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# Anti-complement activity in salivary glands and midgut of Chagas disease vector, *Panstrongylus megistus* (Hemiptera, Triatominae)

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## ABSTRACT

The triatomine insect *Panstrongylus megistus*, one of the most important Chagas disease vectors in Brazil, presents salivary molecules pharmacologically active to counteract homeostatic responses from the host, including inhibitors of the human complement system, a major effector of immune responses. The aim of the present study was to investigate the effect of *P. megistus* salivary gland extract (SGE) on the complement system from different host species and characterize the inhibitory effect of SGE and intestinal contents on human complement. Glands and midguts from fourth instar nymphs were used. Hemolytic assays were performed with sheep erythrocytes as complement activators by using human, rats and chickens sera in the presence or absence of SGE. An ELISA assay was carried out detect deposition of the C3b component on IgG- or agarose-sensitized microplates, in the presence or absence of SGE or midgut contents. *P. megistus* SGE was able to significantly inhibit the complement of the three studied species (human, rat and chiken). Both, SGE and midgut contents inhibited C3b deposition in either the classical or the alternative pathways. As conclusions, SGE and midgut from *P. megistus* possess anti-complement activity. The inhibitors are effective against different host species and act on the initial steps of the complement system cascade. These inhibitors may have a role in blood feeding and *Trypanosoma cruzi* transmission by the vector.

**KEYWORDS:** *Panstrongylus megistus.* Salivary glands. Midgut. Complement system.

## INTRODUCTION

Triatomines (Hemiptera, Reduvidae), also known as kissing bugs, are obligatory hematophagous insects widely distributed in the Americas[1]. They present great relevance in public health as they are the natural vectors of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease, a potentially life-threatening illness responsible for over 10,000 deaths every year[2]. The transmission occurs during the hematophagy process, when the infected insect ingests blood until fully ingurgitate and defecates infective metacyclic trypomastigote forms of the parasite next to the bite site. Natural infection takes place when the host scratches the region and the parasite enters the blood stream through the bite lesion[3].

During a blood meal, the introduction and presence of the insect mouthparts in the skin triggers a homeostatic response in the vertebrate host, hampering the feeding process[4]. In order to overcome these responses and complete a sucessfull hematophagy, the triatomine insect injects its saliva into the bite site, which has been

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reported to contain pharmacologically active components, such as vasodilators[5], anticoagulants[6], antiplatelets[7] and immune regulators[8,9]. Among the later, inhibitors of the complement system have been reported[10,11].

The complement system is the key component of innate and adaptative immune responses directly involved in induction of inflammation, microbial killing, apoptosis and clearance of immune complexes[12]. The complement system is composed of about thirty proteins which are soluble in plasma or attach to cell surfaces. This system can be initiated by three pathways (classical, lectin and alternative). Classical pathway was the first described and is triggered by antigen-antibody complexes when recognized by the soluble C1q component while the lectin pathway was more recently discovered and is activated when mannose binding lectin (MBL), ficolins or collectins detect carbohydrate ligands that are specifically present on microbial surfaces[13]. The alternative pathway was the second one described despite being the evolutionarily oldest pathway, is activated by spontaneous cleavage of C3, the most abundant complement component in blood, generating C3b, its activated form that can recognize and covalenty bind to foreign elements, such as pathogens. The three pathways converge to a common point with the activation of C3 molecules, which occurs through the proteolytic cleavage promoted by C3 convertases. The cascade activation subsequently promotes the assembly of the membrane attack complex (MAC), which is responsible for membrane lysis and cell death[14].

Complement system inhibitors have been described in salivary glands and midgut of several unrelated species of hematophagous arthropods, such as anopheline mosquitoes[15,16], sand flies[17], tsetse flies[18], ticks[19], scabies mites[20] and triatomine insects[10,11], highlighting their importance for bloodfeeders. The main roles of these inhibitors in the feeding process are to decrease inflammatory responses at the bite site, diminishing the host's perception of the vector and to protect the intestinal epithelium of the vector against MAC-mediated damage[11,15]. Since the insect midgut is mostly composed of only one layer of cells[21] and complement remains active in the ingested blood[11,15], activation of MAC could lead to intestinal disruption and the death of the insect.

*Panstrongylus megistus* is an important Chagas disease vector well distributed in Latin America, especially in Brazil, where it is considered the most broadly distributed species throughout the country[22]. It is commonly associated with animals such as rodents, birds and marsupials, although in some parts of Brazil it occurs mainly in artificial ecotopes, with great capacity to invade and colonize human domicilies[23,24]. Moreover, *P. megistus* presents higher levels of anthropophily and natural infection with *T. cruzi* as compared to other triatomine vector species[25], which accentuates its epidemiological role as a vector. Regardless of these facts, there are only a few studies focusing on its interaction with the parasite and vertebrate hosts.

In the present work, we investigated anti-complement activity in salivary glands extracts (SGE) and midgut contents of *P. megistus*. A discussion on the mechanism of action and possible role of these inhibitors in *T. cruzi* survival inside the vector's gut is provided.

## MATERIALS AND METHODS

### Ethics statement

The experimental procedures used in this study were approved by the Ethics Committee in Animal Experimentation at the Federal University of Piaui (CEUA/UFPI), under the study protocol Nº 079/14.

### Triatomine insects maintenance, salivary glands and intestinal contents preparation

The *Panstrongylus megistus* triatomine insects used in this study were obtained from a colony maintained at 28 ºC and 65% relative humidity at the Parasitology and Microbiology Departament of the Federal University of Piaui, Teresina, Brazil. For colony maintenance, the insects were fed weekly on chickens (*Gallus gallus*). Salivary glands and midguts were dissected from starved fourth instar nymphs, gently washed in saline solution (0.9% NaCl) and transferred to microcentrifuge tubes containing specific buffers, on ice. For the hemolytic assays, glands were stored in $GHB^{2+}$ buffer (5 mM HEPES, 145 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% gelatin, pH 7.4), while for the deposition assays, they were kept in HNCM buffer (4 mM HEPES, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) for testing the classical pathway and in HMEBN buffer (5 mM HEPES, 7 mM $MgCl_2$, 10 mM EGTA, BSA 5 mg/mL, 140 mM NaCl, pH 7.4) for the alternative pathway assays. *Panstrongylus megistus* possess two glands composed of three subunits (D1, D2 and D3) each[26]. The subunits D1 and D2 are considered a single gland andwere used in the assays. The preparations were sonicated for 5 s and centrifuged at 10,000 *g* for 3 min at room temperature. The supernatants were used in the assays.

Each soluble intestinal sample contains a pool of anterior midguts from starving fourth instar nymphs 10 to 20 days after molting. After washing in saline solution, midguts were transferred to 300 mL of HNCM or HMEBN (to assay the classical or the alternative pathway, respectively). The intestines were opened with stylets to retrieve their contents.

After centrifugation at 14,000 *g* for 7 min at 4 ºC, adequate amounts of the supernatant were used in the assays.

### Obtention of serum and erythrocytes

Pools of normal human sera (NHS) were obtained from healthy volunteers. Rat (*Rattus norvegicus*) and chicken (*Gallus gallus*) sera were collected as previously described[17]. Serum samples were aliquoted and maintained at -80 ºC until use. Sheep erythrocytes were obtained by puncture of the jugular vein of an animal kept at the Parasitology and Microbiology Department (UFPI).

### Hemolytic assays

Antibody-sensitized sheep red blood cells and normal human serum (NHS) were prepared as described by Mendes-Sousa *et al.*[17]. To measure the classic pathway inhibition, 25 µL of 1:60 NHS in $GHB^{2+}$ solution was mixed with 12.5 µL of the same buffer containing SGE in 1.5 mL microcentrifuge tubes. Then, 25 µL of IgG-sensitized sheep erythrocytes suspended in $GHB^{2+}$ solution at 2 x $10^8$ cells/mL were added to the tubes and incubated at 37 ºC for 30 min. Two-hundred and fifty microliters of cold saline solution were added after incubation, and the tubes were centrifuged at 1,700 x *g* for 1 min. Two hundred microliters of each supernatant were transferred to a microplate (96 wells), and read in a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 415 nm. Assays were also performed with rat and chicken sera diluted 1:100 and 1:10 in $GHB^{2+}$, respectively.

The hemolytic assays were carried out in duplicates, and three controls were used in each test: total hemolysis, in which 250 µL of cold distilled water were added instead of cold saline; negative control, in which sera were substituted by the same volume of $GHB^{2+}$; and a positive control, in which sera were added without EGS. The means of duplicates were calculated for each independent experiment, and the means of the respective negative controls were subtracted. Results were transformed into a percentage of hemolysis, with the positive control corresponding to 100% of activity.

### C3b-deposition assays

The C3b-deposition assays were reproduced from Barros *et al.*[11]. For the classical pathway experiments, a 96-well ELISA plate (COSTAR®, Corning, USA) was sensitized overnight inside a humid chamber with 50 µL of coating buffer (35 mM $Na_2CO_3$, 15 mM $NaHCO_3$, pH 9.6) containing 2 µg of purified human IgG. Following the plate sensitization, the wells were blocked with 200 µL of the blocking buffer 1 (10 mM Tris, 140 mM NaCl and 3% BSA, pH 7.4), followed by a second blockage with 200 µL of the blocking buffer 2 (10 mM Tris, 140 mM NaCl, 3% BSA, 0.05% Tween-20 and 5 mM $CaCl_2$, pH 7.4), for 30 min, under constant agitation, at room temperature. One microliter of NHS was pre-mixed in a microcentrifuge tube to adequate amounts of the samples containing the SGE or intestinal contents. The final volume was adjusted to 100 µL with HNCM. Each preparation was then transferred to sensitized wells and incubated 30 min at 37 ºC under agitation. Wells without glands or midguts samples and without serum samples were used as positive and negative controls, respectively.

The activation of the classical pathway by plate surface-adhered IgG promotes the covalent binding of C3b molecules that can be quantified by specific antibodies. After sera incubation, the wells were washed twice with 200 µL of washing buffer (10 mM Tris, 140 mM NaCl and 0.1% BSA, pH 7.4) and 50 µL of goat anti-C3b antibody (Sigma, St. Louis, USA) diluted 1:1,000 in HN solution (10 mM HEPES and 140 mM NaCl, pH 7.4) were added to the wells, then the plate was incubated for 30 min under agitation (160 rpm) at room temperature. The wells were washed twice as described before, and 50 µL of rabbit anti-goat antibody conjugated with peroxidase diluted 1:1,500 in HN solution were added to the plate, which was incubated again for 30 min. The wells were filled with 200 µL of developing buffer (50 mM sodium citrate, 50 mM $Na_2HPO_4$, 1 mg/mL o- phenylenediamine (Sigma, St. Louis, USA) and 0.075 % $H_2O_2$, pH 5.0) and the plate was read in a microplate reader at 450 nm and 37 °C for 10 min in the kinetic mode (one read every 30 s). All of the assays were performed in triplicate, with at least three independent repetitions. The means of triplicates were calculated for each independent experiment, and the means of the respective negative controls were subtracted. The results were converted into percentages of C3b deposition, with the positive control corresponding to 100% of activity.

For the alternative pathway assays, the plates were covered with 100 µL of an aqueous 0.1% agarose solution and left to dry overnight. Seven microliters of NHS were pre-mixed with different amounts of the samples containing inhibitors and the final volume adjusted to 100 µL with HMEBN buffer. This buffer contains EGTA, which chelates $Ca^{2+}$ ions necessary for triggering the classical and the lectin pathways. As a consequence, only the alternative pathway was activated. The mixtures were transfered to the agarose-coated wells and the plate was incubated at 37 ºC for 30 min for complement activation. The wells were washed twice with HMEBN buffer and incubated with 50 µL of anti-C3b

antibody diluted to 1:2,000 in a solution containing 10 mM HEPES, 140 mM NaCl and 1 mg/mL BSA (pH 7.4). After two more washing steps 50 µL of anti-goat peroxidase-conjugated IgG diluted to 1:5,000 in the same buffer were added to the wells, and the plate was incubated for 30 min under agitation. The rest of the assay followed the same steps described for the classical pathway.

### Statistical analysis

All statistical tests and graphs were performed using the GraphPad Prism version 6. Data normality was assessed using the Kolmogorov-Smirnov test. The one-way ANOVA followed by the Tukey's test were used to analyze normally distributed results. Significance was set at $p < 0.05$.

## RESULTS

### Hemolytic assays

Hemolytic assays were performed to confirm the effect of *P. megistus* SGE on the lytic activity of the human complement and to investigate its effect on rats and chickens complement systems. As observed in Figure 1, all SGE concentrations (0.5, 1 and 2 glands) were able to inhibit the lysis of sheep erythrocytes from the three studied host species. Even the lowest concentration of SGE (related to 0.5 salivary gland) decreased more than 70% the hemolysis by human and rat sera, while only the amounts of SGE from 1 and 2 glands significantly inhibited the hemolysis by the chickens classical pathway.

### C3b-deposition assays

The inhibitory effect of the SGE on the human classical pathway has also been demonstrated by C3b-deposition assays on IgG-coated microplates. The C3b deposition was significantly inhibited in the presence of SGE from one or two glands ($p < 0.05$) (Figure 2). Moreover, using agarose-coated wells as an activating surface for the alternative pathway, we detected a significant decrease in the deposition of the component C3b, in the presence of SGE (Figure 2). Even in the presence of SGE from half gland, there was a significant difference in the deposition of the complement

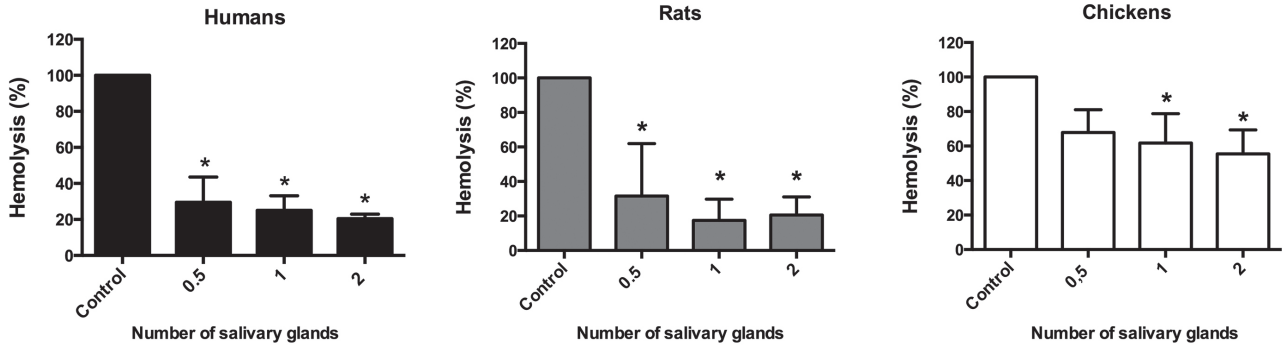


**Figure 1 -** Effect of *P. megistus* salivary glands extracts (SGE) on the lytic activity of the complement system from humans, rats and chickens. Results are expressed as the mean percentage of hemolysis + Standard Deviation (SD). Statistical analyses were performed using ANOVA followed by the Tukey test. Differences were considered significant when $p < 0.05$ (*).

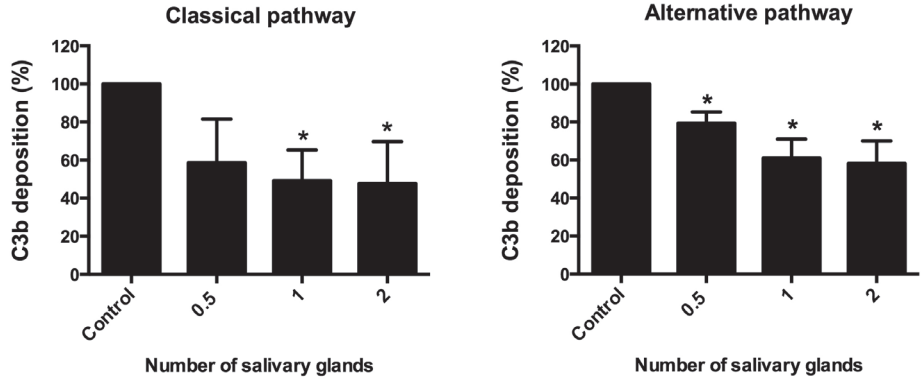


**Figure 2 -** Effect of *P. megistus* salivary glands extracts (SGE) on the classical and the alternative pathways-mediated C3b deposition. Results are expressed as the mean percentage of C3b deposition + SD. Statistical analyses were performed using ANOVA followed by the Tukey test. Differences were considered significant when $p < 0.05$ (*).

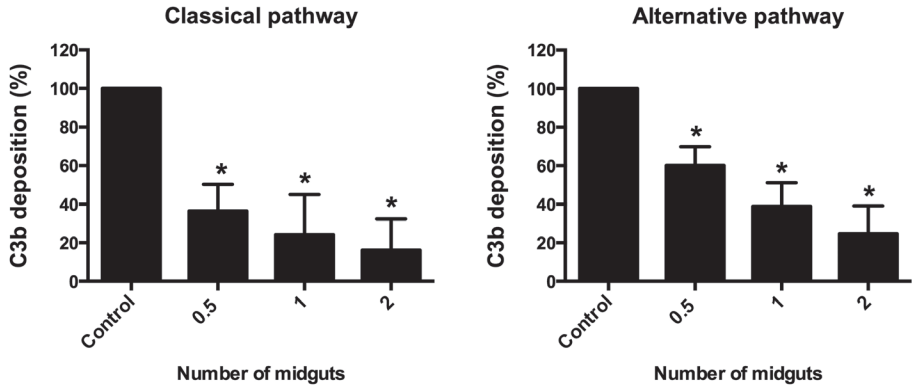


**Figure 3 -** Effect of *P. megistus* midgut contents on the classical and the alternative pathways-mediated C3b deposition. Results are expressed as the mean percentage of C3b deposition + SD. Statistical analyses were performed using ANOVA followed by the Tukey test. Differences were considered significant when $p < 0.05$ (*).

component as compared to controls (alternative pathway) ($p < 0.05$) (Figure 2).

C3b-deposition assays have also been performed to test the inhibitory effect of midgut contents on the complement system. As demonstrated in Figure 3, both, the classical and the alternative pathways were significantly inhibited in the presence of all tested concentrations of midgut contents ($p < 0.05$).

## DISCUSSION

In the present study, we reported the inhibition of the complement system by salivary glands extracts and midgut contents of the triatomine insect *P. megistus*. The anti-complement activity in *P. megistus* was first described by Cavalcante *et al*.[10], who demonstrated inhibition of the lytic activity of the human complement system by SGE. In this study, we confirmed those results. However, considering that *P. megistus* feeds on a wide range of vertebrate hosts[23], being exposed to the deleterious potential of their complement systems, we have also investigated if the SGE could act on rodent and avian complement systems as well, which are usual bloodmeal sources for the vector but present an epidemiological role in Chagas disease transmission. Rats are common *T. cruzi* reservoirs, acting as sources of infection to *P. megistus*[27]. On the other hand, chickens are refractory to the parasite infection but are regularly reported as blood sources to *P. megistus*, thus are involved in the maintenance of vector populations[23]. As expected, *P. megistus* SGE significantly inhibited the lytic activity of rats and chickens, reflecting the importance of these inhibitors to the blood feeding process.

The anti-complement activity has also been demonstrated by C3b-deposition assays, in which we observed inhibition of both, the classical and the alternative pathways of the human complement system. Since C3b deposition was diminished in the presence of SGE, it suggests that the inhibitor(s) act at a point in the cascade before C3 activation or directly on the C3 component itself. Barros *et al*.[11] demonstrated that SGE from triatomines of the genus *Rhodnius* and *Triatoma* can also inhibit the deposition of C3b from both pathways and that only the *Triatoma* genus has also inhibited the C4b deposition in the classical pathway, indicating different mechanisms of action among insects. In fact, recently, Weinberger *et al*.[28], cloned and expressed a salivary secreted protein (TiCRT) from *T. infestans* that binds to C1q, the first component of the classical pathway, inhibiting the subsequent activation of the C4 component. By acting at the beginning of the classical pathway cascade, in addition to protecting the vector midgut, this inhibitor is also responsible for the down-regulation of inflammation at the bite site as it may prevent the generation of C3a and C5a subproducts, which are potent anaphylatoxins that are responsible for recruiting leukocytes to the bite site[14]. Further studies are required to discover if *P. megistus* possess a single salivary inhibitor that strikes both pathways or specific molecules directed to classical and alternative pathways, as it has been recently reported for the phlebotomine sand fly, *Lutzomyia longipalpis*[29,30].

In addition to salivary inhibitors, *P. megistus* has also shown anti-complement activity in the midgut. Their contents were able to significantly inhibit both, the classical and the alternative pathways. Salivary and midgut inhibitors could act together in the protection of intestinal cells against MAC activation, since the perimicrovillar membrane, a cover of triatomine insects midgut' microvilli that could act as a protector, is poorly developed in unfed insects[31]. *Barros et al*.[11] have reported complement inhibitors in midgut contents of kissing bugs from the *Triatoma* and *Rhodnius* genera, which have also inhibited both, the classical and the alternative pathways. Intestinal inhibitors have also been described in midgut contents from sand flies[17], scabies

mites[20] and tsetse flies[18], reflecting their importance in the midgut protection.

Besides the protection of the insect's midgut cells against ingested complement systems, the salivary and intestinal inhibitors could be exploited by *T. cruzi* inside the vector. The infective trypomastigote form of the parasite is resistant to complement-mediated lysis as it presents several inhibitors on its surface, some of them considered virulence factors among *T. cruzi* strains. However, *T. cruzi* epimastigote stages inside the triatomine digestive tract are highly susceptible to complement[32]. Epimastigotes are non-infective and replicative forms of the parasite inside the vector. As complement retains its activity in the ingested blood[11] and therefore could kill epimastigotes when the insects take a second blood meal, the salivary and intestinal inhibitors must have an extremely important role in the protection and maintenance of *T. cruzi* life cycle. In fact, Ooi *et al*.[18] demonstrated that intestinal complement inhibitors from the tsetse fly *Glossina morsitans* can protect procyclic *T. brucei*, the causative agent of African trypanosomiasis, from complement-mediated lysis and are of great importance for the successful establishment of this trypanosome infections in the vector midgut.

The results reported in the present study are of relevance for a better comprehension of Chagas disease biology. As the complement inhibitors present in *P. megistus* salivary glands and midgut act on different vertebrate hosts, especially in mammals, and they could protect both, the insect and the parasite from complement-mediated damage, they may present potential as candidate molecules for a transmission-blocking vaccine.

## AUTHORS' CONTRIBUTION

AFM-S, VCB: conceived and designed the experiments; EARF, MAM: performed the experiments; AFM-S, VCB, EARF, MAM: analyzed the data; AFM-S, VCB: wrote the paper.

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