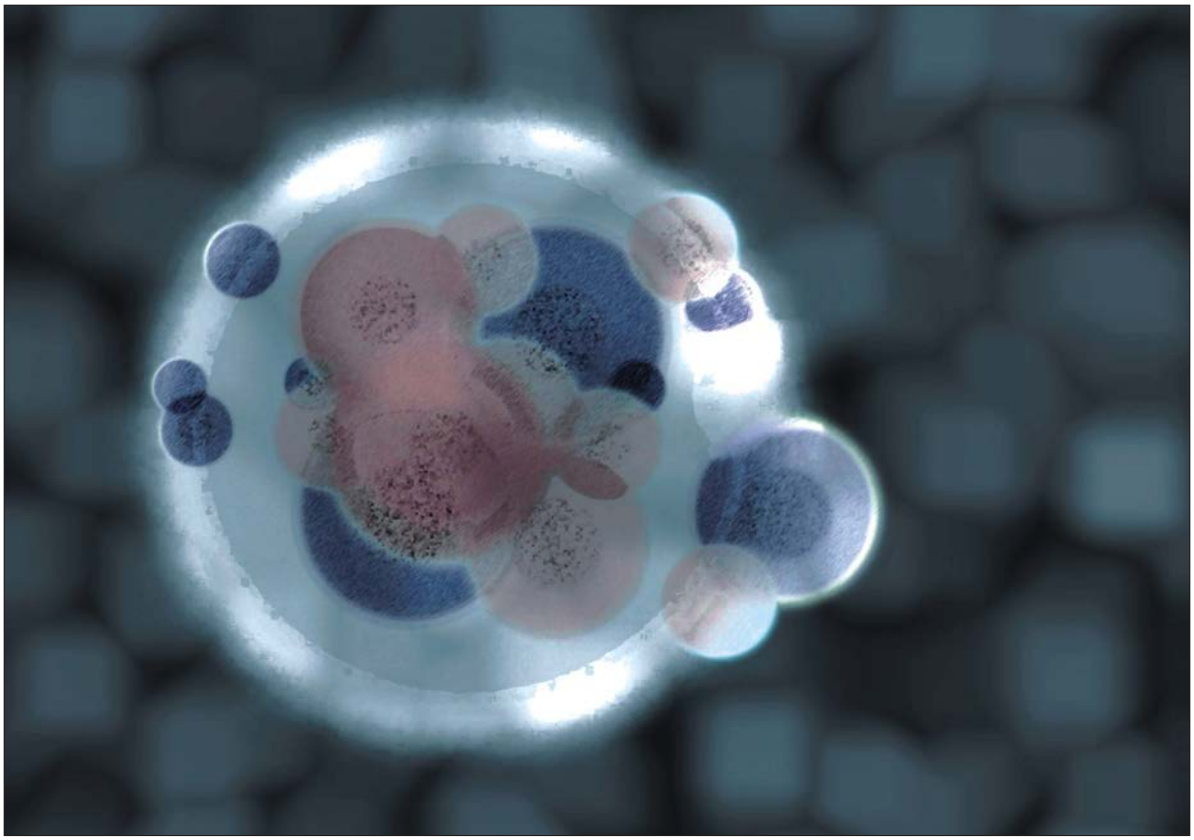

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Comparative Toxic Effects of the Venoms from Three Wasp Species of the Genus *Polybia* (Hymenoptera, Vespidae)

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Abstract: In this study we have investigated hemolytic, edematogenic and neurotoxic effects of the crude venoms of three *Polybia* species; *P. occidentalis, P. paulista* and *P. ignobilis*. In addition, protein contents and dry weights of these wasp's venom reservoirs have also been compared. *P. ignobilis* presented the highest protein content and highest dry weight per venom reservoir. When injected in the hind paw of rats relatively to the other two, it evoked a stronger edematogenic effect. The venom of *P. paulista* showed the most potent hemolytic activity on human washed red blood cells. When tested on the erythrocytes of different species (rat, pigeon, ox, sheep, snake and horse), the three venoms showed marked differences in hemolytic activity according to the blood species. However, this variability appeared to be similar for the three venoms. Moreover, the intracerebroventricular injection of each of all three venoms caused tonic clonic seizures and death to rats. Present data suggest that although the three species are closely phylogenetic related, there must be pharmacological differences among the venom compounds that should be better investigated.

Key words: Biological control, edema, hemolysis, neurotoxicity, *Polybia*, wasp venom

INTRODUCTION

Hymenoptera venoms are complex mixtures of active substances with multiple sites of action and general biological concern[1,2]. The biological activity of Hymenoptera venoms can be classified as neurotoxic, hemolytic, digestive, hemorrhagic and allergenic and can be caused by a variety of chemical classes of compounds including proteins, peptides, biogenic amines and amino acids[3,4].

It is believed that the toxicity of the Vespinae and Polistinae venoms is partially due to their enzymatic content, which may include phospholipases A and B, hyaluronidases, acid phosphatases, proteases and nucleotidases with several possible functions[5,6]. Besides such enzymes, small peptides, like kinins and mastoparans also account for the allergenic, cytotoxic and inflammatory effects of wasp stings, shown to be lethal to certain individuals[7-9]. Also, it has been shown that multiple stings can provoke neurotoxic effects such as: lesions on peripheral and central nervous systems, neuritis or encephalitis[10].

The genus *Polybia* is commonly found in American Neo-tropical areas and contains 56 species[11]. *Polybia* nests usually present a cellulose-like aspect, which led them to be commonly referred to as paper wasps. Some of these wasps are used as biological control of plagues in South American crops, where this use is based upon findings that some *Polybia* wasps are predators of plagues insects, such as ants, termites, hemipterans and lepidopterans [12,13].

In a comparative study it was verified that the chromatographic profiles obtained by high-performance gel filtration of the venoms of *P. paulista, P. occidentalis* and *P. ignobilis*, showed 13 peaks, with recognizable differences between each species studied[14]. Some of the components of *P. paulista* venom were further identified and named polybitoxins 1-4. They are phospholipases showing potent hemolytic effects[6]. The toxin contents of the venoms of the two other wasp species remain unknown and their toxic effects have not been assayed. Therefore, the aim of the present study was to compare the toxicity of *P. paulista, P. occidentalis* and *P. ignobilis* on mammalian nervous tissue, on the

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erythrocytes of different animals, as well as the edematogenic effects of the three venoms when injected into the rat's hind paw.

MATERIALS AND METHODS

Venom extracts: Workers of *P. paulista* and *P. occidentalis* were collected at the University Campus of Ribeirão Preto and of *P. ignobilis* in Luis Antonio, both in the State of São Paulo, Brazil. All wasps were identified by Prof. Sidney Matheus (Entomology Laboratory, USP). They were sacrificed by freezing to -20°C and their venom reservoirs removed, crushed in Milli-Q grade water and centrifuged at 5000 g for 10 min, at 4°C. Supernatants were carefully collected, lyophilized and weighed. Crude venoms of *P. paulista* (PpCv), *P. occidentalis* (PoCv) and *P. ignobilis* (PiCv) were diluted in 15 mM saline to perform the bioassays. Protein contents were estimated by the Lowry method[15].

Animals: Male Wistar rats 220-250 g were kept in wire-mesh cages in a room maintained at a 12 h dark/light cycle (lights on at 7:00 am) and given food and water *ad libitum*. Conditions of luminosity and temperature (22°C) were kept constant in housing and experimental rooms.

All experimental procedures involving animals were conducted in accordance with Brazilian Society of Neurosciences rule's, following guidelines for animal care prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

Surgery and Neurotoxicity Assays: Rats were anesthetized with sodium thiopental, 40 mg kg^{-1} (Cristalia, Brazil) for unilateral stereotaxic implantation of a 10 mm stainless steel guide cannula in the right lateral ventricle. The target coordinates used were 1.0 mm from bregma, 1.6 mm lateral from the midline and 3.3 mm ventral from the surface of the skull, according to the Atlas of Paxinos and Watson[16]. The cannula was attached to the skull with acrylic resin, anchored with stainless steel screws and temporarily sealed with a stainless steel wire to protect it from obstruction. The animals were allowed resting for 5-7 days to recover from surgery.

Prior to the assays, rats (n=6 per group) were placed in an arena (circular enclosure 60 cm in diameter and 50 cm high), for a 10 min period of acclimatization and then injected i.c.v. with the crude venom solutions, using a Hamilton syringe moved by an infusion pump (Insight, Brazil), at a speed of 3 μL min^{-1}. Subjects were filmed for 20 min and subsequently observed for 2 h.

The concentrations of PiCv (22; 44; 88; 132 μg μL^{-1}) PpCv (15; 31; 62; 124 μg μL^{-1}) and PoCv (8; 17; 34; 68 μg μL^{-1}) were chosen using the criteria of number of glands (1.5; 3; 6 and 12 glands for each concentration).

At the end of the experimental period the rats were anaesthetized with sodium pentobarbital (Thiopenthal, 45 mg kg^{-1} body weight, i.p.) and perfused through the left ventricle with saline solution followed by paraformaldehyde 4%. Next, they were injected with 3 μL of toluidine blue dye had their brains carefully removed and manually sliced, in order to check the correct site of the injection. LD_{50}'s and 95% confidence intervals were determined by Probit analysis according to Finey[17].

Hemolysis: Direct hemolytic activity of crude venoms was determined on washed human red blood cells, according to Ho and Ko[18], with modifications, in order to establish the ED_{50}'s and ED_{100}'s values. Lyophilized venoms were dissolved in 0.4 mL 15 mM saline and incubated with red blood cells (50%) at 37°C for 60 min. Hemolysis was interrupted by the addition of cold (4°C), buffered saline, to a final volume of 1 mL. The degree of hemolysis was determined by measuring released hemoglobin, at a wavelength of 545 nm. The three wasp venom extracts were then assayed following the same protocol, on washed red blood cells (50%) of ox (*Bos taurus*), sheep (*Ovis aries*), snake (*Crotalus durissus terrificus*), rat (*Rattus norvergicus*), pigeon (*Columba livia*) and horse (*Equus caballus*), using the ED_{50}'s and ED_{100}'s determined previously using human blood. The results obtained were analyzed by one-way analysis of variance (ANOVA), using $p<0.05$ and $p<0.001$ as indexes of significance.

Rat paw edema: Edema was induced by a subplantar injection of venom extracts into the left hind paw of sodium thiopental-anesthetized rats (n=6 per group). The right hind paw was injected with 15 mM saline to serve as control. Both paws were then measured with a plethysmometer (Ugo Basile, Italy). Edema was determined from the size of the swelling caused by the venoms minus that caused in the controls. Results were analyzed by ANOVA on ranks, using $p<0.05$ and $p<0.001$ as indexes of significance.

RESULTS

Neurotoxicity Assays: The crude venoms of the three wasps tested at their highest dose, evoked an initial 20 min period of low locomotor activity, followed by

Table 1: LD_{50} and proteic content of the venoms of *P. ignobilis*, *P. occidentalis* and *P. paulista*

Species	mg protein/ venom reservoir	Dry wt. of venom per worker (mg)	LD_{50} (i.c.v) (mg protein/rat)	LD_{50} (i.c.v.) (reservoir/rat)
P. ignobilis	0.044	0.045	0.079	1.80
P. occidentalis	0.017	0.029	0.061	3.60
P. paulista	0.031	0.035	0.111	3.36

Data are expressed as mg protein/reservoir. Lethal dose (LD_{50}) for 50% of the animals injected via the intracerebroventricular (i.c.v.) route. Data are expressed as mg protein/rat and reservoir/rat

Table 2: Hemolytic effects of the venoms of *P. ignobilis, P. paulista* and *P. occidentalis*

	Hemolysis (%)					
	P. paulista		*P. ignobilis*		*P. occidentalis*	
Species of erythrocytes	ED_{50}	ED_{100}	ED_{50}	ED_{100}	ED_{50}	ED_{100}
Rat	84±10.5	100±9.9	80±9.5	100±9.4	91±9.0	100±0.0
Pigeon	8±0.3	11±0.3	9±0.2	13±0.6	8±0.8	9±0.8
Ox	1.8±0.4	2.5±0.2	2.0±0.3	2.2±0.2	1.1±0.6	2.7±0.4
Sheep	10±2.9	11±2.5	10.2±2.8	10±2.3	10±2.5	11±2.4
Snake	0.6±0.2	1.4±0.0	0.6±0.3	0.7±0.2	0.7±0.2	0.9±0.5
Horse	2.7±0.2	3.7±1.1	2.5±0.7	4.3±2.2	1.7±0.3	2.5±0.5

Data are expressed as % of hemolysis in relation to controls containing blood samples of each animal, incubated in 15 mM saline

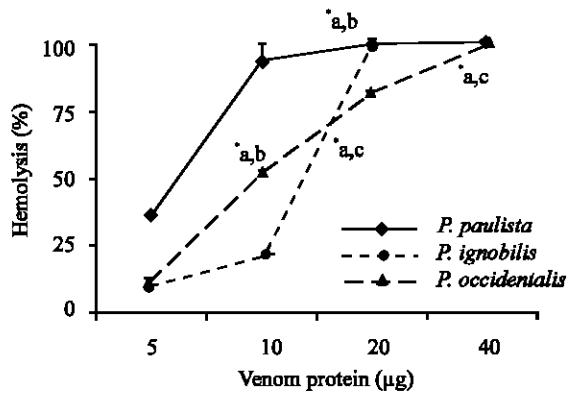

Fig. 1: Hemolysis of human red blood cells incubated with the venoms of *P. ignobilis* (a), *P. occidentalis* (b) and *P. paulista* (c) for 60 min at 37°C. Data are expressed as % hemolysis±SEM for each venom concentration as measured by one-way ANOVA. * $p<0.05$

a 30 min period of agitation. During the high activity period a defensive-like behavior, characterized by wild running, was observed in some of the animals. After approximately 60 min the animals showed a 20 min period of generalized tonic clonic seizures followed by *status epilepticus* and death. Although the number of dead animals varied with dose, neither the magnitude of the seizures, nor the periods of latency until their onset, was affected by the four doses tested.

The LD_{50}'s of the three wasp venoms, as well as their protein contents/worker are shown on Table 1.

Hemolysis: Figure 1 shows that the three venoms had hemolytic activity in human blood cells *in vitro*, the venom of *P. paulista* apparently being the most potent among them. ED_{50}'s were 0.01, 0.006 and 0.01 mg protein for, respectively, PoCv, PpCv and PiCv; ED_{100} 's were 0.020, 0.020 and 0.040 mg of protein in the same order, respectively.

Table 2 shows the different hemolytic effects of ED_{50} and ED_{100} of PoCv, PpCv and PiCv on blood samples from different species. Listed in order of their sensitivity to the venoms, the species responded as follows: rat>human>sheep>pigeon>horse=ox>snake. The three venoms showed marked differences in hemolytic activity according to the species yielding the blood. However, this variability appeared to be similar for the three venoms, each venom showing the highest effectiveness for rat blood.

Rat paw edema: Figure 2A shows that the lowest dose of PoCv tested evoked edema within the first five min following injection; this response was not different from that produced by the highest dose of the venom tested. It gradually decreased from 10 min on, until the end of the experiment. The injections of 0.02 and 0.04 mg of PoCv, caused the same maximal edema response.

Figure 2B shows that each dose of PiCv induced an edema of similar extent within 30 min following application; after this period, responses increased according to the dose applied. The edema induced by the highest dose increased from 30 min on, until the end of the experiment, when a significant difference among the effects of the three doses was noted.

Figure 2C shows that the injection of three increasingly higher doses of PpCv induced the same paw swelling within the first 5 min. The effects of the lowest dose did not change from 5 min until the end of the

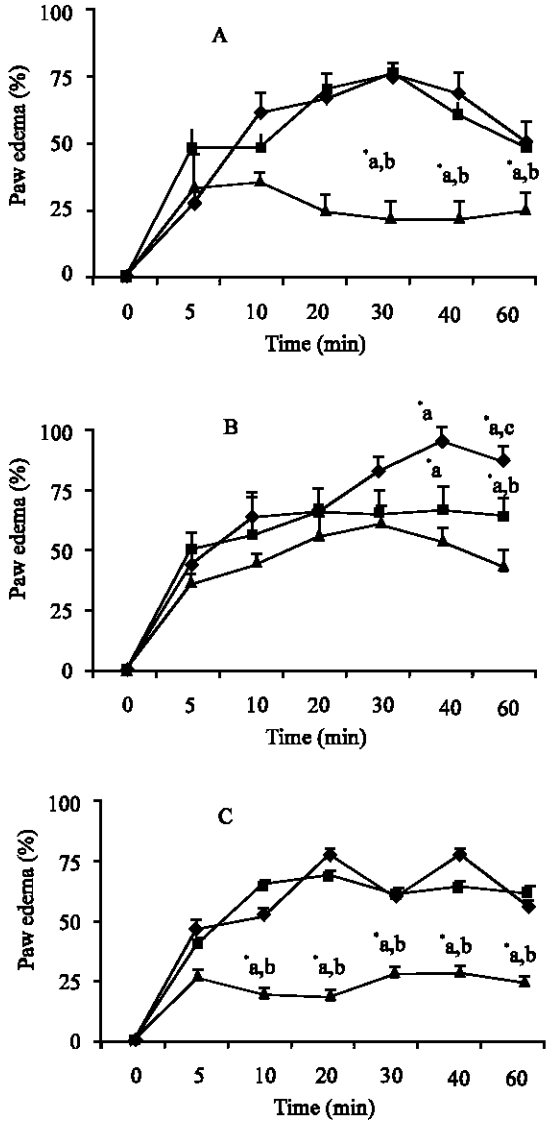

Fig. 2: Edematogenic effects of subplantar injections of three doses of the venoms of *P. occidentalis* (A), *P. ignobilis* (B) and *P. paulista* (c) followed over an interval of 0 to 60 min. Data are expressed as percentages of paw edema compared to control paws injected with saline. (◆, a) 0.04, (■, b) 0.02 and (▲, c) 0.01 mg of protein. Data were analyzed with repeated measures ANOVA, * $p<0.05$

experiment. The two highest doses of this venom induced similar results during the whole experiment.

Figures 2A-C shows that a maximal edema response was verified 40 min following the injections of the venoms. At this period, PiCv was more potent than the other two venoms, when tested at the lowest dose (0.01 mg).

DISCUSSION

The first record of human death attributed to envenomation by wasp or hornet sting was that of King Menes of Egypt in the 26th century B.C.[19]. As wasp venoms contain several biologically active substances and responses to these toxins are usually confounded with immune system disorders, it is particularly difficult to assess venom toxicity[20]. However, studies on the biological activity of crude Hymenoptera venoms are particularly interesting to establish venom induced toxicity to humans as well as other animals.

Polistinae venoms have been only recently studied; among their few characterized toxins, are the phospholipase agelotoxins, probably the most potent hemolysins found in animal venoms[21]. Also, previous work had described the polybitoxins from *P. paulista* venom, which are more potent phospholipases than those of the venom from the honeybee (*Apis mellifera*) and the *Naja naja atra* and *Naja nigricolis* snakes[6].

The present results demonstrate that the three *Polybia* venoms have potent hemolytic activity, *P. paulista* venom having the highest cytotoxic activity on washed human red blood cells. The hemolytic activities of the three *Polybia* venoms were higher than those of the wasps from genera Vespula and Vespa[1]. Half a mg of the proteins from the venoms of *Vespula germanica*, *Vespula vulgaris* and *Vespa crabro* lysed only 2% of human erythrocytes. However, crude *A. mellifera* venom lysed 100% of human red blood cells at a concentration of less than 0.01 mg of venom protein, indicating that the honeybee venom is more potent than the venoms of *Polybia* wasps[1].

Erythrocytes from several different species were used in our study in order to establish the degree of effectiveness of the wasp venoms on red blood cells of different animal species. The order of potency indicates that *Polybia* venoms are more effective to lyse rat and human blood and show almost no effectiveness to the snake blood. Present data are in agreement with the findings of Bernheimer and *et al.*[22] who verified that the venoms of wasps of the genera Vespa and Polistes have their highest hemolytic activity on human and rat blood cells.

The results obtained in the paw edema experiments indicated that the edematogenic toxins of *P. occidentalis* and *P. paulista* venoms were less potent than those of *Vespula vulgaris*. Griesbacher *et al.*[23] injected 0.01 mg of *V. vulgaris* venom into the hind paws of rats and observed an increase of 40% in their volume compared to saline injected paws. The same concentration of *P. ignobilis* venom induced a 50% increase in paw volume in our experiments.

Neurotoxicity assays demonstrated that the three wasps could cause similar neurotoxic effects. Approximately one hour after having been injected with lethal doses of venoms, rats presented generalized tonic-clonic seizures with hind limb extension, apnea and death. These effects apparently were due to high molecular weight venom components, since ultrafiltration (cut at 3000 Da), eliminated these proconvulsant effects (unpublished data).

Finally, it should be noted that there existed a considerable difference in the volume of wasp venom reservoirs as well as in their protein contents. For instance, in order to achieve the lysis of 100% red blood cells, 2.3, 0.65 and 0.45 venom reservoirs of *P. occidentalis*, *P. paulista* and *P. ignobilis*, respectively had to be used. These differences should be extrapolated to other bioassays and become particularly important for the choice of which wasp species would be less toxic to humans and other animals when employed for biological control of plagues in agriculture.

Polybia wasps are widely used as natural pest controls in Neotropical plantations of among others, melons[24], oranges[25], tobacco[26] and tomatoes [27]. In the field, wasps may reduce the damage caused by arthropod plagues to crops by about 70%. In some cases the use of traditional insecticides is restricted to events of population explosion. The use of *Polybia* wasps as biological controls also offers advantages regarding ease of transportation and handling of their colonies. Thus present data on the toxicity of the crude venoms of some *Polybia* wasps, which have been in common use as biological controls.

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