Professional hunters collected the snakes from the wild throughout the Kingdom Saudi Arabia. The animals were kept in the serpentarium facility at the previously mentioned place. A specialized team of the Venomology Unit was responsible for scientific classification, milking of specimens, lyophilization and storage of the venom.

The venom was dissolved in saline (final concentration: 10mg/ml) and immediately stored at -20 °C until used. The antivenom utilized in this study was obtained from Al-Hayatt Company, Riyadh, Saudi Arabia. It was produced in hyperimmunized horses by using a mixture of the venoms from *Echis carinatus, Echis coloratus, Bitis arietans, Cerastes cerastes, Naja haje,* and *Walterinnesia aegyptia*. The antivenom was dialyzed to remove the preservatives.

### Drugs and solutions

Dipyridamole (DPM) was obtained from Sigma, USA, and quinacrine (QNC) was obtained from I.C.N. Biochemicals, Aurora, Ohio, USA.

### Animals

Male Wistar rats weighing 145-155g (mean = 150g) were used in all the experiments. These animals were provided by the Armed Forces Hospital, Research Center (Animal House Services). All experiments were carried out according to the methods described by Faria *et al.* (22) and modified by Al-Asmari (4). Animals were injected subcutaneously in their subplantar region of the right hind paw, with 0.1 ml NhaV (0.625-10µg/paw). The left paws were used as control, and received the same volume (0.1ml) of sterile saline. Edema was measured after 0.25, 0.5, 2, 4, 6, and 24 h by using a Plethysmometer (Model 7150, Ugo Basile, Italy). The results were expressed as mean differences between the final and initial volumes of the injected paws. We could not inject a dose higher than 10µg/paw into the rats because it was lethal.

### Influence of three substances on the NhaV-induced edema

Three groups of rats (n = 6) were pretreated with three different classes of substances: 1) dipyridamole (40mg/kg, 50mg/kg, 60mg/kg and 70mg/kg), was administered intraperitonially (i.p.), 30 min before the injection of NhaV (5µg/paw). 2) quinacrine (40mg/kg, 50mg/kg, 60mg/kg and 70mg/kg), was administered i.p., 30 min

before the injection of NhaV (5µg/paw). The venom dose of 5µg/paw was equivalent to 0.033mg/kg body weight of the animals. 3) the commercial antivenom was locally injected (2, 4, 6 and 8mg/kg), together with the same dose of venom, after 30 min incubation at 37°C.

In a separate group of experiments, the commercial antivenom was intravenously administered (up to 6mg/kg) immediately before the subplantar injection of NhaV.

### *Statistical analysis*

Data were presented as the mean ± SEM, evaluated by analysis of variance (ANOVA) and followed by a Bonferroni test (SPSS Program). A p-value of less than 0.05 was considered significant.


## RESULTS

### *Effects of NhaV on rat paw edema*

Five µg/paw of NhaV caused significant increase (n = 6; $p<0.001$) in paw edema, compared to the saline-injected controls.

Subplantar injection of NhaV (0.625-10µg/paw) produced a dose and time-dependent non-hemorrhagic edema (Figure 1A). The maximal response was observed 15 min after the venom injection, gradually decreasing in 24 h (Figure 1B). For additional experiments, 5µg/paw of NhaV was used.

### *Modulation of venom-induced edema with PLA$_2$ and adenosine transport inhibitors*

Higher doses of QNC (60 and 70mg/kg) significantly attenuated the venom-induced edema ($p<0.001$). At 40mg/kg, the effect on edema was insignificant (Figure 2). Treating the animals with DPM (60 and 70mg/kg) also caused a significant ($p<0.001$) reduction in the venom-induced edema (Figure 3).

Local injection of antivenom (6 and 8 mg/kg) caused a significant ($p<0.001$) reduction in the NhaV-induced paw edema in a dose-dependent manner. Two mg/kg failed to reduce the edema (Figure 4). The commercial antivenom (up to 6mg/kg), intravenously administered, failed to modify the edema induced by injecting NhaV into the paws of the animals.
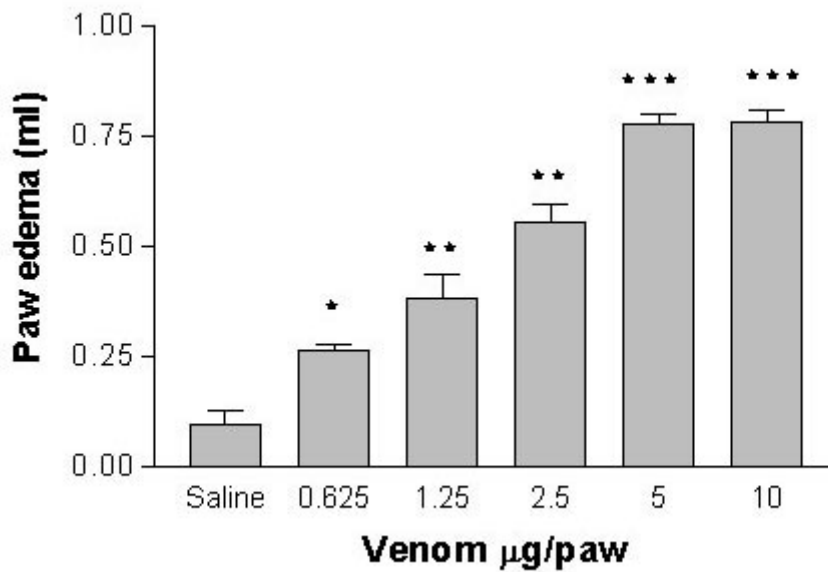
Figure 1A**:** Intensity of the rat paw edema induced by NhaV varying according to different doses (0.625–10µg/paw) of venom. The control group received saline (0.1ml) in the same experimental conditions. Each column represents the mean ± S.E.M. of six rats.

\* $p < 0.05$ (weakly significant); \*\* $p < 0.01$ (significant); and \*\*\* $p < 0.001$ (very significant); (ANOVA and Bonferroni posttest).
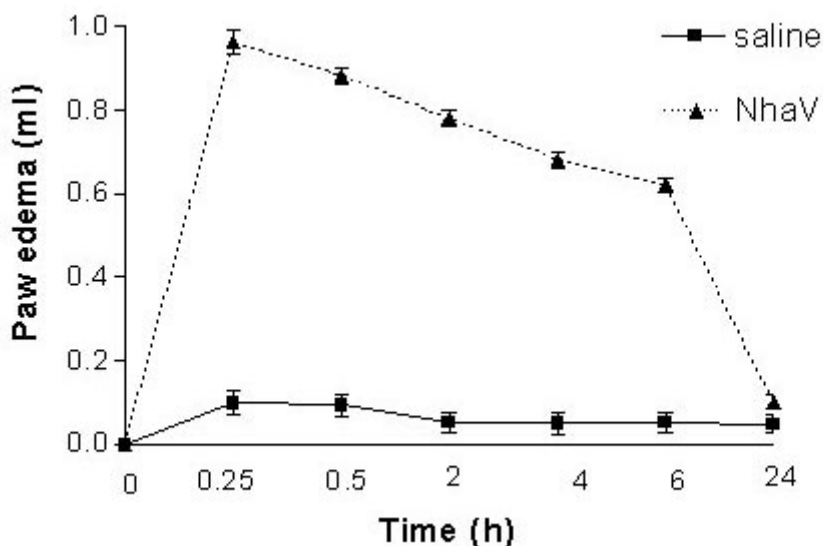


Figure 1B: Time-course of the rat paw edema induced by NhaV (15min to 24h) using 5µg/paw of venom (▲), compared to saline (■). The control group received saline (0.1ml) in the same experimental conditions.
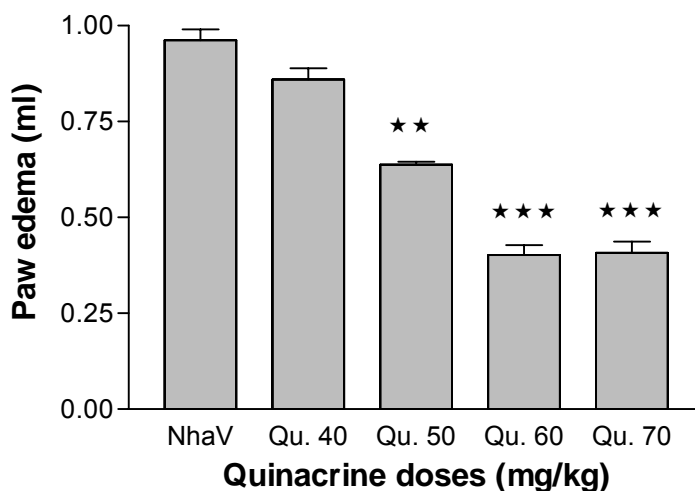
Figure 2: Effect of quinacrine (40, 50, 60, 70mg/kg, i.p. administered 30 min before the venom injection) on the rat paw edema induced by NhaV (5µg/paw). The control group received NhaV alone (5µg/paw), instead of drugs, in the same experimental conditions. Each column represents the mean ± S.E.M. of six rats.

* $p < 0.05$ (weakly significant); ** $p < 0.01$ (significant); and *** $p < 0.001$ (very significant); (ANOVA and Bonferroni posttest).
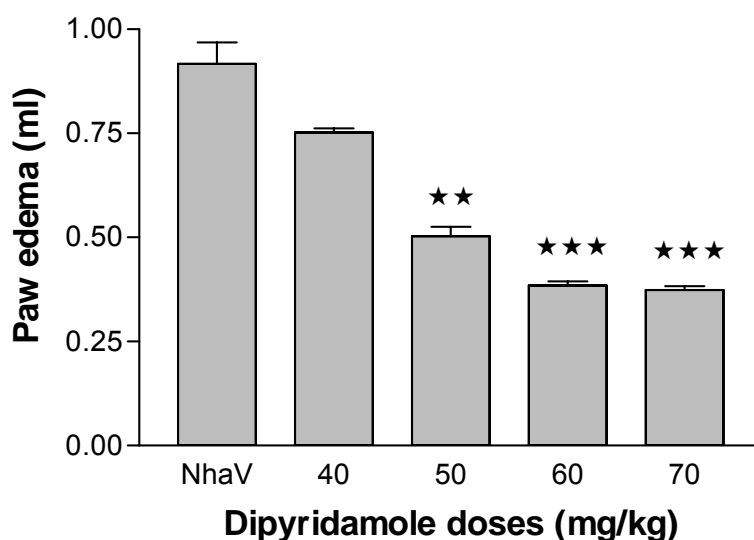


Figure 3: Effect of dipyridamole (40, 50, 60, 80mg/kg, i.p. administered 30 min before the venom injection) on the rat paw edema induced by NhaV (5µg/paw). The control group received NhaV alone (5µg/paw), instead of drugs, in the same experimental conditions. Each column represents the mean ± S.E.M. of six rats.

* $p < 0.05$ (weakly significant); ** $p < 0.01$ (significant); and *** $p < 0.001$ (very significant); (ANOVA and Bonferroni posttest).
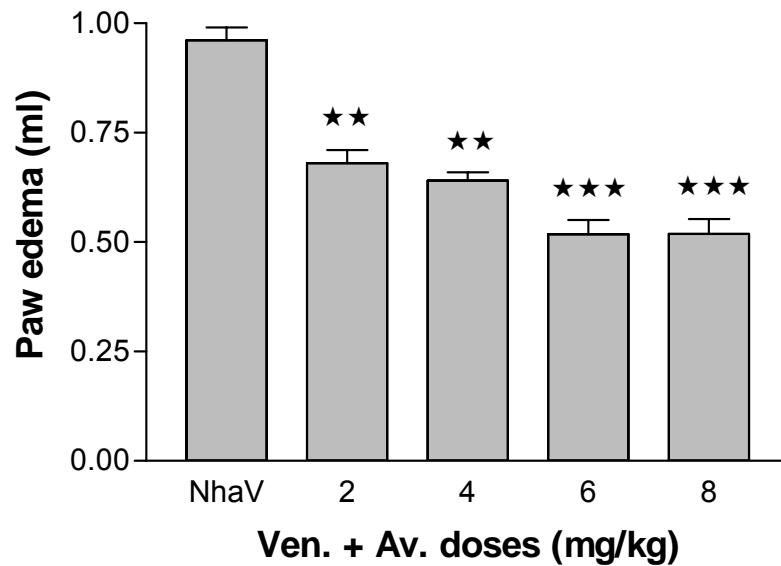
Figure 4: Edematous effects on rat paw by coupling and incubating the antivenom (0.4, 0.8 and 1.6mg/kg) with NhaV (5μg/paw) for 30 min The control group received NhaV alone (5μg/paw), instead of drugs, in the same experimental conditions. Each column represents the mean ± S.E.M. of six rats.

* $p<0.05$ (weakly significant); ** $p<0.01$ (significant); and *** $p<0.001$ (very significant); (ANOVA and Bonferroni posttest).

## DISCUSSION

Previous studies on cobra (Elapidae family) venoms were mainly directed to their neurocardiotoxic effects on experimental animals, due to the nature of the major components present in these venoms. Different toxic effects produced by cobra venoms were due to their contents of neurotoxins, cardiotoxins, activated complement factors, and enzyme toxins (64). Common initial envenoming signs were reported as hypoglycemia (1), general metabolic disturbances (44), muscular dystrophy (49), nephrotoxicity (30), and induction of cytotoxicity (15). Although there are some studies (16, 27) on the venoms local effects, such as myonecrosis and hemorrhage (mainly caused by viper venoms), none of them investigated the edematogenic effect of NhaV, which is well-known only for other Elapids.

The present study shows that subplantar injection of NhaV can cause a significant paw edema in rats. Envenoming also produced edema and altered vascular permeability in mouse hind paw (42, 69). Following these responses, there was an abundant leukocyte infiltration and consequent hemorrhage due to the high doses of

venom (42). Since hemorrhage appeared right after the venom injection and interfered in the development of the inflammatory edema (13, 22, 45), most of the previous works were performed with pre-heated viper venoms. The components of cobra venom differ from those of viper venom, usually in the main causative factors like $PLA_2$.

Further works on the NhaV enzymatic (phospholipase $A_2$ [$PLA_2$, E.C.3.1.1.4], hyaluronidase [E.C.4.2.2.1], L-amino-acid-oxidase [LAAO, E.C.1.4.3.2], phosphodiesterase [PDE, E.C.3.1.4.1], and other proteolytic enymes) and biological activity (lethality, neuro and myotoxicity) were tested and speculated by Al-Asmari (2). Increased vascular permeability and blood flow play an important role in the edema formation (17, 75, 78). The capacity of potentiating inflammatory edema in response to different inflammatory mediators is well-known for some exogenously applied vasodilators, such as calcitonin gene-related peptide (CGRP), prostaglandin $E_2$, and prostacyclin (76). Inverse comparisons, based on the use of edema inhibitors, showed that the drugs tested in this work could reverse the edema induced by NhaV. This venom, alone, induced rat paw edema without causing hemorrhage, since it is free of metalloproteinase activity. Furthermore, NhaV had no proteolytic activity when L-BAPNA and DMCasein were used (2). This can refer to a direct or indirect relationship between the local blood flow and the intensity of the edema, compared to other workers (12, 22, 42).

Dipyridamole is a potent inhibitor of platelet aggregation. It suppresses adenosine cellular uptake into erythrocytes, endothelial cells, and platelets, increasing the levels of circulating adenosine and intraplatelet cAMP (19, 48, 79). It also inhibits PDE, an enzyme responsible for the breakdown of cGMP, which has anti-aggregating effect. The edema reduction might be due to DPM effect on PDE and other mediators responsible for inducing inflammation.

Quinacrine is a direct inhibitor of $PLA_2$ action (7, 23, 65). It has been shown to act as a noncompetitive inhibitor of the nicotinic acetylcholine receptor (nAChR), though this mechanism is still controversial (60). Phospholipase $A_2$ leads to an increase in capillary permeability and to myonecrosis by the disruption of lipid membranes (*in vitro* and *in vivo*) that end in hemolysis, as an indirect effect (38, 54). It is also involved in membrane signal transduction, cellular function, and inflammatory responses (7). Lipid membrane hydrolysis by $PLA_2$ results in membrane perturbation, cell degranulation, and stripping of receptors in the cell surface (46, 56). The

enzymatic effect on arachidonic acid provides a number of inflammatory mediators via cyclooxygenase or lipoxygenase action (22). The complement system is activated by the cobra venom factor (CVF) infusion (34) through an alternative pathway. Cobra venom factor and zymosan-activated plasma (ZAP) increased the extravascular accumulation of radiolabeled albumin in rats, mice (61, 68) and rabbits (73). Moreover, the CVF used in some previous studies contained $PLA_2$, which may affect the hemodynamic and permeability responses (58) by the generation of arachidonic acid metabolites (51).

It is important to refer to the other uses of QNC (also known as mepacrine) as antimalarial agent (53), cell membrane stabilizer, scavenger of oxygen-derived free radicals (ODFR) and inhibitor of tumor necrotic factor (TNF) (59, 62), and potent $PLA_2$ inhibitor (20). Though the elapidic venom usually shows low hyaluronidase activity, *Naja haje* venom showed a considerable activity of this enzyme (2). Corticosteroids also acted directly on leukocytes and other types of cells, inhibiting the release of cytokines and other inflammatory mediators (11). Gutierrez *et al.* (28) and Lomonte *et al.* (43) had a similar report on the kinetics and cell composition of the inflammatory infiltrate observed in rat footpad, where many proteases such as kallikrein (which produces bradykinin and kallidin), are found as active factors (21, 26). These kinins are potent vasodilators that increase capillary permeability and are easily produced in the tissues after injury, being the most important agents in edema formation (26,33). The primary difference between our findings and those of other researchers, like Faria *et al.* (22), was that NhaV induced edema without potentiators. Secondly, some NhaV components decreased the muscle contraction by blocking the nerve conduction postsynaptically.

Finally, we attempted to examine the ability of polyspecific antivenom in neutralizing the edematogenic activity of NhaV. The use of antivenom, intravenoulsly, proved to be ineffective against the rat paw edema induced by NhaV. When a mixture of venom and antivenom was injected into the rat paws, an inhibitory effect was observed. This situation could raise questions about using antivenom in the treatment of victims. Benbassat and Shalev (14) investigated this fact by reviewing reported data on the effect of other snake antivenoms *in vitro*, on laboratory animals, and on humans. These authors also re-examined alternative methods of treatment in order to assess the efficacy of using antivenom. Tilbury *et al.* (67), reporting about a different species, also speculated and reviewed this situation.

In regard to the edema, it could be suggested that the amount of antibodies produced against the edematogenic component(s) of NhaV was very low (highly diluted antiserum) to neutralize them. This finding is in agreement with other works (4, 5, 22). Further studies, attempting to purify the edematogenic component(s) present in this venom, are necessary to elucidate this aspect.

## ACKNOWLEDGEMENTS

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