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Pre- and post- morphological evaluation of the kidneys of male Wistar rats inoculated with *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidade) salivary gland extract

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Abstract

Controlling the population of ticks is a subject of great concern to researchers, due to the parasitic nature of these ectoparasites and their capacity to transmit pathogens to the human being and other vertebrate animals. In this scenario, some authors have studied the salivary glands of *Rhipicephalus sanguineus*, having demonstrated the morphophysiological importance of these organs. The saliva plays a role in the modulation of the host's immune-inflammatory and hemostatic systems; in addition, it is considered a potential reservoir of several functions. In this sense, the present study had the objective to establish, analyze and correlate the morpho-physiological behavior of the cells and tissues from the kidneys of male Wistar rats pre/post inoculated with *R. sanguineus* salivary gland extract, in addition to verify the possible toxic action of this saliva on the kidney. For this, the rats were divided into 5 groups with 4 animals each: **CG** (control, no exposure); **PBS1** (one injection of PBS 1X), **PBS2** (two injections of PBS 1X), **SGE1** (one injection of salivary gland extract 0.04µg/µL) and **SGE2** (two injections of salivary gland extract 0.04µg/µL). After the exposures, the kidneys were subjected to histological/histochemical techniques: HE, toluidine blue and xylidine Ponceau. Each inoculated rat was weighed one day before the first inoculation and after 21 days, the blood count analysis was performed. The results showed that the exposures, either to PBS or to the SGE caused small alterations: a) emergence of fat plates in the glomerular region b) presence of nuclei with higher heterochromatin concentration in the cells of both regions. The data indicated that, despite these small morphological alterations, no systemic toxicity signs were observed, which was confirmed by the blood clinical analysis and the body weight measurements that were conducted before and after the treatment. In this context, it was possible to conclude that in general both **PBS** and **SGE** did not cause damage to renal tissues.

Keywords bioactive, control, pathogen, *R. sanguineus*, salivary gland, ticks

Introduction

Ticks are ectoparasites arthropod of great medical and veterinary importance, once they cause significant damages to the host, either through the feeding process or by transmitting pathogenic agents. These ectoparasites are obligatory hematophagus and vectors of arboviruses, rickettsioses, spirochetosis, and protozoa; affecting domestic and wild animals, and the human beings as well [1-3]. According to Balashov [4], in ticks,

the combined action of the mouthparts and the saliva produced by the salivary glands (vital organs for their biological success of the ectoparasite) enable the feeding process. The salivary glands have different and fundamental functions, and among them, one is the production of substances to ensure the fixation on the host and nutrition [5]. In addition, these glands act as storage sites for the pathogens transmitted to the host through the inoculation of the saliva [2].

The salivary glands of the species *R. sanguineus* (Latreille, 1806) (Acari: Ixodidae) have been studied by Furquim since 2007 [6], who demonstrated the morphological and physiological complexity of these structures and established a new classification for their cells, aggregating a larger number of types (in acinus II) in the glands of both males and females. Specifically in the females, the salivary glands are composed of acini types I, II and III [6-7]. In the male individuals, these glands comprise acini types I, II, III and IV [8-10]. Acini type I are agranular, and are involved in the hydric balance of the ectoparasite, while types II and III (granular), act on the process of fixation, feeding and osmoregulation in the phase of high blood consumption [6]. The function of acini type IV (granular) has not been established in the literature; however, they seem to act on the reproductive process of the ectoparasites [11]. In *R. sanguineus* females, the acini II are constituted of secretory cells type **a, b, c1, c2, c3, c4, c5** and **c6**, while acini III are comprised of cells type **d, e** and **f** [6-7]. Exclusively in the males, acini II present cells *c7* and *c8*. Acini IV are solely formed by cells **g** [8, 10].

According to Camargo-Mathias et al. [12], the saliva of the ticks, a complex mixture, as is the organ where it is produced, ensures the nutrition and survival of the ectoparasite [13-14].

For this reason, several studies have been developed aiming to identify and isolate the bioactive molecules of the ticks' saliva, which function as vasodilators, anticoagulants, immune-suppressants and anti-inflammatory agents [15]. It is suggested that these molecules have developed over the co-evolution of parasite-host and would be crucial to overcome the host's biological barriers, allowing the tick to complete the feeding process and advance towards the developmental stages [16].

The search for substances with the potential to modulate cancer cell proliferations is ongoing, once these cells are aggressive and difficult to be controlled. The methods used to control cancer cells brought significant consequences to those subjected to them. In this sense, bioactives obtained from plants or animals have been considered as promising alternatives to handle this problem, which affects great part of the world population [14, 17-18].

To have a blood meal, the ticks insert their highly specialized mouthparts on the host's skin [2]. This penetration damages the capillaries and small blood vessels in the region, forming an ideal place for the tick to feed [19].

The rupture of the blood vessels stimulates the production and the release of components that recruit humoral and cellular inflammatory mediators. The injuries caused to the host, expose the tissues and adjacent cells, which in turn activate a number of inflammatory responses [20]. The host's immunological response against the tick involves cells producing antigens, cytokines, lymphocytes B and T, and granulocytes, along with other cells and molecules [21].

The kidneys, vital organs for the mammals, are bean-shaped organs, each with a convex and a concave side, covered by connective tissues. Each kidney is subdivided into a cortical (peripheral) and a medullar (central) region. The nephrons, functional units located in the cortical region, are comprised of a Malpighian corpuscle, a proximal convoluted tubule, thin and thick portions of Henle loop and a distal convoluted tubule. The kidneys regulate the chemical composition of the body's internal environment through the processes of filtration, active/passive absorption, and secretion. Furthermore, these organs transfer elements from the blood to be eliminated with the urine [22].

Abreu et al. [23] reported that the extract obtained from *R. sanguineus* salivary glands inhibited the invasion of tumor cells in Wistar rat muscles; however, further investigation is needed in order to investigate how the extract would act on the physiology of these individuals, mainly on the kidneys, as these organs are critical for the survival of the animals.

Thus, the present study analyzed the *in vivo* effects of one and two injections of *R. sanguineus* salivary gland extract on male Wistar rats, studying the morphology of the renal tissue using histological

and histochemical techniques along with clinical analysis of the blood and weight measurement (pre and post inoculation), in order to verify possible alterations.

Methodology

Biological material

This study was approved by the Ethics Committee on Animal Use (ECAU), UNESP, twenty male Wistar rats aged 46 days were divided into 5 groups comprised of 4 rats each. The animals were kept in collective polypropylene cages (40cm x 34cm x 18cm), receiving extruded food (Labina® Purina®) ad Purina®) ad libitum drinking water from the same source (a water bottle for each cage).

The five study groups were established as follows: a) Control (CG): non-inoculated individuals; b) PBS1: individuals inoculated once with 300 µg/µL of saline phosphate buffer 1x – PBS, containing the diluted extracts; c) PBS2: injected twice with 300 µg/µL of PBS, at a 4-day interval between the injections; d) Treatment 1 (SGE1): inoculated once with 300 µg/µL of salivary gland extract (SGE), 0.04 µg/µL and e) Treatment 2 (SGE2): inoculated twice with 300 µg/µL of SGE, at a 4-day interval between the injections.

Obtaining the female tick salivary glands and the glandular extract (SGE)

Male and female fasting ticks were used; these were obtained from *R. sanguineus* colonies kept especially for the purpose of this study in a BOD chamber under controlled conditions (29°C, 80% humidity and 12h photoperiod) in the Animal Facility in the Department of Biology, UNESP, Rio Claro, SP, Brazil. Male and female ticks were placed together on the host rabbits, once only after mating the females start feeding on hosts. After two days feeding, the females were collected and dissected to have the salivary glands removed. According to Camargo-Mathias; Furquim [7], the component of saliva undergoes modifications throughout the glandular cycle,, because the bioactives synthesized with two days feeding are in part different from those synthesized with four and six days feeding. Another important point is that the salivary glands of *R. sanguineus* females with two days feeding present the f cells of the acini III in full secretory activity, which does not occur with other periods of the glandular cycle.

The salivary glands were placed into microcentrifuge tubes containing 200µL of phosphate buffer and macerated. The tubes were then centrifuged for 30 min at 10,000×g, and the collected supernatants were placed into previously sterilized microtubes. The extract was filtered in a vertical laminar flow hood using sterile PVDF Durapore filters, 0.45µm pore size, 13mm (Millipore). Then, the proteins were quantified according to the modified Bradford method [24], using bovine serum albumin (BSA). The analyses were performed under spectrophotometer Thermo BioMate 3 (Madison USA), wavelength 595nm.

The concentration of 0.04 µg/µL of salivary gland extract, used in this work, was obtained by diluting of extract in sodium phosphate buffer pH 7.4, where concentration of the extract was according to Abreu et al. [23].

Inoculation with PBS and SGE

The inoculations with 300 µg/µL of PBS in the individuals from PBS1 and PBS2 and 300 µg/µL of SGE (0.04 µg/µL) in the rats belonging to the SGE1 and SGE2 were performed by a veterinarian, using an 1mL syringe, in a sterilized room at the Animal Facility of the Biology Department, Biosciences Institute, UNESP campus Rio Claro, SP, Brazil.

The first PBS (groups PBS1 and PBS2) and SGE (groups SGE1 and SGE2) doses were injected in the posterior right leg of the animals, now 103 days old; while the second doses (groups PBS2 and SGE2), applied after 4 days, were inoculated in the left posterior leg of the individuals.

Blood collection, kidneys removal and euthanasia of the individuals

The blood used in the blood count analysis was collected from the heart. The left kidney of each animal was removed, fragmented, and prepared for the application of morphological techniques (histology and histochemistry).

Then, the animals were euthanized by a veterinarian, using ketamine and xylazine. These procedures were performed in the surgical center of the veterinary clinics Polivet, Rio Claro, SP, Brazil.

Blood count
Blood samples were collected from the heart of all the animals (124 days old), using a 1mL-syringe. The animals were anesthetized with ketamine and xylazine by a veterinarian in Polivet clinic, Rio Claro, SP, Brazil. The blood was transferred to labelled BD Vacutainer® tubes containing EDTA. The results were evaluated in the Veterinary Diagnosis Center (CedVet), Rio Claro, SP, Brazil.

Body weight
All the rats were weighed twice, using scale Marte, model LC1, in the Animal Facility of the Biology Department, Bioscience Institute, UNESP, Rio Claro, SP, Brazil. The animals were weighed at the age of 97 days (without receiving any treatment – PBS or SGE) and 124 days, when they were euthanized.

Statistical analysis
The animals were weighed, and the results were presented as mean ± EPM and submitted to multiple comparison variance analysis (ANOVA), followed by Bonferroni test. Program GraphPad Prisma (GraphPadPrism Software Inc, 2007) was used to perform the analysis.

Light Microscopy Analysis
Histology and histochemistry
For the application of the histological (HE) and histochemical (toluidine blue and xylidine Ponceau) techniques, fragments of the kidneys were fixed in aqueous Bouin's solution for 72 hours, dehydrated in alcohol series 70-95% for 15 minutes each bath and transferred to resin for 48 hours. The material was then included in Leica resin and the blocks were polymerized in incubator (37ºC), and sectioned using microtome Leica RM 2145.

The 3µm-thick sections were mounted on glass slides and dried in incubator at 37ºC for the application of the following techniques:

Harris hematoxylin and aqueous eosin to stain acid and base structures, respectively [25]
The sections were stained with Harris hematoxylin for 10 minutes, washed in running tap water for 5 minutes to allow the reaction and remove excess hematoxylin. The sections were stained with eosin for 5 minutes followed by washing with running tap water and later, left to get air dried.

Toluidine Blue to detect DNA and RNA [26].
The sections were stained with toluidine blue for 3 minutes, washed in running tap water and left to get air dried.

Xylidine Ponceau to detect total protein [27].
The sections were stained with xylidine Ponceau for 30 minutes, washed in distilled water and left to get air-dried. After the application of the techniques, the slides were mounted in Canada balsam and dried in incubator at 37ºC for further analysis and further documented in photomicroscope MOTIC BA 300.

Results and Discussion

The kidneys of the animals belonging to the control group (CG) served as reference to detect and demonstrate the morphological alterations occurred in the kidneys from the individuals subjected to different treatments; therefore, the histological and histochemical results regarding this group are listed first. The present study analyzed the cortical region of the organ, as the medullar region did not suffer any morphological alterations.

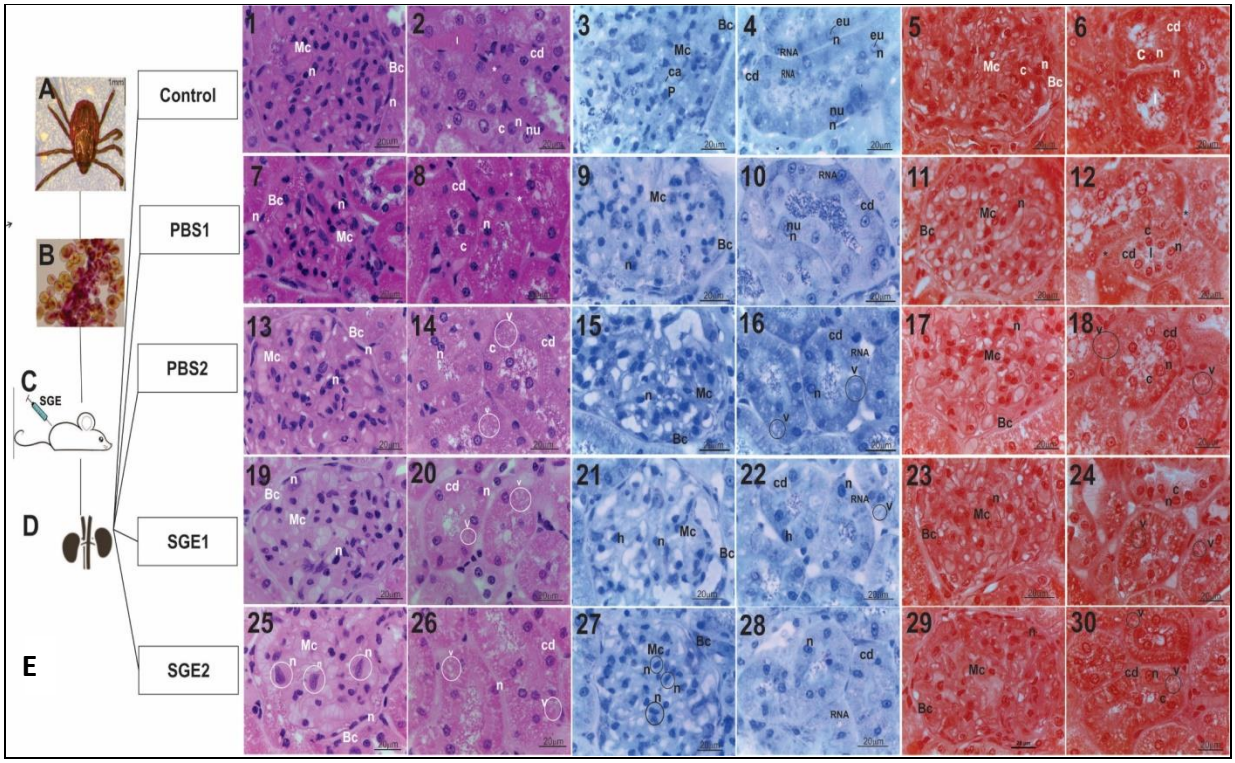


Figure 1. (A) Rhipicephalus sanguineus female tick, (B) Full montage of salivary gland using acid phosphatase technique, (C) SGE (salivary gland extract) (D) Scheme of Wistar rat treated with SGE, (E) Scheme of rat kidneys.

1-30: Histological sections of male rat kidneys pre/post inoculated with *Rhipicephalus sanguineus* salivary gland extract, submitted to the following stains or reactions:
HE: CG (Figures 1, 2)**, PBS1** (Figures 7, 8)**, PBS2** (Figures 13,14)**, SGE1** (Figures 19, 20)**, SGE2** (Figures 25, 26).
TB: CG (Figures 3,4)**, PBS1** (Figures 9, 10)**, PBS2** (Figures 15, 16)**, SGE1** (Figures 21, 22)**, SGE2** (Figures 27, 28)**.**
XP: CG (Figures 5, 6) **PBS1** (Figures 11, 12)**, PBS2** (Figures 17, 18)**, SGE1** (Figures 23, 24)**, SGE2** (Figures 29, 30)**.**

CG (Control Group): non-inoculated individuals**, PBS1=** individuals inoculated once with 300 µg/µL of saline phosphate buffer 1x–PBS, containing the diluted extracts**, PBS2=** injected twice with 300 µg/µL of PBS, at a 4-day interval between the injections**, SGE1= Treatment 1 (SGE1):** inoculated once with 300 µg/µL of salivary gland extract (SGE), 0.04 µg/µL, **SGE2=** inoculated twice with 300 µg/µL of SGE, at a 4-day interval between the injections

HE= Hematoxylin/Eosin, **TB=** Toluidine Blue, **XP=** Xylidine Ponceau

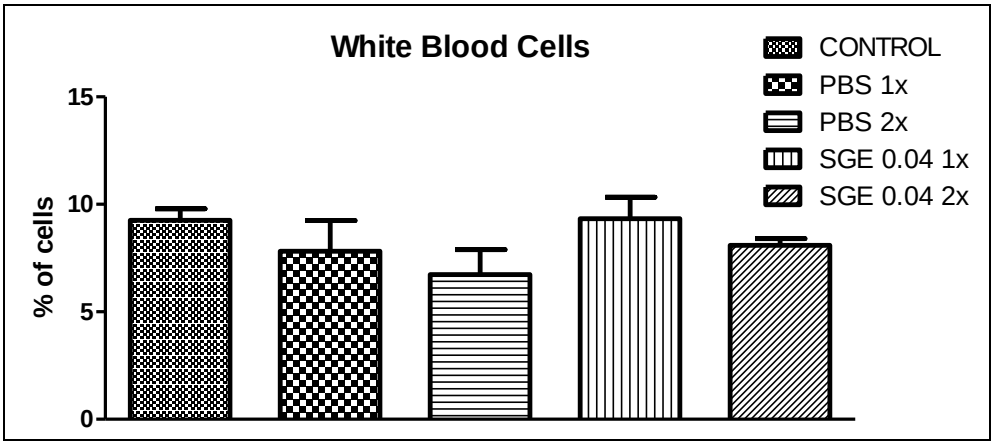
Bc= Bowman's capsule, **c=** cytoplasm, **ca=** capillary, **cd=** collector duct, **DNA=** Deoxyribonucleic acid, **eu=** euchromatin, **h=** heterochromatin, **l=** lumen, **Mc=** Malpighian corpuscle, **n=** nucleus, **nu=** nucleolus, **P=** podocyte, **RNA=** Ribonucleic acid, **v=** vacuolation, ***=** intertubular space**.**

Control group (CG)

The renal tissue of these animals – not exposed to PBS or SGE – was intact, constituted of the cortical and medullar regions. In the cortical region, the Malpighian corpuscles (Figure 1-1) showed intact morphology; i.e., preserved elements, and the Bowman's capsule (Figure 1-1), with flattened cells and nuclei (Figure 1-1). The latter were strongly stained by hematoxylin, with the presence of heterochromatin (Figure 1-1).

The proximal and distal convoluted tubules were transversal and obliquely sectioned (Figure 1-2), with cubic cells and spherical hematoxylin-stained nuclei and nucleoli (Figure 1-2). The tissue was intact (Figure 1-2), and the cytoplasm, the lumen of the tubules and the intertubular spaces showed basic character material (Figure 1B).

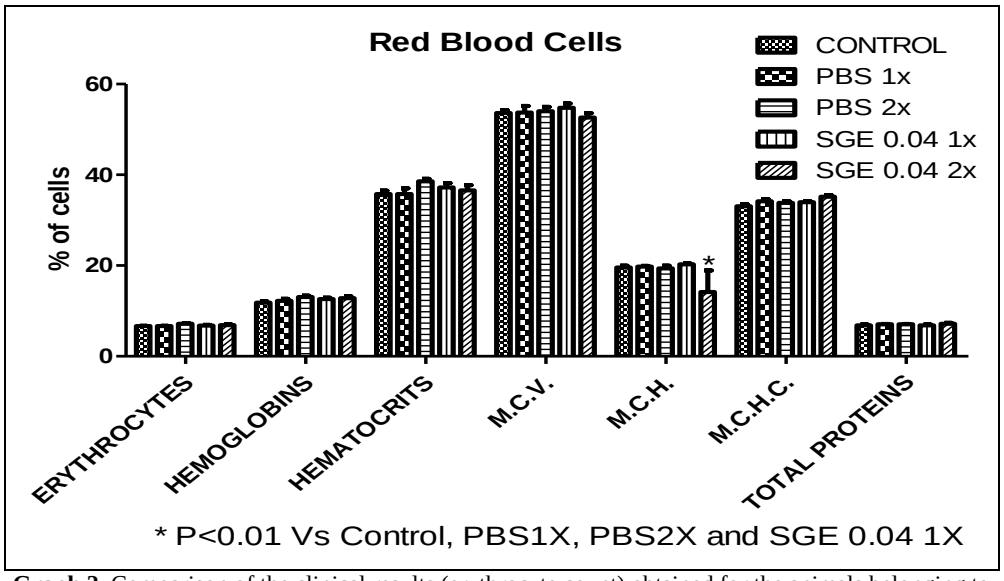
The toluidine blue technique applied in the kidney histological sections showed intact glomerular capillaries in the interior of the Malpighian corpuscles (Figure 1-3). Some nuclei were detected through light



Graph 1. Comparison of the clinical results (leukocytes count) of the animals belonging to CG and the ones from the groups PBS1, **PBS2**, **SGE1** and **SGE2** (n=4)
CG (Control Group): non-inoculated individuals,
PBS1= individuals inoculated once with 300 µg/µL of saline phosphate buffer 1x–PBS, containing the diluted extracts,
PBS2= injected twice with 300 µg/µL of PBS, at a 4-day interval between the injections,
SGE1= **Treatment 1 (SGE1):** inoculated once with 300 µg/µL of salivary gland extract (SGE), 0.04 µg/µL,
SGE2= inoculated twice with 300 µg/µL of SGE, at a 4-day interval between the injections

microscopy, and strongly stained DNA were observed (Figure 1-3). The cells of the convoluted tubules were evident; the nuclei showed dispersed chromatin (euchromatin) and stained nucleolar DNA (Figure 1-4). The cytoplasm of these cells were moderately stained, with the presence of RNAs (Figure 2B).

The xylidine Ponceau technique was used to detect total protein, showing the renal corpuscle (Figure 1-5) and the convoluted tubules with strongly stained nuclei (Figure 1-5) and cytoplasm (Figure 1-6).



Graph 2. Comparison of the clinical results (erythrocyte count) obtained for the animals belonging to the group CG and the ones from the groups **PBS1**, **PBS2**, **SGE1** and **SGE2** (n=4)
CG (Control Group): non-inoculated individuals**,**

PBS1= individuals inoculated once with 300 µg/µL of saline phosphate buffer 1x–PBS, containing the diluted extracts**,**
PBS2= injected twice with 300 µg/µL of PBS, at a 4-day interval between the injections,
SGE1= Treatment 1 (SGE1): inoculated once with 300 µg/µL of salivary gland extract (SGE), 0.04 µg/µL
SGE2= inoculated twice with 300 µg/µL of SGE, at a 4-day interval between the injections

Group PBS1 (PBS 1X)

The kidneys of the animals belonging to this group were intact, and the nuclei of the Bowman's capsule cells (Figure 1-7) and of the renal glomerulus did not display any alterations (Figure 1-7). However, the nuclei of the convoluted tubule cells were intensely stained (Figure 1-8). The inter-tubular spaces were larger (Figure 1-8) in comparison with the ones from the individuals belonging to CG (Figure 1-2).

Toluidine blue technique showed that the nuclei of the glomerular cells (Figure 1-9) and of the convoluted tubules (Figure 1-10) presented similar morphology to the CG (Figures 1-3 and 1-4). However, the cytoplasm of the cells from the tubules displayed slight RNA staining (Figure 1-10).

In the Malpighian corpuscle cells (Figure 1-11), in the convoluted tubules (Figure 1-12) under their light (Figure 1-12) and in the inter tubule spaces (Figure 1-12) total proteins were moderately stained in comparison with the CG (Figures 1-5 and 6).

Group PBS2 (PBS 2X)

The results of the analysis of the kidneys from the individuals subjected to two PBS (Figures 1-13, 14, 15 and 16) applications showed some cellular alterations in comparison with the CG (Figures1-1, 2, 3 and 4).

The total protein (Figures 1-17 and 18) staining result was similar to those described for the kidney cells of the individuals belonging to the group PBS1 (Figures 1-11 and 12).

Treatment group 1 (SGE1 1X)

The morphology of the cells from the individuals belonging to this group (Figures 1G-H) was similar to one of the group PBS2 (Figures 1-13 and 14). These results were confirmed by the application of toluidine blue (Figures 1-21 and 22) and xylidine Ponceau (Figures 1-23 and 24) techniques. The nuclei of the glomerulus (Figures 1-21) and convoluted tubule (Figure 1-22) cells presented higher concentration of heterochromatin, stained by toluidine blue (Figures 1-21 and 22).

Treatment group 2 (SGE2 2X)

Morphological alterations in the shape and size of several nuclei were observed in the glomerular region of the kidneys of the individuals this group (Figure 1-25). These results were confirmed by the application of toluidine blue (Figure 1-27). The results of contorted tubule cells (Figures 1-26, and 28) were similar to those presented in the PBS2 group (Figures 1-14 and 16).

Total protein (Figures 1-29 and 30) staining revealed the same pattern of the kidneys from the individuals belonging to CG (Figures 1-5 and 6).

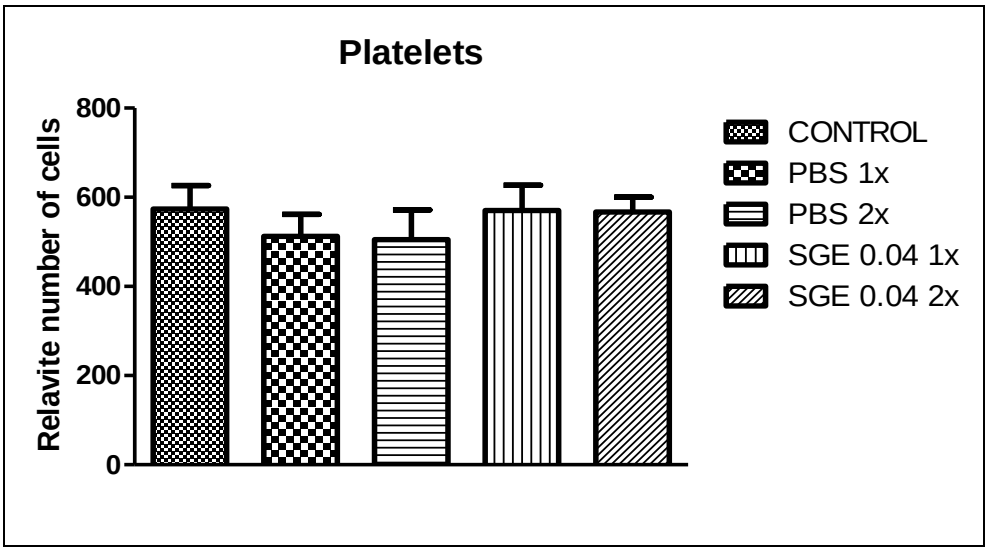
Blood count

The results of the blood count clinical analysis (CG, PBS1, PBS2, SGE1 and SGE2) were plotted in graphs for the comparison of the parameters; i.e., leukocytes (Graph 1), erythrocytes (Graph 2) and platelets (Graph 3).

There were no significant differences when the means were compared, showing that neither the PBS used to dilute the extracts, nor the extracts in different dilutions were able to affect the production of blood constituents in the inoculated rats.

Body weight

The body weight of the rats from all the groups was measured and the results of the averages (Graph 4), showed that all the animals gained weight throughout the experiment; in addition, none of them died.



Graph 3. Comparison of the clinical results (platelet relative number) obtained from the animals belonging to group **CG** and the ones from the groups **PBS1**, **PBS2**, **SGE1** and **SGE2** (n=4)
CG (Control Group): non-inoculated individuals**,**
PBS1= individuals inoculated once with 300 µg/µL of saline phosphate buffer 1x–PBS, containing the diluted extracts,
PBS2= injected twice with 300 µg/µL of PBS, at a 4-day interval between the injections,
SGE1= Treatment 1 (SGE1): inoculated once with 300 µg/µL of salivary gland extract (SGE), 0.04 µg/µL,
SGE2= inoculated twice with 300 µg/µL of SGE, at a 4-day interval between the injections

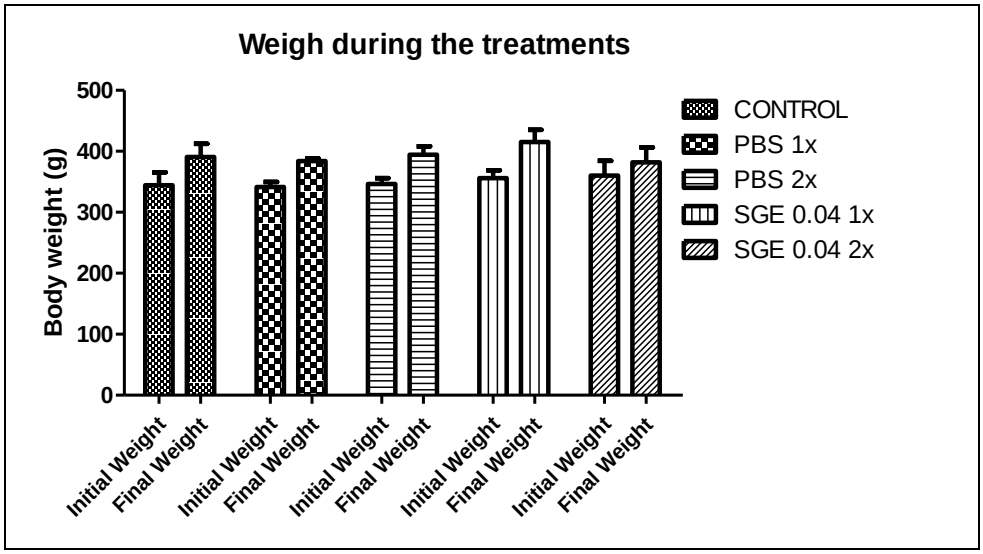
Discussion

Abreu et al. [23] have recently demonstrated that the salivary secretions of female *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) ticks fed for two days on host rabbits have the potential to inhibit the invasion of tumor cells in the muscles of Wistar rats inoculated with one 0.04µg/µL injection. These authors, using bright field microscopy and TEM techniques, found that, in addition to preventing the invasion of tumor cells in the rat muscles, the extract would not cause histopathological damage to the liver. The same authors emphasized that, although it had been demonstrated that *R. sanguineus* salivary gland extracts had the capacity to inhibit the multiplication of tumor cells, there were no studies showing the occurrence of alterations in the physiology of the individuals exposed, mainly in the kidneys, encouraging the other authors to investigate the tissues.

In this study, the morphological data was obtained through bioassays using male Wistar rats inoculated with one or two 300µg/µL injections of salivary gland extract (concentration of 0.04µg/µL) obtained from female *R. sanguineus* ticks fed for two days on host rabbits. Each rat was weighed one day before the first inoculation and 21 days after the last one (individuals inoculated twice), and a blood count analysis was performed for each animal, in order to detect any signs of systemic toxicity.

Overall, the results showed that the kidneys of the individuals belonging to the control group (CG) – not exposed to the phosphate buffered saline 1, 2X-PBS or salivary gland extracts- showed intact renal tissue, with very evident cortical and medullar regions, noting that in this study only the cortical region was considered, as the medullar one did not show any alterations. The Malpighian corpuscles were intact and the Bowman's capsule was preserved according to the expected result, since the individuals were not exposed to any test substance.

The histology and histochemical analysis of the individuals exposed to PBS (PBS1 and PBS2), buffer used to dilute the extracts, showed that those inoculated with one injection displayed the kidneys with intact tissue (as the individuals belonging to control group), as well as regular distribution of the cells; therefore, the buffer was not able to disorganize the histological constitution of the organ.



Graph 4. Body weight averages **(CG, PBS1, PBS2, SGE1, and SGE2)** obtained in the beginning and in the end ofthe bioassay

CG (Control Group): non-inoculated individuals**,**
PBS1= individuals inoculated once with 300 µg/µL of saline phosphate buffer 1x–PBS, containing the diluted extracts,
PBS2= injected twice with 300 µg/µL of PBS, at a 4-day interval between the injections,
SGE1= Treatment 1 (SGE1): inoculated once with 300 µg/µL of salivary gland extract (SGE), 0.04 µg/µL,
SGE2= inoculated twice with 300 µg/µL of SGE, at a 4-day interval between the injections

In the collecting intertubules spaces were observed, and more frequently in the group PBS2, which shows different moments of renal absorption between rats from different groups.

The comparison between the results for the animals belonging to treatment groups SGE1 and SGE2, which received one and two injections containing 300µg/µL of R. sanguineus salivary gland extract, 0.04µg/µL, respectively, showed that the ones that were once inoculated displayed renal tissue cells with similar morphology to the group PBS2. In addition to cell alterations, there was loss of metachromasy in the cytoplasm of the convoluted tubule cells, revealed by the weak toluidine staining in this compartment of the cell. The loss of cytoplasmic metachromasy could indicate a decrease in the synthesis of protein in the cells, as RNA is responsible for the cytoplasmic metachromasy [28]. In this sense, we could observe that, despite the weak RNA staining by Toluidine blue, the protein synthesis was not affected, as this organic product was strongly stained by xylidine Ponceau in the groups SGE1 and SGE2.

In the kidneys of the individuals belonging to group SGE2, the morphological alterations (shape and size) occurred in several nuclei located in the glomerular region indicating the cell death [29, 30, 31], that is the natural process of programmed tissue renewal.

The hematologic tests performed in this study showed that one or two injections of PBS (PBS1 and PBS2) and of the salivary gland extracts (SGE1 and SGE2) did not cause significant alterations; i.e., the results are within the reference standards. Regarding the body weight parameter, the results showed that the substances tested would not interfere in the metabolism of the animals, causing them to lose weight during the experiment. The results showed that, if some systemic toxicity was caused by the PBS or the SGEs, it was very slight. According to the norms provided by Guideline 423 about acute toxicity (OECD - Organisation for Economic Cooperation and Development), the systemic toxicity in the organisms would be defined by the alterations in the hematological parameters [32], and in the animal body weight [33].

Thus, this study confirmed and complemented the information obtained by Abreu et al., [23] that demonstrated that the bioactives present in *R. sanguineus* salivary glands would potentially inhibit the invasion Walker 256 in Wistar rat muscles, without affecting the general metabolism. The results confirmed

that this extract (concentration of 0.04µg/µL), when applied once or twice in the individuals of the treatment groups SGE1 and SGE2, respectively, did not cause pathological damage in the kidneys of the individuals. The injections containing PBS 1X (groups PBS1 and PBS2) showed some morphological alterations in the glomerular and collecting ducts regions; however, the integrity of the organ was preserved, as the protein synthesis was not affected. This data was confirmed by the hematological and weight parameters, which were unaltered throughout the experiment, showing that there were no signs of systemic toxicity in the analyzed animals.

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