PHARMACOLOGICAL CHARACTERIZATION OF RAT PAW EDEMA INDUCED BY *Cerastes gasperettii* (*cerastes*) VENOM

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ABSTRACT: Inflammatory response induced by the venom of the Arabian sand viper *Cerastes gasperettii* was studied by measuring rat hind-paw edema. *Cerastes gasperettii* venom (CgV, 3.75-240 µg/paw), heated for 30s at 97°C, caused a marked dose and time-dependent edema in rat paw. Response was maximal 2h after venom administration and ceased within 24h. Heated CgV was routinely used in our experiments at the dose of 120 µg/paw. Among all the drugs and antivenoms tested, cyproheptadine and 5-nitroindazole were the most effective in inhibiting edema formation. Aprotinin, mepyramine, dexamethasone, diclofenac, dipyridamole, N^{ω}-nitro-L-arginine, quinacrine, and nordihydroguaiaretic acid showed statistically ($p<0.001$) significant inhibitory effect, but with variations in their inhibition degree. Equine polyspecific and rabbit monospecific antivenoms significantly ($p<0.001$) reduced edema when locally administered (subplantar) but were ineffective when intravenously injected. We can conclude that the principal inflammatory mediators were serotonin, histamine, adenosine transport factors, phosphodiesterase (PDE), cyclooxygenase, lipoxygenase and phospholipase A_2 (PLA_2), in addition to other prostaglandins and cytokines.

KEY WORDS: inflammatory mediators, *Cerastes gasperettii* venom, edema, antagonist, antivenom.

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INTRODUCTION

The Kingdom of Saudi Arabia and surrounding areas are inhabited by several venomous snakes of medical importance. Among them are the families Elapidae, Hydrophiidae, Viperidae and Atractaspididae, in addition to the innocuous family Colubridae, which includes weakly venomous, back-fanged representatives (19). From the medical point of view, vipers, with their highly sophisticated venom delivery system, are the most important snakes in Arabia since they are responsible for most of the snakebites affecting humans, and the sand viper (*Cerastes cerastes gasperettii*) is the most frequent of all (20).

Vipers are widely distributed throughout Africa and Eurasia, including Senegal in West Africa, North Africa, South Africa, Kenya in the East-Central Africa, and also Turkmenistan, Uzbekistan, Aral Sea, and Sri Lanka in the Indian subcontinent (15, 20, 54). *Cerastes cerastes* mainly inhabits the North Africa (Algeria, Sudan, Chad and Sinai), and also Asia (Palestine, Jordan, Iraq and Iran). It is largely distributed in Arabia, except for areas of more than 1,500m in height (20). According to the records of the Ministry of Health, the incidence of snakebites in Saudi Arabia is approximately 2,494 cases per year.

Following envenoming, spread of the various venom components depends to a large extent on their molecular weights. Thus, Reid and Theakston (44) showed that most constituents of viper venoms have high molecular weights and are slowly absorbed by the lymphatic system to produce maximum serum levels within 6-24 hours. Pathophysiological picture after viper envenomation includes systemic and local effects. Local manifestations are mainly represented by edema, pain, hemorrhage and necrosis, which may result in prolonged or permanent disability (54).

True vipers of the Old World and pit vipers of the New World present venoms rich in proteolytic enzymes. Many coagulant and anticoagulant factors like ancrod, crotalase, batroxobin, and α and β-fibrinogenases have been isolated and purified from their venoms (14, 25, 36, 42, 47, 57). Knowledge on modulators of the coagulation factors of venoms from the *Cerastes* genus is scarce; however, Mohamed *et al.* (40), studying *Cerastes cerastes,* which is homologous to the recently classified species *Cerastes gasperettii* from the Arabian Peninsula, referred to the existence of a factor with a thrombin-like coagulant effect. Later, Mohamed and El-Damarawy (39) showed that the venoms of two species (*Cerastes* and *Vipera*) of the genus *Cerastes* do possess fibrinogenolytic and fibrinolytic activities. Labib *et al.*

(31) found out that some venom fractions of these two species were procoagulant at low concentrations and anticoagulant at high concentrations.

Several researchers have studied the biochemical and pharmacological effects of venoms collected from various vipers (38, 49, 50, 51). However, to the best of our knowledge, the inflammatory effects of the venom from *Cerastes gasperettii*, especially those inhabiting the Arabian Peninsula, have not been investigated so far. The present study reflected the dose and time-related inflammatory effects of CgV using the rat paw edema model and aimed to examine the capability of CgV to induce paw edema in rats and the employment of various drugs that could neutralize it and unveil the pharmacological mechanisms underlying these effects.

MATERIALS AND METHODS

Reagents

Cyproheptadine and dexamethasone were purchased from Merck & Co., Inc. (USA), and aprotinin from Buyer AG (Germany). Mepyramine, diclofenac, dipyridamole, and N^ω-nitro-L-arginine were obtained from Sigma Chemical Co. (USA). Quinacrine was obtained from ICN Biochemicals (USA), 5-Nitroindazole from Aldrich Chemical Company, Inc. (USA), and Nordihydroguaiaretic acid [NDGA] from Fluka Chemie AG (Switzerland).

Venom and antivenom

Cerastes gasperettii venom (CgV) was obtained from the National Serpentarium, Riyadh, Saudi Arabia. It was dissolved in saline and stored at -20°C until used.

Two types of antivenoms [rabbit monospecific IgG and equine polyspecific $F(ab)_2$] were used in this study. Equine antivenom, a national product, was obtained from Al-Hayatt Company, Riyadh, Saudi Arabia. It was raised in horses using a mixture of venoms from *Echis carinatus*, *Echis coloratus*, *Bitis arietans*, *Cerastes cerastes*, *Naja haje*, and *Walterinnesia aegyptia* and was dialyzed to remove preservatives. Monospecific antivenom was raised in rabbits and was produced in our laboratory.

Measurement of rat paw edema

Male Wistar rats weighing 170-190g (mean weight = 180 g) were used for all the experiments and were provided by the Armed Forces Hospital, Research Center (Animal House Services), Riyadh, Saudi Arabia. All experiments were carried out

according to the methods described by Faria *et al.* (14) and Al Asmari (3, 4). Animals were divided into seven groups; each rat was injected into the subplantar region of its right hind paw with serial doses (3.75-240 µg/paw) of 0.1 ml heated (30 s, 97°C) CgV. The contralateral paw received the same volume (0.1 ml) of sterile saline for control. Paw volume was measured with a Plethysmometer (Model 7141, Ugo, Basile, Italy). Results of the dose-dependent edema measurements were expressed as mean differences between the volumes of pre and post injected paws. Such measurements were carried out in order to determine the minimum edematous CgV dose that could produce the highest edema level (plateau). To determine the time the edema took to achieve its peak and consequently subside, measurements were performed at 0.25, 1, 2, 4, 6, and 24 h after venom injection.

Influence of various substances on CgV-induced edema

In another set of experiment, drugs were titrated to determine their minimim (optimum) edematous effect. Groups of six rats each were pretreated with serial doses of a drug or antivenom as follows: Group A – mepyramine (3-24 mg/kg), 15 min before venom; Group B – diclofenac (5-80 mg/kg), 30 min before venom; Group C – cyproheptadine (1.5-24 mg/kg), 15 min before venom; Group D – dexamethasone (0.25-2 mg/kg), 2h before venom; Group E – aprotinin (1×10^3-4×10^3 KIU/kg), 30 min before venom; Group F – dipyridamole (30-120 mg/kg), 30 min before venom; Group G – N^ω-nitro-L-arginine (25-200 mg/kg), 30 min before venom; Group H – quinacrine (25-200 mg/kg), 30 min before venom; Group I – 5-Nitroindazole (20-80 mg/kg), 30 min before venom; Group J – nordihydroguaiaretic acid (NDGA, 20-80 mg/kg), 30 min before venom. All drugs were intraperitoneally injected into rats.

Different doses (0.5-16 mg/kg) of equine (commercial) and rabbit antivenoms (mixed with 120 µg CgV and incubated for 30 min at 37°C) were also intravenously or locally administered to animals.

Then, the animals received subplantar injection of 120 µg/paw CgV (0.49 mg/kg) and an extra positive control group received heated 120 µg/paw (0.49 mg/kg) CgV alone in the same experimental conditions; edema measurements were carried out accordingly.

Statistical analysis

Data were presented as means ± SEM and were analyzed by ANOVA (GraphPad Instat and Prism Programs) followed by the Dunnett's Multiple Comparison Tests. Values of $p<0.05$ were considered significant (99% confidence interval).

RESULTS

Effect of *Cerastes gasperettii* venom on rat paw edema

Heated CgV (3.75-240 µg/paw) induced dose (Figure 1A) and time-dependent (Figure 1B) non-hemorrhagic edema. The minimum edematous CgV dose that induced the highest edema level was 120 µg/paw (Figure 1A). Maximal time-dependent response was observed 2h after venom injection, gradually decreasing from 4 to 6h and subsiding after 24h (Figure 1B). According to these results, the dose of 120 µg/paw was routinely used for all further experiments.

Pharmacological modulation of heated *Cerastes gasperettii* venom on rat paw induced edema

All drugs used were titrated against 120µg/paw heated CgV and the minimum effective dose that induced the maximum edema decrease (% inhibition) was determined (Table 1).

Comparative analysis of drugs and antivenoms inhibitory effect

The minimum effective dose of all the tested drugs and antivenoms was highly ($p<0.001$) significant in inhibiting CgV-induced edema compared with the positive control (Table 1). Drugs were classified into groups according to their percentage of edema inhibition. The most effective group of drugs included cyproheptadine (a histamine H_1 and serotonin 5-HT$_2$ receptor antagonist) with 59.86% edema inhibition, and 5-Nitroindazole (a neuronal nitric oxide synthase [nNOs] inhibitor) with 56.64% (Table 1). The second most effective group was represented by mepyramine (a histamine H_1 receptor antagonist) with 48.25% inhibition, dipyridamole (an adenosine transport inhibitor) with 43.90%, and the two types of antivenoms [rabbit monospecific IgG and the equine polyspecific F(ab)$_2$] with 43.00% and 44.00% edema inhibition, respectively. Diclofenac (a cyclooxygenase inhibitor) with 39.32%, N^{ω}-nitro-L-arginine (a nitric oxide synthase inhibitor) with 38.20%, and quinacrine (a PLA$_2$ inhibitor) with 37.74% edema inhibition represented the third most effective

group. The fourth group included dexamethasone (an indirect PLA_2 inhibitor) with 33.60%, aprotinin (a proteinase [kallikrein] inhibitor) with 30.45%, and NDGA (a cyclooxygenase and lipoxygenase inhibitor) with 27.85% edema inhibition.

Local administration of mixtures of CgV and the antivenoms significantly ($p<0.001$) reduced edema, as reported above (second group), compared with their intravenous administration which failed to cause any significant neutralization.
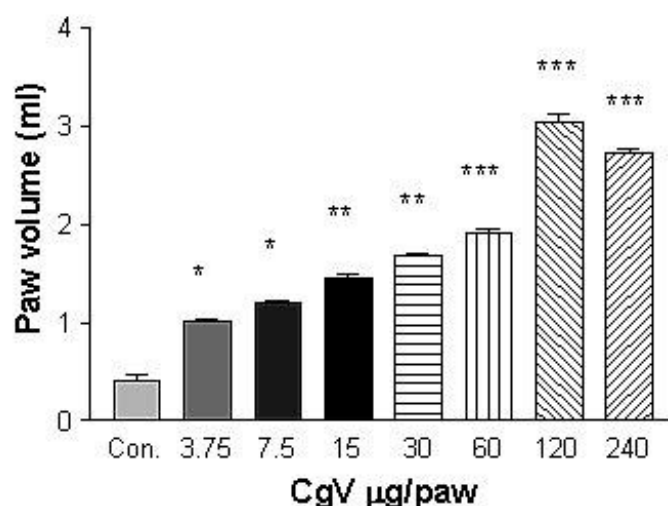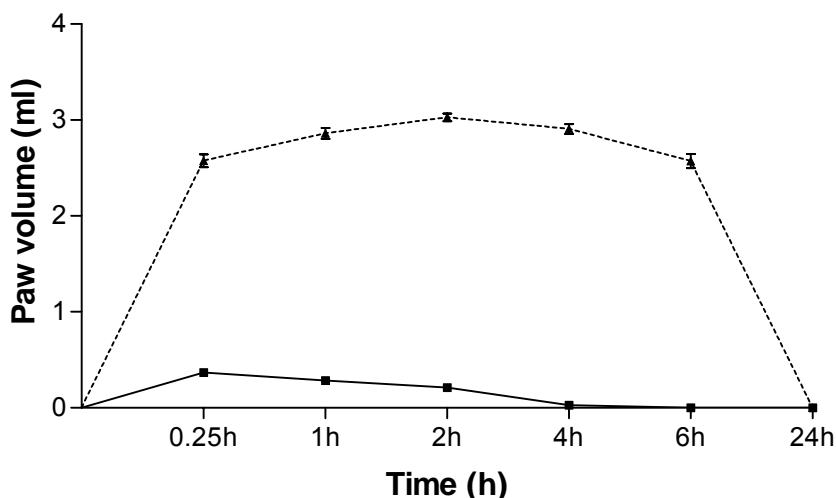

Fig. 1A

Fig. 1B

Figure 1: Edema induced by injecting the right hind paw of rats with heated *Cerastes gasperettii* venom. The contralateral paw was used as control and received 0.1ml saline in the same experimental conditions. Figure 1A shows that the intensity of edema increases with increasing venom doses from 3.75 µg to 240 µg/paw. Each column represents mean ± SEM of six rats. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, compared with saline. Figure 1B shows the time-dependent edema (from 0.25 to 24h) using 120 µg/paw of venom (---▲---), compared with saline (—■—).
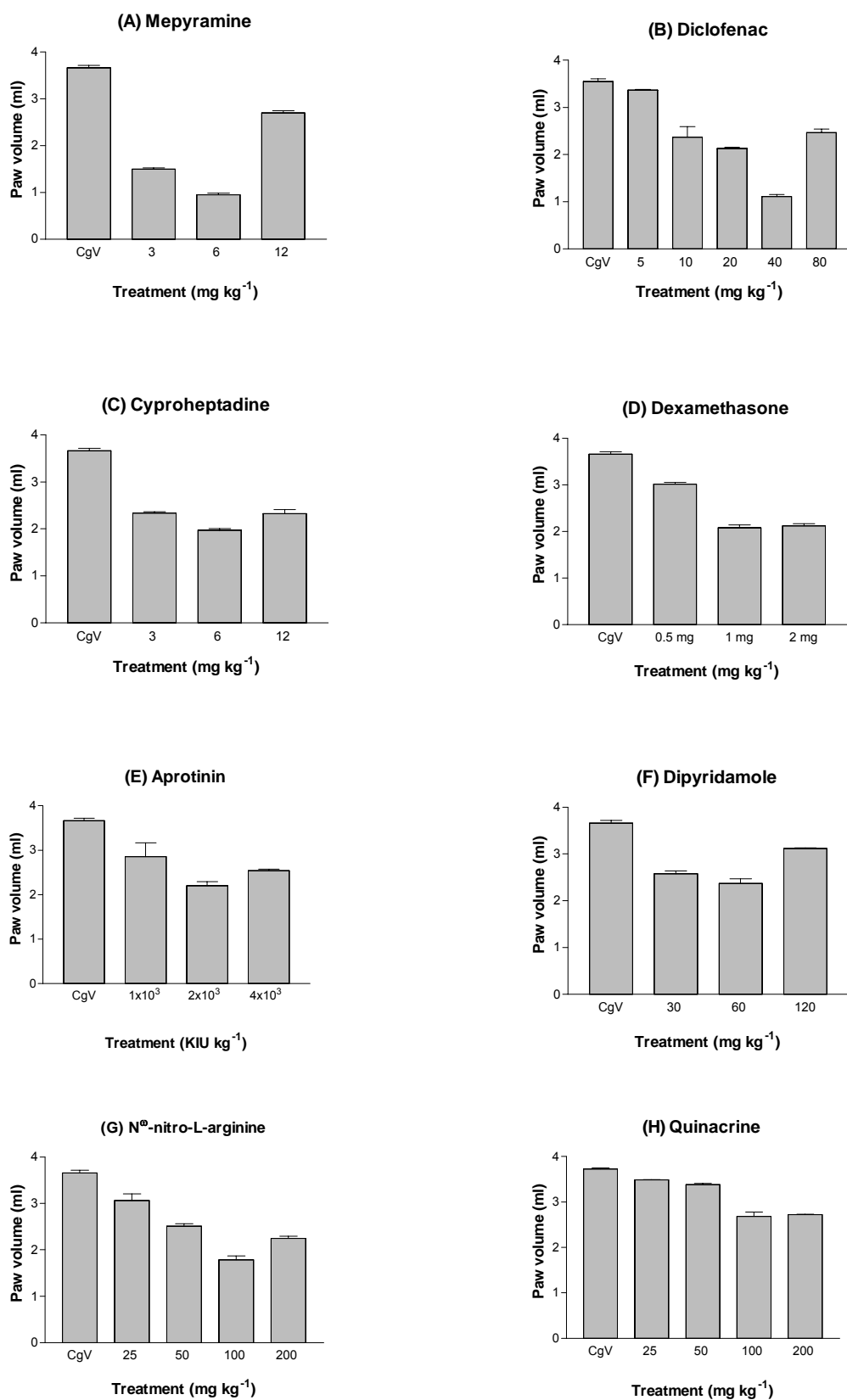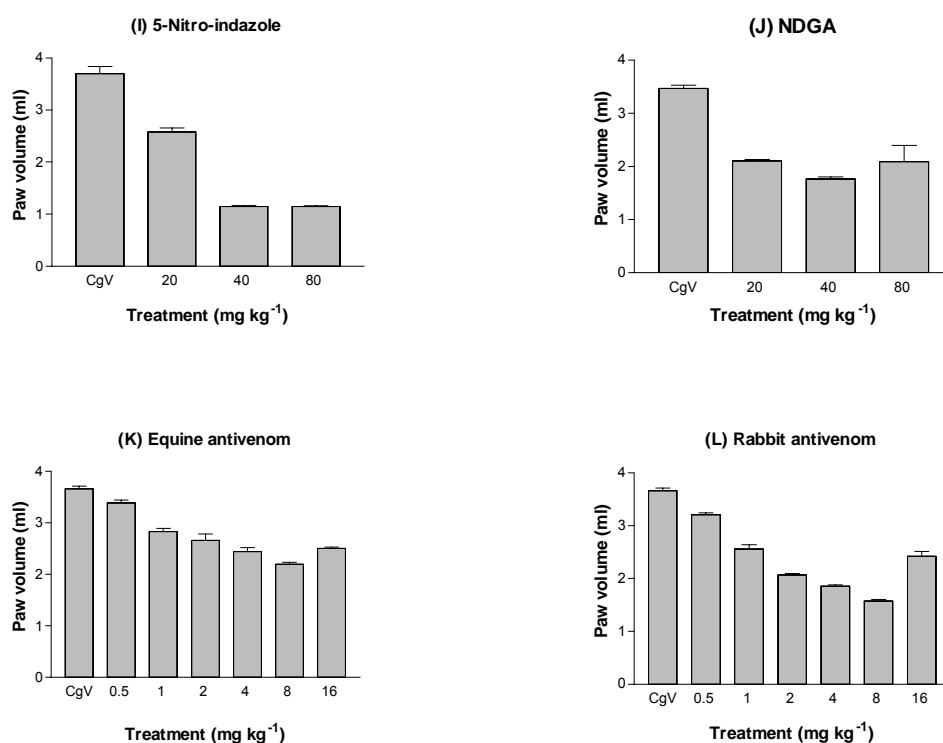
Fig. 2 (A-H)

Fig. 2 (I-L)

Figure 2: Inhibitory effects of several drugs and antivenoms. All of them were titrated against 120 µg/paw (0.49 mg/kg) *Cerastes gasperettii* venom to detect their minimum effective dose. Serial double dilutions of each drug or antivenom were according to the following ranges: (A) mepyramine, 3-24 mg/kg, 15 min before venom; (C) cyproheptadine, 1.5-24 mg/kg, 15 min before venom; (D) dexamethasone, 0.25-2 mg/kg, 2 h before venom; (B) diclofenac, 5-80 mg/kg, 30 min before venom; (E) aprotinin, 1×10^3-4×10^3 KIU/kg, 30 min before venom; (F) dipyridamole, 30-120 mg/kg, 30 min before venom; (G) N^{ω}-nitro-L-arginine, 25-200 mg/kg, 30 min before venom; (H) quinacrine, 25-200 mg/kg, 30 min before venom; (I) 5-Nitroindazole, 20-80 mg/kg, 30 min before venom; (J) nordihydroguaiaretic acid (NDGA), 20-80 mg/kg, 30 min before venom. All drugs were intraperitoneally administered to rats. Equine and rabbit antivenoms (0.5-16 mg/kg) were administered intravenously (results not shown) or locally (mixed with 120 µg CgV and incubated for 30 min at 37°C). A positive control group received heated 120 µg/paw (0.49 mg/kg) CgV alone in the same experimental conditions. Each column represents mean ± SEM of six rats.

Table. 1. Inhibitory effect of drugs and antivenoms on rat paw edema induced by *Cerastes gasperettii* venom (120µg/paw).

Group	Drugs/Antivenoms	Optimum dose (intraperitoneal injection)	Edema inhibition (%)
A	Mepyramine	6 mg/kg	48.25
B	Diclofenac	40 mg/kg	39.32
C	Cyproheptadine	6 mg/kg	59.86
D	Dexamethasone	1 mg/kg	33.60
E	Aprotinin	2×10^3 KIU	30.45
F	Dipyridamole	60 mg/kg	43.90
G	N^{ω}-nitro-L-arginine	100 mg/kg	38.20
H	Quinacrine	100 mg/kg	37.74
I	5-Nitroindazole	40 mg/kg	56.64
J	Nordihydroguaiaretic acid	40 mg/kg	27.85
K	Polyspecific equine antivenom	8 mg/kg	40.00
L	Monospecific rabbit antivenom	8 mg/kg	43.00

DISCUSSION

Snake venom components, especially those of viper venoms, activate, inhibit or liberate enzymes by destroying cellular organelles (1, 37). The different toxic effects of viper venoms are due to their proteolytic and lipolytic enzymes (48). Common initial signs of envenoming are hypoglycemia (2), general metabolic disturbance (35), muscular dystrophy (41), nephrotoxicity (28) and cytotoxicity (10).

The present study shows that subplantar injection of heated CgV in rats caused a significant paw edema similar to that induced by other viper venoms, which altered the vascular permeability in mice hind paws (13, 53), causing abundant leukocyte infiltration, and hemorrhage with higher venom doses (13). Since hemorrhage has been reported to appear soon after venom injection and to interfere with the inflammatory edema development (8, 14), in our study we employed heated venom in all the tests.

Development of edema induced by CgV was different from that observed with other vipers (including *Echis coloratus* and *Echis pyramidum*) venoms; CgV-edema volume achieved its peak in a longer time (3, 4).

Our results agreed with those of Lomonte (33), who reported that in his study edema lasted for more than 12 h.

Edema induced by CgV diminished after 2 hours while that induced by other viper or crotalid venoms like *Bothrops jararaca* venom (BjV) was still significant even after 6 hours (52). Also, mice injected with low doses of BjV (1 µg) presented edemas lasting for more than 8 hours. Similar results were observed in rats (53). Long-acting edema was also reported by Theakston (49), who attributed the slow absorption (maximum level, 6-12h) of viper venoms to their high molecular weights. Therefore, it could be assumed that venoms of different viper species trigger and maintain edema by different mechanisms, depending on their components' molecular weights.

Similarly to what has been observed with BjV (53), CgV-induced edema might be caused by the release of pharmacologically active substances at the injection site.

Increased vascular permeability and increased blood flow have been shown to play an important role in edema formation (11, 55, 56). Our results showed that CgV induced rat paw edema, which might indicate a direct or an indirect relationship between local blood flow and edema intensity, compared with the studies of Faria *et al.* (14), De Araujo *et al.* (13), and Antunes *et al.* (6).

To further understand the pharmacological mechanisms involved in the CgV-induced rat paw edema, we used different treatments.

Although all the tested drugs and antivenoms were statistically significant and effective, there were variations in their efficiency. The drugs cyproheptadine and 5-nitroindazole were the most effective in reducing edema, confirming their important roles in *in vivo* mast cell degranulation (12, 14) and in the increase of cytokine production (27).

Cyproheptadine leads to blockade of serotonin receptors; similarly methysergide was reported to decrease the edematogenic effects of many phlogistic agents such as sephadex (18) and collagenase (46). Ishii *et al.* (29) indicated that edema induced by arachidonic acid was slightly reduced by methysergide in mice. However, it is necessary to recall that edema induced by other crotalid venoms in rats (53) and mice (43) was not reversed by methysergide. The present results indicate that serotonin and histamine had an important role in decreasing CgV-induced edema, since cyproheptadine, which is a histamine (H_1) and serotonin (5-HT) receptor antagonist, significantly reduced rat paw volume. Histamine seems to be an important autocoid in the formation of edema induced by many phlogistic agents. However, it has been reported that histamine might not be involved in edema induced by BjV in rats (53) and mice (43) since the use of antihistamine did not cause any

significant change, which does not agree with the present results since mepyramine, an antagonist of H_1 receptors, significantly reduced CgV-induced edema. Therefore, differences between the edematogenic effects induced by CgV and those caused by other viper and crotalid venoms like BjV and *Echis coloratus* and *Echis pyramidum* venoms (3, 4) could be attributed to the release of other autocoids.

Nitric oxide synthase (NOs) inhibitors have been used as tools to establish the role of nitric oxide (NO) in edema induced by different phlogistic agents (7). Many experimental data demonstrated that NO has an important role in inflammation, however, mechanisms of the NO contribution to such process are not completely elucidated yet. It might participate in the edema pathophysiology by several mechanisms such as increasing prostaglandin biosynthesis at the inflammatory site (45) and cytokine production (27). Nitric oxide has been shown to play a very important role in edema induced by carrageenin in rats (26, 32, 45) and mice (27). In rats, NO was involved in the acute inflammatory response following subplantar injection of bradykinin and serotonin (21). It was also involved in neurogenic inflammation induced by topical application of mustard oil on rat skin (32).

The present results also demonstrated that NO might play a role in reducing CgV-edema, since antagonists (5-Nitroindazole and N^{ω}-nitro-L-arginine) significantly inhibited its formation. Knowledge about histamine and serotonin release due to CgV, in addition to activation of NO synthases, could clarify important steps in the edema formation.

Mepyramine, dipyridamole, rabbit IgG and equine $F(ab)_2$ antivenoms were the second most effective in inhibiting edema, although the antivenoms failed to reverse venom-induced edema when intravenously administered, which may indicate the antivenom local action in countering myonecrosis. *Cerastes gasperettii* venom local effects (e.g. myonecrosis) and edematogenic effects have not been sufficiently studied so far, therefore employing viper antivenoms in the treatment of systemic symptoms could be doubtful. Benbassat and Shalev (9) investigated these situations by reviewing data on the *in vitro* and *in vivo* effects of viper venoms (on laboratory animals and humans) and reexamined alternative treatment methods in order to assess the efficacy of using antivenoms. Tilbury *et al.* (52) also speculated and reviewed this argument.

Mepyramine action is a second confirmation of the histamine release due to CgV. Dipyridamole is a potent inhibitor of platelet aggregation and suppresses adenosine

cellular uptake by erythrocytes. It is also responsible for the inhibition of phosphodiesterase (PDE), which, together with several other effects on other mediators responsible for inflammation induction, may reduce edema (58).

The third most effective drugs in inhibiting edema were diclofenac, N^{ω}-nitro-L-arginine and quinacrine, another NOs inhibitor that confirmed the specificity of CgV in inducing edema. Inhibition of PLA_2 by quinacrine is a significant marker of induction of this mediator. Antagonists of the arachidonic acid metabolism such as cyclooxygenase and corticosteroids were shown to reduce edema induced by toxic proteins isolated from some insects (16, 24). The present results demonstrated the possible involvement of NOs, PLA_2 and arachidonate metabolites in the edema formation, since antagonistic drugs (the third most effective group) also reduced rat paw volume.

The fourth most effective drugs included aprotinin, dexamethasone and NDGA. Significant reduction of venom-induced paw edema by dexamethasone was expected since corticosteroids were known to indirectly inhibit PLA_2 action (17). Furthermore, corticosteroids directly acted on leucocytes and other cell types inhibiting the release of cytokines and other inflammatory mediators (5). Similar actions were observed in the kinetics and cell composition of the inflammatory infiltrate in rat paw (23, 34). Aprotinin inhibits many proteases such as kallikrein, whose products, bradykinin and kallidin, are active inflammatory inducers (16, 22). These kinins are potent vasodilators that increase the capillary permeability and are easily produced in tissues after injury; thus, being cardinal agents in the edema formation (22, 30). The primary difference between the present findings and those of De Faria *et al.* (14) was that CgV induced significant edema without the need of potentiators. Secondly, NDGA and dexamethasone reduced CgV-induced edema, which indicated that these drugs might be involved in blocking or reducing mast cell degranulation and activation by CgV. Furthermore, H_1 and serotonin $5\text{-}HT_2$ receptor antagonists significantly reduced CgV-induced rat paw edema. Contradictory results were obtained with other venoms (13, 43, 53).

In conclusion, CgV induced edema 15 min after venom injection; peak volume was achieved after 2 h, which diminished and ceased within 24 h. There is release of active amines such as serotonin, histamine, arachidonic acid metabolites and NO at the injection site, since antagonistic drugs like cyproheptadine, mepyramine, NDGA, dexamethasone and NOs inhibitors reduced the edematogenic response at various

doses. As cyproheptadine and 5-nitroindazole were the most active inhibitors of paw edema formation, we could suggest that at least serotonin, histamine and nNOs are important mediators and of high priority in edema induced by CgV.

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