

Original Article

## EFFECT OF CROTALUS ATROX VENOM ON PERITONEAL AND SPLEEN CELL AND MEDIATORS PRODUCTION

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### ABSTRACT

**Objective:** Snake venom is a complex mixture of many substances such as toxins, enzymes, growth factors, activators and inhibitors with a wide spectrum of biological activities. The envenomation caused by *Crotalus atrox* venom (*VCat*) in human is characterized by clinical signs and symptoms resulting from excessive systemic host response. It has been proposed that immune injury is the central mechanism of envenomation. In this study, we investigated the effect of *VCat* on peritoneal and spleen cells number, splenic index and mediators production from groups of female CD1 mice.

**Materials:** Quantitative analysis of peritoneal and spleen cells was carried out by determined by hemocytometer. The mediators production such as IL-6 and IL-10 levels were determined by enzyme-like immunosorbent assay, whereas TNF and nitrite levels were detected by standard assay with L929 cells and colorimetric Griess reactive, respectively.

**Results:** The present study showed that mice exposed to *VCat* presented the following: a) significant reduction in peritoneal cells number; b) severe reduction in spleen cells number and pronounced atrophy of spleen volume, c) marked increase in mediators production in the first times after exposition and d) the ratios of TNF/IL-10 showed predominantly an anti-inflammatory response for groups of animals injected with acute doses.

**Conclusion:** In conclusion the peritoneal and spleen cells were found to be severely reduced by *VCat*. The pronounced reduction of the cells number was an important indicator that this venom affects a wide range of immune cell types. The extent of damage to the immune cells appeared to be related to the length of severe envenomation.

**Keywords:** Venom, Macrophages, Spleen index, Mediators.

### INTRODUCTION

Snake venom is a complex mixture of many substances such as toxins, enzymes, growth factors, activators and inhibitors with a wide spectrum of biological activities. The effect of *Crotalus atrox* venom on human is characterized by cardiovascular system, respiratory system, somatic nerve system, and skeletal muscle [1]. The local effects caused by this venom are hemorrhage, myonecrosis, edema, pain and inflammation [2-5]. Inflammation is produced by immune cells within the tissue, releasing specific mediators which control local circulation and cell activities. The immune system is a complex, multicomponent system that comprises lymphoid organs, cells and molecules that interact to provide a coordinate immune response. The organs of the immune system are bone marrow, thymus, lymph nodes and spleen. Macrophages are released from bone marrow migrate to many tissues and undergo to final differentiation into specific type of resident macrophage. They are concentrated in lungs, liver, lymphnodes, spleen and in the serosal membrane of pleural and peritoneal cavity. Macrophage function is to remove cellular debris generated during normal tissue function while following tissue injury or infection. These cells can respond rapidly to different insults and become activated and have many activities such as lymphocyte activation, tissue damage and microbicidal activity through the production of several cytokines, superoxide and nitric oxide. The production of pro-inflammatory cytokines by resident macrophages in turn promotes the recruitment of neutrophils and inflammatory monocytes/macrophages to the inflamed region [6]. Integrated actions among these immune phagocytes help to eliminate pathogens, repair damaged, tissues, initiate an adaptative immune response, and most importantly restore tissue homeostasis [6]. The spleen is an important immunologic filter of the blood. It is made up of B cells, T cells, macrophages, dendritic cells, natural killer cells and red blood cells [7]. From an evolutionary point of view, the function of removing abnormal of foreign material determines

the clinical consequences of hyper- or hyposplenism. Hypersplenism occurs when the size of the spleen is increased by cells or tissue components or by vascular engorgement. This augments its filtering function and even normal blood cells experience a delayed transit and temporary sequestration. Hyposplenism is characterized by splenic function reduced in certain illness or eliminated by splenectomy. It may or may not be associated with a reduction in splenic size. In addition, to capturing foreign materials such as antigens from the blood that passes through of spleen, migratory macrophages and dendritic cells bring antigens to the spleen via the bloodstream. An immune response is initiated when the macrophages or dendritic cells present the antigen to the appropriate B or T cells. In the spleen, B cells become activated and produce large amounts of antibody. Also, old red blood cells are destroyed in the spleen. In the spleen, there are two regions with intense phagocytic activity, the marginal zone, which is responsible for elimination of inert particles such as bacteria and virus [8], and the red pulp which is engaged in removal of senescent and aberrant red blood cells [8,9]. Blood enters the spleen through the splenic artery, which branches and gives rise to arterioles and capillaries.

Lymphocytes circulate among lymphoid organs throughout the body. Upon engagement of surface molecules with structures having complementary conformation, lymphocytes become activated. After activation T helper cells (Th) differentiate and proliferate into Th1 and Th2 effectors cells [10, 11]. While Th1 cells are characterized by the prevalent production of pro-inflammatory cytokines as, interleukin-2 (IL-2), interferon-gamma (IFN- $\gamma$ ) and tumor necrosis factor-alpha (TNF- $\alpha$ ) which can potential the disease [12], the Th2 cells produce anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13, which have been associated with remissions of the disease. Th1/Th2 cytokines are responsible of the cross-regulate the development and function of each subset [13]. It has been reported that abnormalities in Th1/Th2 cytokine balance may account for the pathophysiology of human autoimmune diseases and in

envenomation processes. In the pathogenesis of envenomation has been included the release of mediators such as cytokines and nitric oxide [14]. The interactions between different cytokines and possibly other components of the immune response take place during severe infections. These are accumulating data suggesting that equilibrium between the Th1 and Th2 cytokines responses is important for the final outcome of victims with severe envenomation [15, 16]. Another inflammatory mediator as a nitric oxide, that is important free radical serving as a second messenger in processes that include maintenance of vasodilator tone and arterial pressure and it has been suggested that cytokine-mediated circulatory shock [17]. The main of the present study is to evaluate alterations of *Crotalus atrox* venom administration on peritoneal and spleen cell number and also on the mediators production.

## MATERIAL AND METHODS

### Chemicals, reagents and buffers

Actinomycin D, orthophenyldiamine (OPD), fetal calf serum (FCS), RPMI-1640 medium were purchased from Sigma (St. Louis, MO, USA), murine anti-IL-6 (clones MP5-20F3 and MP5-32C11), recombinant IL-6, murine anti-IL-10 (clones JES5-16E3 and SXC-1), recombinant IL-10 were purchased from BD Biosciences Pharmingen (USA), and recombinant TNF was purchased from Boehringer Mannheim (Mannheim, Germany).

**Venom:** Lyophilized venom of *Crotalus atrox* (*VCat*) was obtained from Dr. R. Zucattelli Mendonca (Instituto Butantann - SP - Brasil), and stored at -20°C. The venom was dissolved in sterile physiological saline [0.85% (w/v) NaCl solution] immediately before use.

**Animals:** Females CD-1 mice (6 - 8 weeks old, weighing 20 to 25 g) were maintained in Bioterio - Facultad de Medicina, UAEM (Cuernavaca, México). The animals were maintained and used under strict ethical conditions according to international recommendations for animal welfare Committee Members, International Society in Toxicology, (1992) [18].

**Lethality assay:** Probits method was used to calculate the lethal dose fifty ( $LD_{50}$ ) of the *VCat* venom. The mice weighing 20 to 25 g were intraperitoneously (i.p.) injected with 0.1 mL of various concentrations of venom and the number of mice those died was counted after 48 h. The number of mice used at each dose was four [19].

**Blood collection:** Groups of female BALB/c mice with 20 - 25 g were injected intra peritoneously (i.p.) with different amounts of *VCat* dissolved in 0.1 mL of saline solution. Control mice received 0.1 mL of saline solution. Different times after injection with *VCat* animals were bled by retro orbital plexo. The blood samples were allowed to stand until they formed a clot and the sera were separated by centrifugation and stored at -20°C until assayed. The sera were used to detect the presence of mediators.

### Cell collection

**Spleen cells:** Groups of mice i.p. injected with different amounts of *VCat*, and after different time the spleens were removed and pressed through a wire mesh, the red blood cells were removed by buffer lysis containing: 155 mM  $NH_4Cl$  (pH 7.4), 10 mM  $NaHCO_3$  and 0.1 mM EDTA. The cells were collected and washed two times by centrifugation at 500 rpm for 5 min. The cells were suspended in RPMI-1640 medium supplemented with 10% FCS and the number of cells was determined by hemocytometer. Cell viability ranged from 90% - 95% and was determined by Trypan blue exclusion [20].

**Spleen index (SI):** Groups of mice with 20 - 25 g were intraperitoneal injected with 0.1, 0.5 and/or 1  $LD_{50}$  of *VCat* and after different times the control or injected animals were weighed sacrificed by cervical dislocations and their spleens were removed and weighed. The splenic index was calculated as follows: [(spleen weight/body weight of treated mice) / (spleen weight/body weight of control mice)] X 100 [21].

**Peritoneal cell:** Groups of female mice were intraperitoneal injected with 0.1, 0.5 and/or 1  $LD_{50}$  of *VCat* and after different times the control and/or exposed to venom were sacrificed by cervical dislocations and their resident peritoneal cells were harvested by

peritoneal lavage [22]. In brief, the peritoneal cavity was injected with 5 mL of cold RPMI-1640. The fluid-distended peritoneal cavity was massaged, and the cells were collected and washed three times by centrifugation at 400 rpm for 5 min. The cells were suspended in RPMI-1640 supplemented with 10% FCS and the number of cells was determined by hemocytometer. Cell viability ranged from 90% - 95% and was determined by Trypan blue exclusion.

### Mediators production

**Cytokines ELISA assay:** The levels of cytokines IL-6 and IL-10 present in serum from BALB/c mice were assayed by a two-site sandwich enzyme-like immunosorbent assay (ELISA) [23]. Briefly, ELISA plates were coated with 100  $\mu L$  (1  $\mu g/mL$ ) of the monoclonal antibodies anti-IL-6 or anti-IL-10 in 0.1 M sodium carbonate buffer (pH 8.2) and incubated for 6 h at room temperature. The wells were then washed with 0.1% phosphate-buffered saline (PBS/Tween-20) and blocked with 100  $\mu L$  of 10% FCS in PBS for 2 h at room temperature. After washing, duplicate sera samples of 50  $\mu L$  were added to each well. After 18 h of incubation at 4°C, the wells were washed and incubated with 100  $\mu L$  (2  $\mu g/mL$ ) of the biotinylated monoclonal antibodies anti-IL-6 or anti-IL-10 as second antibodies for 45 min at room temperature. After a final wash, the reaction was developed by the addition of OPD to each well. Optical densities were measured at 405 nm in a microplate reader. The cytokine content of each sample was read from a standard curve established with the appropriate recombinant cytokines (expressed in nanograms per milliliter). The minimum levels of each cytokine detectable in the conditions of the assays were 10 pg/mL for IL-10 and IL-6.

**Biological assay:** To measure the cytotoxicity of TNF present in sera samples, a standard assay with L929 cells, a fibroblast continuous cell line was used as described previously by Ruff & Gifford (1980) [24]. The percentage cytotoxicity was calculated as follows:  $(A_{control} - A_{sample}/A_{control}) \times 100$ . Titres were calculated as the reciprocal of the dilution of the sample in which 50% of the cells in the monolayers were lysed. TNF activity is expressed as pg/mL estimated from the ratio of a 50% cytotoxic dose of the test to that of standard mouse recombinant TNF.

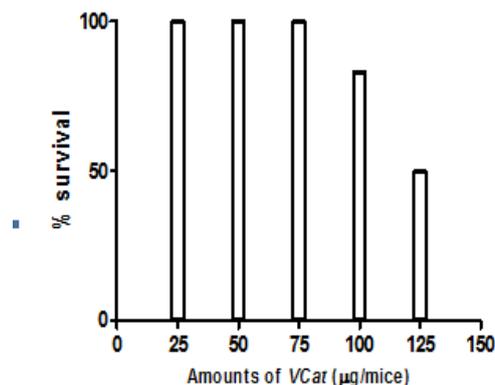


Fig. 1: Effect of *VCat* on mortality is correct the other is repeated

### Nitrite determination

The nitrite levels in female mice sera as an indication of NO production were determined as previously described [25]. Briefly, 40  $\mu L$  of each mice sera sample were incubated in a 96-well, flat-bottomed plate with 40  $\mu L$  of the reduction solution (NADPH, 1.25 ng/mL; FAD 10.4 ng/mL;  $KH_2PO_4$ , 0.125 M) containing 0.5U of  $NO_2$  reductase for 2 h at 37°C. After this time, 80  $\mu L$  of Griess reagent (0.1% naphthylendiamine hydrochloride, 1% sulphonylamide, 3%  $H_3PO_4$ ) were added to each well. The optical densities were measured at 540nm in a microplate reader and nitrite concentrations were determined using a standard curve of  $NaNO_3$  ranging from 1.25 to 275 nM (expressed as mMol/mL).

**Statistical analysis**

The statistical significance of differences between the groups was obtained using analysis of variance (ANOVA) complemented by Dennett's test  $p < 0.05$  and  $p < 0.01$  considered to be significant.

**RESULTS**

**Effect of VCat on mice sensibility**

To determine the sensibility of animals, different amounts of venom were injected i.p. in CD1 female mice and the deaths occurring during 24 hours were recorded and the LD<sub>50</sub> value were calculated. The intraperitoneal LD<sub>50</sub> mean values of venom were 125 µg/100µL (Fig. 1).

**Effect of VCat on peritoneal cells**

In order to determine the effect of VCat on peritoneal cells, groups of animals were injected with different amounts of venom and after distinct times were sacrificed and the after peritoneal cavities lavage the cells were collected. The all amounts of VCat were able to decrease the number of peritoneal cells in the first 2 hours (Fig. 2). For groups of mice exposed to 0.1 LD<sub>50</sub> of VCat by 4 up to 24 hours the number of peritoneal cells returns a similar numbers observed in control group (Fig. 2). In contrast, the number of peritoneal cells from mice injected with 0.5 and/or 1.0 LD<sub>50</sub> of VCat was significantly lower when compared with those obtained in control group (Fig. 2).

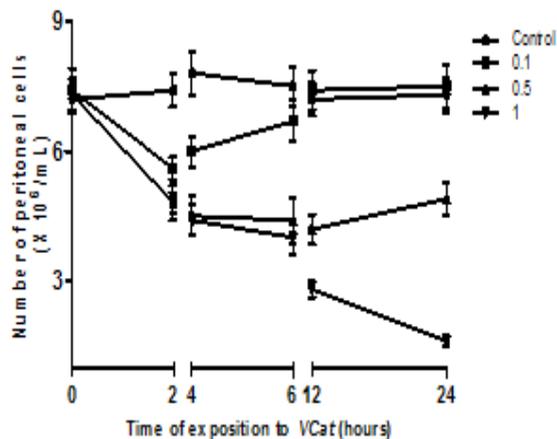


Fig. 2: Number of peritoneal cells.

**Effect of V Cat on spleen cells and splenic index**

To determine the effect of VCat on spleen cells and spleen index, groups of animals were i.p. injected with different amounts of VCat and after distinct times the net weight were measured and sacrifice to remove their spleen and the cells were collected. The number of spleen cells from mice injected with different amounts of VCat decreased in first 2 hours (Fig. 3). The number of spleen cells for groups injected with 0.1 LD<sub>50</sub> returns a similar numbers observed for control groups after 12 up to 24 hours. In contrast, the number of spleen cells from mice injected with 0.5 and 1.0 LD<sub>50</sub> of VCat was significantly lower when compared when the number obtained for in control groups ( $p < 0.01$ ) (Fig. 3). The spleens from mice injected with 0.5 and 1.0 LD<sub>50</sub> of VCat were consistently smaller than the spleens obtained from control group, which was reflected in the lower number of cells obtain from them.

As indicated in Figure 4 the venom caused a significant reduction in spleen index ( $p < 0.01$ ). The spleen index showed a clear correlation the spleen weight. In groups of mice injected with VCat the spleen volume decrease, for groups injected with 0.1 the spleen index decreased in the first 4 hours (Fig. 4). However for the groups of mice injected with 0.5 and/or 1 LD<sub>50</sub> of VCat the spleen index were significantly lower when compared with those obtained for control groups ( $p < 0.01$ ) (Fig. 4).

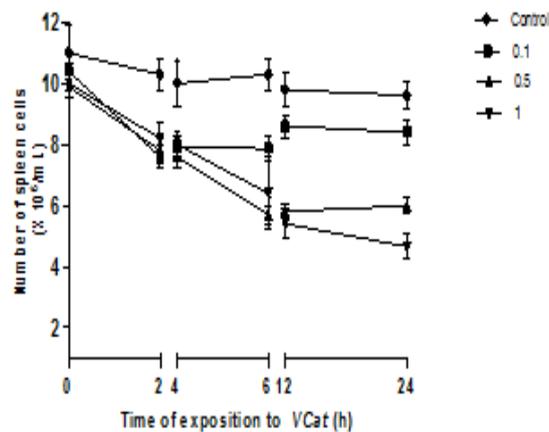


Fig. 3: Number of spleen cells.

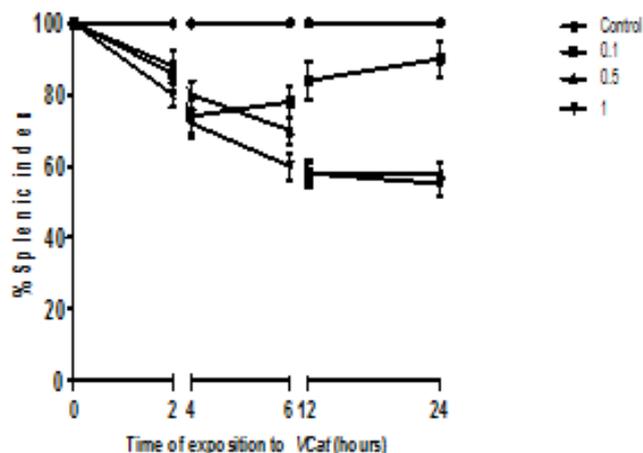


Fig. 4: Splenic index.

**Kinetics of mediators production**

To determine mediators production, groups of female mice CD1 with 20–25 g of body weight were injected intraperitoneously with different amounts of VCat and bled after distinct time intervals and used for to evaluate the levels of cytokines and NO. Mice injected with saline solution had low levels of all mediators assayed in the serum. Figure 5 shows that the different amounts VCat was able to cytokine and NO production. Levels significantly higher of TNF were observed in sera from mice injected with 0.5 and 1.0 LD<sub>50</sub> of VCat for 6 h when compared with those observed in sera from control group ( $p < 0.01$ ) (Fig. 5). The highest levels of IL-6 were observed between 4-6 h for groups treated with 0.5 and 1.0 LD<sub>50</sub> of VCat (Fig. 5). No difference was observed in IL-10 levels between control animals and groups of mice treated with 0.1 LD<sub>50</sub> of VCat. In contrast, the levels were increasing with the time after injection with 0.5 and 1.0 LD<sub>50</sub> of VCat. Levels significantly higher of IL-10 were observed in groups of mice exposed to 0.5 and 1.0 LD<sub>50</sub> of VCat ( $p > 0.01$ ). In these amounts of VCat the peak maximum of IL-10 production was observed at 24 h after injection (Fig. 5). The levels of nitrite in sera from mice treated with different amounts of VCat were increased up to 2 h, decaying thereafter. NO levels were significantly highest in sera from groups of mice injected with 0.5 and 1.0 LD<sub>50</sub> of VCat when compared with those observed in control group ( $p < 0.01$ ) (Fig. 5).

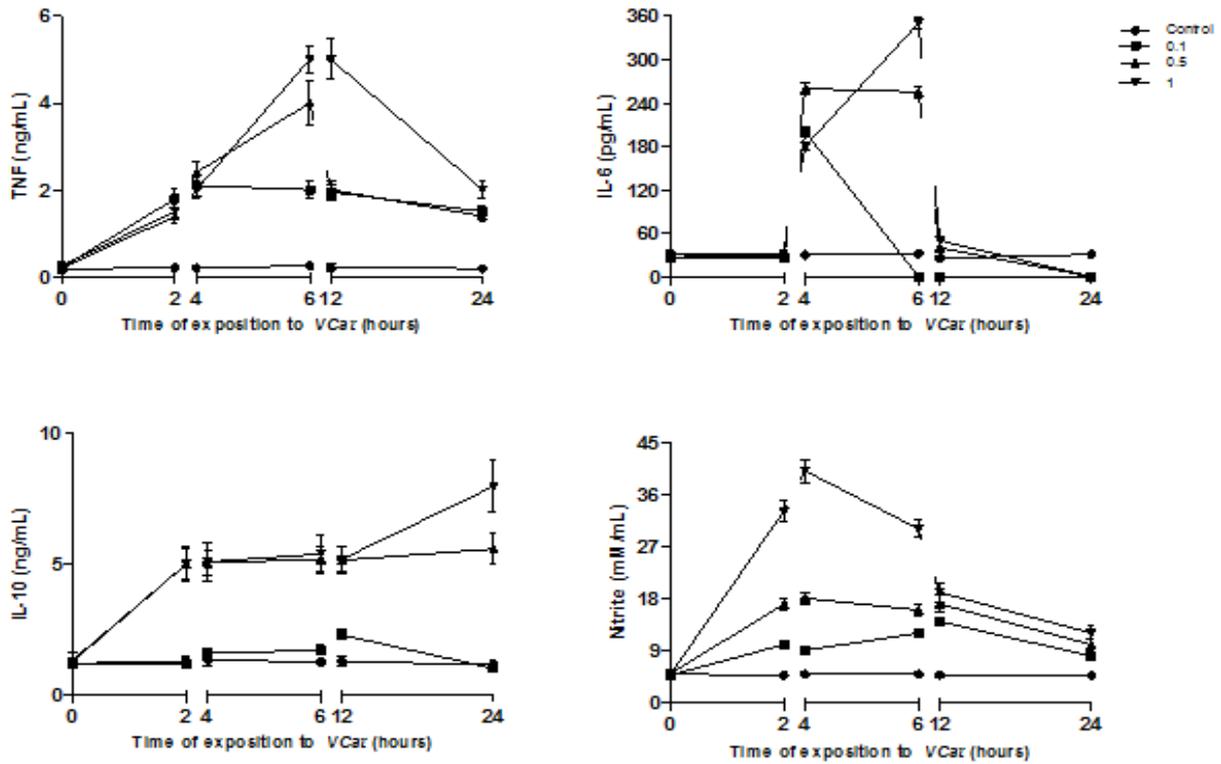


Fig. 5: Mediators production.

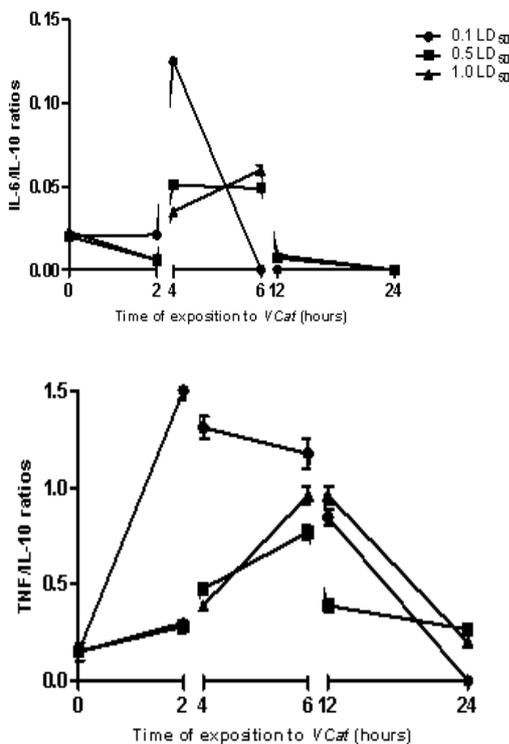


Fig. 6: IL-6/IL-10 and TNF/IL-10 ratios.

**Balance of pro- and anti-inflammatory cytokines**

To evaluate the balance of pro- and anti-inflammatory cytokines TNF/IL-10 ratios were determined. Figure 6 illustrates the IL-6/IL-10 and TNF/IL-10 ratios. The highest IL-6/IL-10 and TNF/IL-10 ratios were observed in groups of mice injected with 0.1 LD<sub>50</sub> of

VCat up to 2 h, decaying thereafter. In contrast, the maximum IL-6/IL-10 and TNF/IL-10 ratios were observed in groups of mice treated with 0.5 and 1.0 LD<sub>50</sub> of VCat after 6 h, decaying thereafter (Fig. 6).

**DISCUSSION**

The overall mechanism of the toxic action of rattlesnake poisoning is somewhat unclear due to the complex nature of crotalid venoms. These venoms are constituted by a variety of toxic proteins and produce serious complications such as neurotoxicity, coagulation disorders, systemic myotoxicity and multiple organ failure [14]. The envenomation process is consequence of the unbalanced immune response to the injury organism. Our experiments were designed to investigate the effects of VCat on the immune system of mice *in vivo*. In the present study we investigated the acute and sub-acute effects of VCat and were designed to study four immune parameters: number of peritoneal and spleen cells, spleen index and mediators production.

In order to establish optimal conditions for peritoneal and/or spleen-venom interactions, the effect of VCat were evaluated *in vivo* and the LD<sub>50</sub> detected in the conditions used in our experiments was 125 µg and was named as acute doses while the sub-acute doses were 0.1 and 0.5 LD<sub>50</sub>. The effect of VCat on cells was evaluated: with 0.1 LD<sub>50</sub> sub-acute dose of VCat was capable to affect peritoneal and spleen cells number 2 h after administration, but the number of these cells recovered by 24 h. In contrast, the injection with 0.5 and/or 1 LD<sub>50</sub> of VCat led to significant decreases in the number of peritoneal and spleen cells. Our data on the effects of treatment with VCat show an important percentage suppression of peritoneal and spleen cells number. The present results suggest that VCat might exhibit direct cytotoxic activity. Macrophages are important in the regulation of immune responses. They are often referred to as scavengers or antigen-presenting cells because they pick up and ingest foreign materials and present these antigens to other cells of the immune system such as T cells and B cells. This is one of the important first steps in the initiation of an immune response.

The present study shows the effects of exposition to *VCat* show a 40% suppression of spleen index over the control, which is in agreement with previous reports that lymphoid organs, including thymus, spleen and mesenteric lymph nodes, show moderate reductions in their weights [20,26]. In this work, we show reductions in spleen weights after 0.5 or 1.0 LD<sub>50</sub> of *VCat* exposition for 24 hs. The different amounts of *VCat* are probably indicative of the diversity of effective concentrations among individual subjects and species. Determination of the time course revealed that the decrease in spleen weight and the spleen index are parallel processes. The number of spleen cells in mice is decreased by *VCat* treatment. The decrease in spleen cell number caused by *VCat* exposition was accompanied with the decrease in spleen weight. This reduction in the spleen index of treated animals suggests that *VCat* is capable to suppress the immune function. Splenic sequestration occur episodes in highest frequency during the first hours of mouse exposition to *VCat*. The spleen is the largest organ in the reticulo-endothelial system. It has been standard practice, for many years, to use splenic size as an indicator of disease activity in a variety of disorders of the reticulo-endothelial system, when spleen is injured causes bleeding.

The results obtained in this study are in agreement with previous reports which show that in crotalid venoms the severe forms is the existence of immune depression is suggested by the alterations of lymphoid tissue [27]. These observations highlight the contributions of cellular proliferation and death to the previously reported changes in spleen. The spleen is a major secondary lymphoid organ, which contains a variety of immune competent cells. The contact of these cells with a specific antigen or mitogens induces a proliferation and differentiation into plasma cells or immune regulatory cells [28]. Hence the spleen can indirectly reflect humoral immunity. Our data demonstrate that systemic administration of *VCat* produced a significant suppression of macrophages and spleen cells at 2 – 6 hs after treatment. These effects and the mechanisms involved have not yet been elucidated. Cardoso et al., (2001) [29], demonstrated marked alterations on peripheral leukocyte distribution, characterized by a drop in the number of lymphocytes and monocytes and an increase in the number of neutrophils, were observed after *C.d.terrificus* venom. This inhibition interferes with both inflammation and immune reactions. Several studies have been reported that most cytokines are a group of immune regulatory proteins involved in a number of physiological processes. During inflammation, the cytokines initiate and amplify the response, others or attenuate it, and some of them cause it to resolve. This includes TNF- $\alpha$ , IL-6, IL-10 and NO production during envenomation process. Differences were observed in the pattern of cytokine production and effects of *VCat* in the serum. Under the conditions used in the present study, we observed that the acute or sub-acute dose of *VCat* may alter mediator production. For example, NO, TNF- $\alpha$  and IL-6 levels peaked after 2 and 6 h, respectively, decaying thereafter. The present study also shows a markedly enhanced in IL-10 production in serum from mice exposed to *VCat* for 2 up to 24 h. IL-10 is an anti-inflammatory cytokine that acts in part by blocking monocytes/macrophage production of inflammatory cytokines such as IL-6 and TNF- $\alpha$  [30]. Some of these differences could also be due to differences in the concentration, rate of clearance, and concentrations of active metabolites of *VCat* in liver or spleen, which are sources for a substantial proportion of cytokines in serum. The cytokines balance indicates the inflammatory status of the cells. Our results showed the IL-6/IL-10 and TNF/IL-10 ratios in serum, which may represent inflammatory status in acute and sub-acute effects of *VCat*. Based on these findings when groups of animals were treated with sub-acute dose of *VCat* the balance was predominantly pro-inflammatory up to 2 h, decaying thereafter. For acute effects with 1.0 LD<sub>50</sub> of *VCat* the balance was predominantly anti-inflammatory in all time. It seems evident that the balance between pro- and anti-inflammatory cytokines was associated with organ function, which was evaluated by spleen weight and spleen index. The exact mechanism by which different amounts of *VCat* suppresses the immune response is difficult to explain at this time. One possible explanation is that *VCat* may perturb the equilibrium and change the functionality of immune reactions, leading to a reduction in pharmacological effects.

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#### CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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