

## BIOCHEMICAL AND BIOLOGICAL PROPERTIES OF *Lonomia obliqua* BRISTLE EXTRACT

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**ABSTRACT:** *Lonomia obliqua* caterpillar is frequently seen in accidents with humans especially in the south of Brazil. Patients develop a hemorrhagic syndrome that can be treated with specific antilonomic serum. A consumptive coagulopathy was found to be the main cause of bleeding complications observed in patients after contact with *L. obliqua*. Studies revealed that *L. obliqua* caterpillar bristle extract (LOCBE) displays a procoagulant activity that leads to intravascular thrombin formation, resulting in a special form of disseminated intravascular coagulation (DIC). Fibrinolysis seems to be secondary to the fibrin production, since no direct fibrinolytic activity was found in LOCBE. Two procoagulant toxins, a factor X activator (Losac) and a prothrombin activator (Lopap), were isolated from LOCBE and characterized. Infusion of Lopap into experimental animals triggered a condition similar to that observed in human envenomation.

**KEY WORDS:** *Lonomia obliqua*, *Lonomia obliqua* bristle extract, hemorrhagic syndrome, disseminated intravascular coagulation.

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## INTRODUCTION

*Lonomia obliqua* is a moth of the Saturniidae family (Lepidoptera), which is called caterpillar at its larval stages with poisonous spines (33). It occurs during spring and summer, especially in the southern part of Brazil. Caterpillars have gregarious habit, live in colonies on trunks or branches of fructiferous trees, and present mimetic characteristics. Human envenomation occurs after accidental contact with the caterpillar bristles, which deliver toxic substances into the victim's skin. The accident is denominated lonomism and may be severe, resulting in a hemorrhagic syndrome characterized by consumptive coagulopathy. Since 1989, a high incidence of accidents by *L. obliqua* has been reported in the southern region of Brazil (13, 20-22, 29) and therefore it has been considered an important public health problem (18).

The first report of a human accident caused by *Lonomia* dates from the early 1900s and describes a hemorrhagic syndrome in a patient who had been in contact with a group of caterpillars (1). However, more detailed studies started around 1960 in Venezuela by Arocha-Piñango and collaborators (2). Since then, various cases of envenomation by *Lonomia achelous* in Venezuela have been reported and studies with its hemolymph have been carried out (3-8, 27).

Lemaire distinguished *Lonomia obliqua* (Walker) of the south of Brazil from those caterpillars of Venezuela and classified it as *Lonomia achelous* (Cramer) (30). Although envenomation by *L. obliqua* and *L. achelous* is followed by similar symptoms associated with a hemorrhagic syndrome, these species may differ in their venom properties and mechanism of action. *Lonomia achelous* presents toxins with fibrinolytic and procoagulant activities (6), and the toxic action of *L. obliqua* is characterized by a predominant procoagulant activity (19, 38). We conducted several *in vivo* and *in vitro* studies with LOCBE and two toxins were identified and characterized so far. In this paper, we present a review of the information available up until now about the toxic properties of *L. obliqua* bristle extract and discuss the clinical aspects of envenomation.

## HUMAN ACCIDENTS

Since 1989 *L. obliqua* has been associated with human accidents in the southern region of Brazil (Rio Grande do Sul, Santa Catarina, and Paraná States), where it is considered epidemic (18, 20, 29). Nevertheless, this species has overspread from its

original places and, recently, new accidental cases were reported in the states of São Paulo (22), Rio de Janeiro (16) and Minas Gerais.

The clinical symptoms of envenomation by *L. obliqua* are very similar to those observed after *L. achelous* envenomation in Venezuela (5). Severity generally depends on the number of larvae, larval stage, and extension of the patient's skin area involved in the accident (18, 48). Initial symptoms are pain, burning sensation, hyperemia, occasional local inflammation at the contact sites, edema, headache, nausea, and vomiting (14, 21, 29, 53). These symptoms are generally followed by systemic reactions associated with a severe coagulopathy and bleeding manifestations. The diagnosis proposed for *L. obliqua* envenomation is characterized by subcutaneous and generalized hemorrhage, including skin, mucosal and visceral bleeding, hematoma, hematuria, gingivorrhagia, ecchymosis, epistaxis, hematemesis, and melena (14, 16, 29, 53). This syndrome usually occurs during the first 12 hours after envenomation and bleeding may appear spontaneously or as a result of mild injuries (22, 53). Major complications such as acute renal failure (13, 20) and intracerebral bleeding may occur leading the patient to death. Renal failure is relatively frequent among patients envenomed by *L. obliqua*, but it is rarely observed in envenomation by *L. achelous* (6, 13, 20). Pathogenesis of this manifestation remains unclear, but it may probably be a result from the deposition of fibrin in glomeruli and renal ischemia (22).

Recently, we have published a study that evaluated coagulation and fibrinolysis in 105 patients after contact with *L. obliqua* (53). It is the unique clinical report that concerns a large number of patients affected by *L. obliqua* envenomation. The other few clinical studies available in literature involve only a limited number of envenomed patients (16, 22, 29) but agree with our findings. In general, patients presented prolonged global coagulation, prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT). Fibrinogen (Fg) was reduced in the majority of envenomed patients, and the extent of defibrinogenation was directly associated with bleeding manifestations. Therefore, Fg level could be used as an efficient marker for evaluating the envenomation severity and for monitoring the treatment. A significant decrease in the levels of factors V, XIII and VIII, prekallikrein (PK), and protein C was also observed when fibrinogen was depleted. No changes were observed in factors X, II and von Willebrand Factor (vWF), protein S, and antithrombin (AT). Platelet count remained normal in most cases. A little change observed in the platelet count

and in the AT level suggests that a different form of clotting activation may be involved in the hemorrhagic syndrome triggered by *L. obliqua* venom.

In addition, it was observed a great production of the coagulation activation markers prothrombin F1+2 and thrombin-antithrombin (TAT) complex, which is associated with the generation of thrombin in envenomed patients. Extremely high D-dimer levels were observed in all patients after contact with the caterpillar, indicating disseminated intravascular coagulation. Tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) levels did not change substantially, while plasminogen and  $\alpha$ 2-antiplasmin levels were significantly reduced in cases of afibrinogenemia. Consumption of these fibrinolytic factors, associated with high levels of D-dimer, indicates intense fibrinolysis. These observations altogether indicate a consumptive coagulopathy and secondary fibrinolysis.

#### *Lonomia obliqua* BLEEDING SYNDROME AND DIC

Disseminated intravascular coagulation (DIC) is a syndrome associated with certain clinical conditions (e.g. trauma, neoplasia, sepsis) and is characterized by a systemic activation of the coagulation system and a loss of the hemostatic balance (45, 47, 51). This syndrome is caused by enhanced and abnormal generation of thrombin (from prothrombin) into circulation, leading to consumption and depletion of clotting and anticlotting factors. Clinical manifestations can vary from bleeding to thrombosis in large and small blood vessels resulting in widespread vascular or microvascular fibrin deposition (28, 31, 47). Clinical and laboratory parameters for DIC diagnosis were algorithmically defined (46) and involved prolonged clotting time, elevated concentration of fibrin degradation products as well as reduced levels of platelets, coagulation proteins, and inhibitors.

The consumptive coagulopathy associated with lonomism seems to be different from that observed in DIC, since in the former there is no reduction of factors XII, II and X, and the platelet number remains unaltered. In contrast, reduction of factors V, VIII and XIII, and PK levels can be attributed to consumption during coagulation, similarly to that of DIC. An increase in the concentration of F1+2 and TAT complex (as in DIC) was reported after *L. obliqua* envenomation (53). However, values of TAT reaching 900 $\mu$ g/l were much higher than those observed in patients with other causes of DIC, in which the levels did not exceed 100 $\mu$ g/l (9, 10, 51, 52). Interestingly, it seems that

AT is not consumed as it is in other types of DIC (12, 51), even though large amounts of thrombin and TAT complex seem to be generated, particularly in patients with a more severe coagulopathy. Therefore, despite the presence of consumptive coagulopathy in *L. obliqua* envenomation, the initiation and maintenance mechanisms of this special form of DIC seems to be different from that observed in other clinical situations. Additional studies are necessary to clarify the pathophysiologic process involved in DIC and the hemorrhagic syndrome common in patients envenomed by the contact with *L. obliqua* bristles.

### **ANTIVENOM THERAPY**

In Venezuela, conventional therapy with antifibrinolytic drugs such as aprotinin and  $\epsilon$ -aminocaproic acid has been successfully used for treating patients envenomed by *L. achelous*, since the major biological activity of its venom is activation of the fibrinolytic system (8). Also, administration of cryoprecipitate or purified fibrinogen apparently contributes to stop clinical evidence of bleeding (6). In contrast, replacement therapy with either whole blood or fresh-frozen plasma has been shown to exacerbate the clinical symptoms of envenomation (4, 5). In Brazil, administration of  $\epsilon$ -aminocaproic acid (17, 40), blood transfusion, replacement of clotting factors (fibrinogen and cryoprecipitates of factor VIII), and dialytic therapy have been used to reverse clinical manifestations of patients envenomed by *L. obliqua* caterpillar (16). Nevertheless, antifibrinolytics or cryoprecipitates have not been effective (40) and thus are not recommended for *L. obliqua* envenomation. Moreover, as in *L. achelous* envenomation, the administration of whole blood or fresh-frozen plasma is not indicated because they exacerbate DIC (4).

In the last years, Butantan Institute, São Paulo, Brazil, developed an antilonomic serum for specific treatment of envenomation caused by contact with *Lonomia obliqua*. This antiserum is obtained by immunizing horses with *L. obliqua* bristle extracts, which triggers the production of specific IgG antibodies (17, 40). The antivenom is composed by purified F(ab')<sub>2</sub> and was shown to be effective in reverting hemostatic disturbances and bleeding observed in humans and experimental animals envenomed by *L. obliqua* (17). Since the antivenom has been standardized and produced in large scale (40), treatment of envenomed patients is now largely based on its administration. Caovilla and Barros investigated the optimal dose of antilonomic serum for immunotherapy (14). Finally, Zanin and collaborators (53)

demonstrated that early diagnosis and an adequate treatment, particularly within the first 12 hours, could prevent severe coagulopathy in a great number of patients. After the introduction of *L. obliqua* antivenom, no more deaths were recorded (40).

## **EXPERIMENTAL ENVENOMATION**

Laboratory findings about the effects of *Lonomia obliqua* bristle extracts on animal models are consistent with data on blood coagulation and fibrinolysis in patients. Blood uncoagulability with prolonged clotting time and bleeding time, as well as Fg depletion, was observed in rats, rabbits and mice and showed to be dose-dependent (29, 35, 37, 40). In addition, Prezoto and collaborators (35) demonstrated that LOCBE is effective in preventing thrombus formation but does not exert thrombolytic activity in pre-formed thrombi. The antithrombotic effect of LOCBE is most probably due to Fg depletion as a consequence of consumptive coagulopathy. Local and systemic effects of LOCBE and its toxins are summarized in Table 1.

Although experimental envenomation by LOCBE caused complete Fg depletion, a 50% decrease of FXIII levels was observed in rats (Table 1). This reduced level of plasma FXIII zymogen in treated rats (25, 35) is apparently similar to that observed in humans envenomed by *L. obliqua* (53). In these cases, FXIII reduction is related to consumption and not to degradation, as indicated by *in vitro* studies, which showed that FXIII levels were unchanged irrespective of whether they were measured in fibrinogen-depleted plasma of LOCBE-treated or untreated rats (25). Furthermore, the clear correlation between FXIII levels and fibrinogen levels demonstrated by our studies on patients and rats reinforces the theory that decreases in FXIII levels induced by *L. obliqua* are associated with consumptive coagulopathy. It contrasts to what occurs in patients envenomed by *L. achelous*, in which FXIII is drastically reduced due to a factor XIII degradation factor present in the venom (27).

## *Lonomia obliqua* VENOM TOXINS AND BIOCHEMISTRY

*In vitro* studies demonstrated that LOCBE induces clot formation by activating the coagulation cascade via both prothrombin and factor X activator activities (19, 29). In our laboratory we purified and characterized a prothrombin activator – Lopap (*Lonomia obliqua* prothrombin activator protease) (39) – and a factor X activator – Losac (*Lonomia obliqua* Stuart-factor activator) (23) – from LOCBE. In addition, some

other toxins were identified in *L. obliqua* bristle extract by classical biochemical methods (Table 2) and by transcriptomic analysis (50).

Losac purification resulted in a single polypeptide chain of about 43kDa; it was the first factor X activator purified from Lepidopter secretion. This enzyme can activate factor X in a concentration-dependent manner forming FXa, which integrates the prothrombinase complex (42). An indirect assay was used to evaluate factor X activator activity of Losac, and it was based on the capability of this enzyme to form factor Xa in the presence of factor X and a chromogenic substrate specific for factor Xa (S-2222). Losac activity was totally inhibited by phenylmethylsulphonylfluoride (PMSF), indicating that this enzyme is a serine-like protease. Partial amino acid sequencing of Losac showed no similarities with any other well-known factor X activator sequences. The biochemical properties of this newly characterized factor X activator are still being investigated.

The prothrombin activator named Lopap is the most studied protein concerning *L. obliqua* bristle extract components. Lopap is a 69-kDa tetrameric protein with a prothrombin activator activity independent of the prothrombinase compounds, although calcium ions increase its activity (39). The purified protein activates prothrombin in a dose-dependent manner and its mechanism of action is similar to that of factor Xa, generating thrombin, which is capable of clotting purified fibrinogen. In an indirect assay using a chromogenic substrate specific for thrombin (S-2238) in the presence of prothrombin and calcium, the extract and purified protein presented a prothrombin activator activity similar to that of factor Xa. Lopap activity is inhibited by antilonomic serum (17) and serine protease inhibitors such as PMSF (38, 39). However, amino acid sequencing of Lopap indicated no homology with prothrombin activators or other serine proteases known, but with lipocalins.

Infusion of Lopap into rats causes a coagulopathy similar to the one that occurs in human envenomation (37), indicating that it could be one of the toxins responsible for the severe consumptive coagulopathy found in patients envenomed by *L. obliqua*. Besides its prothrombin activator activity, we have shown that Lopap acts on endothelial cells responses inducing expression of mediators involved in clotting, in inflammation and in the fibrinolytic system, as well as affecting cell viability and mechanisms of anti-apoptosis (15, 24).

*In vitro* experiments demonstrated that *L. obliqua* venom mainly presents procoagulant activity and is not capable of activating the fibrinolytic system or

degrading cross-linked fibrin (25, 29). These results agree with *in vivo* studies reported by Prezoto and collaborators (35), who showed that LOCBE is not capable of lysing pre-formed thrombi.

Some authors showed that *L. obliqua* venom has fibrinolytic activity (25, 34, 49), which hardly occurs with high concentrations of bristles extract and long incubation time with the fibrinogen molecule. Prolongation of the fibrinogen clotting time could be observed at a 1:2 stoichiometry, an uncommon situation during envenomation. In addition, the products generated are different from those induced by plasmin, and no clot lysis is observed in fibrin plate (25). All data found up until now indicate that fibrinolysis is not caused by *L. obliqua* venom but seems to be secondary to DIC in the envenomation syndrome.

## **CONCLUSION**

The data presented here reinforce the idea that the pathogenic mechanisms involved in the hemorrhagic syndrome observed in patients envenomed either by *L. obliqua* or by *L. achelous* are related to venom proteins that have different specific activities in each species. While in envenomation by *L. achelous* hemorrhage is due to enzymes that degrade FXIII and proteins that activate fibrinolysis, in envenomation by *L. obliqua* bleeding manifestations seem to be related to a consumptive coagulopathy induced by the presence of procoagulant proteins. Therefore, therapy should be conducted according to the main mechanism involved in each case. Antifibrinolytic agents are not recommended for *L. obliqua* accidents and the treatment should be based on the administration of antivenom, which is an effective way to reverse hemostatic disturbances and bleeding.



Table 1: *In vivo* effects of *Lonomia obliqua* bristle extract.

Effect observed	Animal model	Venom fraction	Observations	References
Blood incoagulability with prolonged clotting time (PT and APTT) and bleeding time; fibrinogen and FXIII depletion	rat, rabbit and mouse	LOCBE	Dose-dependent effect	25, 35, 40
	mouse and rat	Lopap	DIC in the rat cremaster microcirculatory network (observed by intravital microscopy)	37, 38
Antithrombotic effect	rabbit and rat	LOCBE	Prevention of thrombus formation, and incapability of inducing lysis of preformed thrombi	35
Lethality	mouse	LOCBE	LD <sub>50</sub> = 0.19mg IV / 18-20g mouse; no death was observed with up to 1mg IP / 18-20g mouse.	40
Acute inflammatory reaction	mouse	LOCBE	Effect observed in genetically selected lines of mice	36
Intravascular hemolysis	rat	LOCBE	Intravascular hemolysis has not been reported in humans after accidental contact with <i>Lonomia obliqua</i>	41
Edematogenic and nociceptive responses	rat	LOCBE	Nociception is inhibited by indomethacin pretreatment and seems to be facilitated by prostaglandin production; edema is reduced by loratadine pretreatment and is probably mediated by prostanoids and histamine	11
Damage in the central nervous system, causing disruption of the blood brain barrier	rat	LOCBE	Intracerebral hemorrhage was rarely observed, occurring 24h after envenomation	43

LOCBE: *Lonomia obliqua* bristle extract

Lopap: *Lonomia obliqua* prothrombin activator protease

PT: Prothrombin time

APTT: Activated partial thromboplastin time

DIC: Disseminated intravascular coagulation

Table 2: *In vitro* activities identified in *Lonomia obliqua* bristle extract.

Activity	Toxin Molecular Weight (Da)	Characteristic	References
Prothrombin activator	Lopap 69,000 (tetrameric)	Serine protease Ca <sup>2+</sup> increases its activity	19, 39
Factor Xa-like	20,746	Amino acid sequence shows 100% homology with the Lopap sequence [gi: 59709575] deposited in the National Center for Biotechnology Information (NCBI) data bank	32  <a href="http://www.ncbi.nlm.nih.gov">http://www.ncbi.nlm.nih.gov</a>
Factor X activator	Losac 43,000	Serine protease Ca <sup>2+</sup> -independent	19, 23
Phospholipase A <sub>2</sub> -like	15,000	Indirect hemolytic activity in human and rats red blood cells	42
αβ-Fibrinogenase-like	Lonofibrase 35,000	Incapable of inducing fibrin clot lysis	25, 34, 49
Hyaluronidase-like	49,000 53,000	They display hydrolase activity as a β- endohexosaminidase; degradation of purified hyaluronic acid, purified chondroitin sulphate, and extracellular matrix	26
Anti-apoptotic*	51,000	Activity on <i>Spodoptera frugiperda</i> (Sf-9) cell culture	44

\* Identified in *L. obliqua* hemolymph.

Lopap: *Lonomia obliqua* prothrombin activator protease.

Losac: *Lonomia obliqua* Stuart-factor activator.

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