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Immunomodulatory and morphophysiological effects of *Rhipicephalus sanguineus* s. l. (Acari: Ixodidae) salivary gland extracts

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ABSTRACT

Rhipicephalus sanguineus s. l. is popularly known as the "brown dog tick" since dogs are its preferential hosts, but the species has been reported to parasitize other mammals, including humans, with significant medical-veterinary importance since it transmits several important pathogenic agents during the feeding period. The tick saliva is a complex mixture that has several functions, including the capability to modulate the hemostatic, inflammatory and immunologic systems of the host, allowing pathogens to settle. Despite knowledge about the immunosuppressive action of tick saliva, little is known about the mechanisms involved in this process and the morphophysiological effects caused by exposure to the salivary gland extract, taking into consideration the different periods of the glandular cycle. Thus, the objective of this study was to analyze the *in vitro* effects of salivary gland extracts obtained from *R. sanguineus* s. l. females fed on host rabbits for two (**SGE2 – Salivary Gland Extracts of 2 days**) and four days (**SGE4 – Salivary Gland Extracts of 4 days**) on J774 cells (monocyte macrophage cell line) and verify the occurrence of morphological and immunomodulatory alterations in these cells when exposed to different concentrations of these extracts. The results showed that: (i) **SGE2** and **SGE4** at the concentration of 4 μg/mL presented cytotoxicity to the J774 cells exposed for 24 and 48 hours; (ii) **SGE2** at the concentrations of 2 μg/mL(48-hour exposure) and 1 μg/mL (24-hour exposure) and **SGE4** at the concentrations of 2 and 1 μg/mL (48-hour exposure) showed proinflammatory activity, confirmed by the increased secretion of NO and proinflammatory cytokine (IL-2), and the presence of morphological characteristics detected by microscopy; and (iii) **SGE2** and **SGE4** at the concentrations of 0.5 and 0.1 μg/mL had immunomodulatory activity, demonstrated by decreases in the secretion of NO and proinflammatory cytokines (IL2, IL-6 and TNF-α) and increase in the synthesis of IL-10, confirmed by the morphophysiological analysis. These unprecedented data are extremely relevant for future research to identify the processes involved in the ectoparasite-host relationship, as well to develop more efficient tick control strategies.

1. Introduction

Ticks are obligatory hematophagous ectoparasites of several organisms, including mammals, birds, reptiles, amphibians and other arachnids, even ticks. These arthropods are widespread, found in all the regions of the globe (Walker et al., 2005; Anderson and Magnarelli, 2008).

The species *Rhipicephalus sanguineus* s. l. is popularly known as the "brown dog tick" since dogs are its hosts. However, the species has been reported to parasitize other mammals, including humans (Dantas-torres et al., 2006; Gray et al., 2013a, 2013b). Furthermore, *R. sanguineus* s. l. ticks have significant veterinary importance (Cardoso et al., 2006; Cunha et al., 2009), since they transmit several important pathogenic agents, such as *Ehrlichia canis*, *Babesia canis*, *Haemobartonella canis* and *Hapatozoon canis* in dogs; and *Rickettsia conorii* (Europe) and *R. rickettsii* (Mexico) in humans (Dantas-Torres, 2008; Gray et al., 2013b).

The salivary glands are vital for the biological success of ticks, since these organs are responsible for the production of substances that ensure the processes of fixation and feeding on the host (Walker et al., 2005). Tick saliva is a complex mixture that has several functions, including the ability to modulate the hemostatic, inflammatory and immunologic systems of the host, by: i) increasing blood flow in the feeding lesion region by secreting vasodilating agents; ii) inoculating anticoagulants to ensure blood fluidity; and iii) inhibiting the host

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inflammatory process by acting as immunosuppressant, allowing the ectoparasite fixation and preventing host rejection (Bowman et al., 1996).

The bioactive substances present in tick saliva contain glycol and lipoprotein elements (Binnington, 1978; Wheeler et al., 1991), such as acid phosphatase, esterase, aminopeptidase, metalloproteinase, prostaglandin, lipocalin, calreticulin (Binnington, 1978; Mulenga et al., 2007; Oliveira et al., 2011) and other molecules with still unknown properties. In addition to its complex composition, tick saliva presents quantitative and qualitative variations regarding glandular cycle and species (Binnington, 1978; Camargo-Mathias, 2013; Xiang et al., 2009). In *R. sanguineus* s. l., the secretion produced by the salivary glands has different characteristics in the beginning (two days), middle (four days) and end (six days) of the secretory cycle (Camargo-Mathias, 2013). Such variations occur due to the host immune system and the fact that the tick saliva components have coevolved, in an 'arms race', against the host defense mechanisms (Kotál et al., 2015).

Despite knowledge about the immunosuppressive action of tick saliva, little is known about the morphophysiological effects caused by exposure to the salivary gland extract, taking into consideration the different periods of the glandular cycle, since there are distinct characteristics. Moreover, studies of this saliva's effects on the host inflammatory immune system have been considered promising for the formulation of new and more effective methods to control this important tick species.

Macrophages are also responsible for the phagocytosis and elimination of invading microorganisms, as well as secretion of signaling proteins (cytokines) that activate and recruit other cells of the immune system, a key role in the induction of inflammation and the success of the response (Abbas et al., 2015). Important interactions have been reported between macrophages, tick saliva or salivary glands, and pathogens, suggesting they play a major role in host defenses against ticks and tick-borne infectious agents (Kotál et al., 2015).

The J774 cell line originated from macrophages isolated from histiocytic sarcoma. This cell line is frequently used in *in vitro* studies of macrophage immunoregulation, since it produces immunological mediators similar to original cells, such as cytokine, nitric oxide (NO) and prostaglandins (D'Acquisto et al., 1997; Justo et al., 2015; Marotta et al., 1992).

The objective of this study was to analyze the *in vitro* effects of salivary gland extracts obtained from *R. sanguineus* s. l. female ticks fed on host rabbits for two (**SGE2**) and four days (**SGE4**) on J774 cells and verify the occurrence of morphological and immunomodulatory alterations in these cells when exposed to different concentrations of these extracts.

2. Material and methods

All the procedures were approved by the Ethics Committee on Animal Use, Biosciences Institute, UNESP –Rio Claro Campus), under protocol no. 9133.

2.1. R. sanguineus s. l. ticks

In this experiment, 200 adult *R. sanguineus* s. l. females were used. The ticks were collected from the colony maintained by the Animal Facilities of the Biology Department of UNESP, Rio Claro Campus, SP, and kept in a BOD incubator under controlled conditions (29 °C, 80% humidity and 12 h photoperiod). The eight female host rabbits (naïve, Botucatu genetic group) were provided by the Central Animal Facility of UNESP, Botucatu Campus.

The infestations were performed according to the protocol stablished by Bechara et al. (1995): feeding chambers were attached to the dorsal region of host rabbits and 25 pairs of ticks were placed on each rabbit. After two and four days, the female ticks were collected, anesthetized by thermal shock (placed in a freezer for 5 min), and had their salivary glands removed for the preparation of **SGE2** and **SGE4** extracts (about 20 salivary gland pairs per extract).

2.2. Salivary gland extracts (SGE2 and SGE4)

After two and/or four days, the female ticks were removed from the hosts by the hypostome region using tweezers and circular movements. The females were mounted on Petri dishes containing saline solution (7.5 g of NaCl, 2.38 g of Na_2HPO_4, 2.72 g of KH_2PO_4 and 1000 mL of distilled water) for dissection and removal of the salivary glands, which were placed in Eppendorf tubes with 200 µL of phosphate buffer, pH 7.4 (separated by feeding period) and kept on ice.

Afterwards, the glands were rinsed twice with the same buffer, centrifuged for 10 min at 2500 rpm, and delicately macerated with a glass rod. The tubes were centrifuged again for 5 min at 2500 rpm and the supernatant was collected under a sterile laminar flow hood and filtered through disposable units (JBR610303, Millex GV, PVDF Durapore membrane, Millipore®, MilliUni), 0.22 µm/13 mm diameter, coupled to a hypodermic syringe, transferred to sterile Eppendorf tubes and submitted to protein quantification (Sedmak and Grossberg, 1977) (Bradford method).

Following protein quantification, the extracts were diluted in autoclaved in phosphate buffer pH 7.4 to obtain the assay concentrations (4, 2, 1, 0.5 and 0.1 µg/mL).

2.3. Cell culture

Cell line J774 (ATCC) was kindly provided by Dr. Wirla Maria da Silva Cunha Tamashiro of the Cellular Immunology and Inflammation Laboratory of the Genetics, Evolution and Bioagents Department of UNICAMP, Campinas, SP. This cell line is a macrophage-like mouse cell line originally isolated from reticulum cell sarcoma. Interleukin 1β is synthesized continuously by this cell line, which is then commonly used as a tool to test anti-inflammatory agents (Justo et al., 2015)

The J774 cells preserved in liquid nitrogen were reactivated and cultured until semi-confluence in complete RPMI 1640 Sigma® (R6504) medium containing HEPES (2.97 g/L), sodium bicarbonate (2 g/L), 2-mercaptoethanol (2 µL/L) and supplemented with 10% fetal bovine serum (FBS), in culture flasks in a humidified incubator with 5% CO_2, at 37 °C. Then the cells were harvested using a cell scraper, washed with RPMI medium, and centrifuged in Falcon tubes at 1500 rpm for 10 min at 4 °C. The pellet was resuspended in RPMI medium and cells were counted in a Neubauer chamber with trypan blue (1:2). The concentration was adjusted and J774 cells were seeded at a density of 2×10^5 cells/well in 96-well plates. The non-adherent cells were removed by washing after 24 h. Lipopolysaccharide (LPS) (1 µg/mL) was added as inflammation stimulus. Then the concentrations (4, 2, 1, 0.5 and 0.1 µg/mL) of **SGE2** (**Stimuli 1** and **3**) or **SGE4** (**Stimuli 2** and **4**) were added to the wells, in quadruplicate. Two control groups were stablished: negative control group (cells on RPMI) and control group (cells on RPMI and stimulated by LPS only). The cells were incubated in the same culture conditions for 24 (**Stimuli 1** and **2**) and 48 h (**Stimuli 3** and **4**).

2.4. Cell viability assay (MTT)

Cell viability was evaluated using a colorimetric assay based on the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). An aliquot of 10 µL of MTT (5 mg/mL) dissolved in 0.02 M phosphate buffered saline (PBS) at pH 7.0 was added to each well of the culture cell plates and the plates were incubated for 4 h at 37 °C. The formazan crystals formed by MTT reduction by living cells were dissolved in 5% SDS in 0.01 N HCl solution and the optical density was measured using a Multiskan MS microplate reader (Labsystems Oy, Helsink, Finland) at 540 nm. Each experiment was performed at least in quadruplicate.

2.5. Measurement of nitrite concentration

The secretion of nitric oxide (NO) in the supernatant of the J774 cells was indirectly determined using a quantitative colorimetric assay based on Griess reaction, according to Griess (1879). The nitrite standard curve was obtained with sodium nitrite standard solution from 320 at 0 μM/L (stock solution of 320,000 μM/L in complete RPMI), used as a basis for sample quantification.

Aliquots of 50 μL of supernatant obtained from the exposure of cell cultures to the different concentrations of **SGE2** and **SGE4** were transferred to 96-well culture plates and 50 μL of Griess reagent/well (1% sulfanilamide, 0.1% N- (1-naphthylethylenediamine) dihydrochloride and 2.5% phosphoric acid) was added. After 10 min at room temperature, the plate was analyzed using the Multiskan MS reader (Labsystems Oy, Helsink, Finland) at 570 nm. This bioassay was performed in quadruplicate.

2.6. Cytokine determination

Supernatants of the J774 cultures were collected for quantification of cytokines, performed using a commercial kit (BD-Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit; BD Biosciences Pharmingen, San Diego, CA), according to the manufacturer's recommendations. The following cytokines were analyzed: IL-2, IL-4, IL-6, IL-10, IL-17 A, IFN-γ and TNF.

The standard curve beads were reconstituted with the wash buffer included in the kit (top standard). After 15 min at room temperature, serial dilutions were performed (1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128; 1:256), as well as a blank tube.

The capture beads provided by the kit were resuspended in capture bead mix. Then the bead mix was added to the standard curve and sample tubes. Next, the Mouse Th1/Th2/Th17 detection reagent was added to each tube. All the tubes were incubated for 3 h in the dark, at room temperature.

After incubation, 1 mL of wash buffer was added to each tube, and the samples were centrifuged at 200 g for 5 min. The supernatant was discarded and the pellet was resuspended in 300 μL of wash buffer. Immediately afterward, the tubes were analyzed and IL-2, IL-4, IL-6, IL-10, IL-17 A, IFN-γ and TNF-α were quantified in culture supernatants of spleen cells by flow cytometry using a Multiplex CBA kit (BD Cytometric Bead Array Th1/Th2/Th17, San Diego, USA) according to the manufacturer's instructions. Cells were acquired in a FACSCalibur cytometer and analyzed with FCAP Array™ software, version 3.0 (BD) at the Laboratory of Cellular Immunology and Inflammation, Department of Genetics, Evolution and Bioagents, UNICAMP, Campinas. The results were generated using the BD CBA analysis software.

2.7. Statistical analysis

The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The significance of differences between treatments was determined by one-way ANOVA, followed by multiple-comparison Bonferroni test. Results were expressed as mean ± standard error mean (SEM). Values were considered significant at $P < 0.05$. All data are representative of at least three independent experiments.

2.8. Morphological analysis

The J774 cells were seeded on glass coverslips at density 2×10^5 cells/well in 24-well plates and incubated in RPMI medium supplemented with 10% SFB at 37 °C with 5% CO_2 for 24 h. Next, 1 μg/mL of LPS was added to each well + the **SGE2** and **SGE4** concentrations (4, 2, 1, 0.5, 0.1 μg/mL). The control wells (only cells or cells with LPS) were added with 100 μL of RPMI medium or 1 μg/mL of LPS + 50 μL of RPMI medium, respectively, and were incubated under the same culture conditions for 24 (**Stimuli 1** and **2**) or 48 h (**Stimuli 3** and **4**).

Following incubation, the supernatant was discarded and 500 μL/well of paraformaldehyde fixative was added to each culture plate. The plates were kept refrigerated overnight. After fixation, the wells were rinsed with sterile phosphate buffer pH 7.4 (300 μL/well). The samples were dehydrated in an ethanol series at 70, 80, 90, 95 and 100%, 5 min each bath.

For cell staining, 300 μL/well of Harris hematoxylin was added, and after 1 min the excess stain was removed and the samples were rinsed under tap water for 5 min, and then with distilled water for 30 s. Afterwards, 300 μL/well of aqueous eosin was added, and after 1 min the samples were rinsed with distilled water for 30 s.

The samples on the glass coverslips were carefully removed from the 24-well plates, washed in xylol, mounted on clean slides with Entellan® (Merck Millipore), and analyzed and documented under a Leica DM750 bright field microscope.

3. Results

3.1. Evaluation of cytotoxicity

The J774 cells were stimulated with LPS and exposed to the extracts **SGE2** and **SGE4** for 24 or 48 h for evaluation of the cytotoxic activity of the extracts at concentrations of 4; 2; 1; 0.5 and 0.1 μg/mL (Fig. 1**A-D**).

The exposure to **SGE2** for 24 h did not present significant cytotoxicity in comparison with the control group (cells exposed to LPS). The results regarding the viability of cells exposed to **SGE2** for 48 h were similar to the control group, except for the concentration of 4 μg/mL, which caused a significant increase in cell death rate, suggesting the toxicity of this concentration (Fig. 1C).

Exposure to different concentrations of **SGE4** did not show significant increase in cell death in comparison with the control group (cells exposed to LPS), either after 24 or 48 h (Fig. 1**B-D**).

The statistical results of the viability test suggested that the extracts **SGE2** and **SGE4** are not toxic to J774 cells, except at the concentration of 4 μg/mL of **SGE2**.

3.2. Evaluation of NO production

In order to determine the anti or proinflammatory effects of the extracts, NO secretion in the supernatant J774 cells stimulated by different concentrations of **SGE2** and **SGE4** extracts during 24 and 48 h was evaluated.

The cells exposed to the **SGE2** for 24 h presented an increase in NO secretion at the different concentrations compared with the control group. All the groups of cells exposed to the extract presented such increase, considered significant in comparison with the control group (Fig. 2A). After 48 h of exposure to **SGE2** at 4 μg/mL, 2 and 1 μg/mL, there was a significant increase in NO secretion in comparison with the control group (Fig. 2C)

The statistical analysis of **SGE4** stimulation for 24 h indicated a significant decrease in NO production by J774 cells compared with the control group only at the concentration of 4 μg/mL (Fig. 2B). In turn, the cells exposed to **SGE4** for 48 h presented significantly higher NO secretion at 4, 2 and 1 μg/mL (Fig. 2D).

The concentrations of 4, 2 and 1 μg/mL of both extracts presented proinflammatory activity after 48 h of exposure. After 24 h, only the concentration of 4 μg/mL of **SGE4** presented higher anti-inflammatory activity in comparison with the control group (Fig. 2**A-D**).

3.3. Quantification of cytokines

The quantities of cytokines IL-2, IL-6, IL-10, and TNF-α was measured in all the supernatant samples.

Stimulation of J774 cells with different concentrations of **SGE2** did not cause significant alterations in secretion of cytokines in comparison

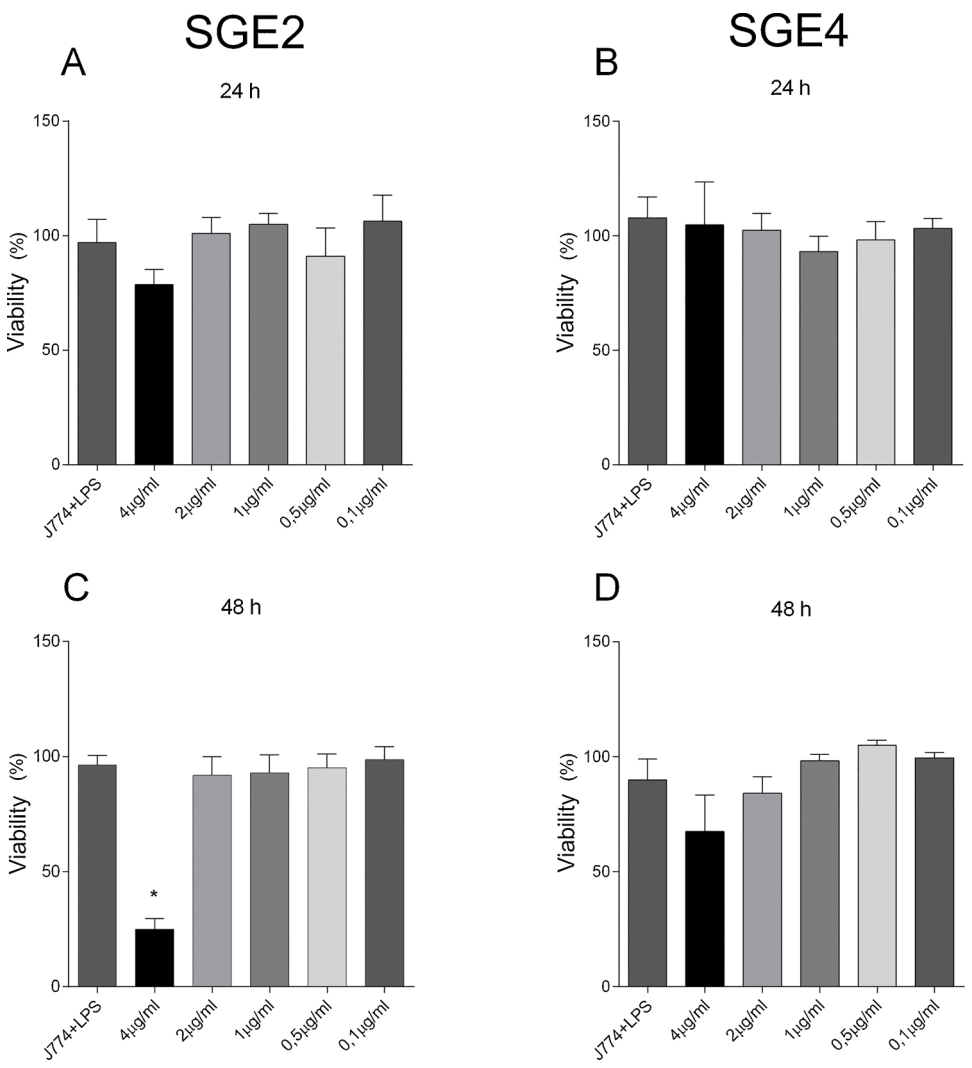

Fig. 1. (A–D): Viability of J774 cells exposed to different concentrations of **SGE2** and **SGE4** for 24 and 48 h. The columns represent the means ± SEM (standard error of the mean) of the data obtained through quadruplicate testing (*) indicates statistically significant differences ($p < 0.05$) in comparison with the control group (J774 + LPS).

with the control group (cells exposed to LPS), either after 24 or 48 h (Fig. 3**A-H**), except for the concentrations of 4 and 2 ug/mL of SGE2, which diminished the secretion of TNF (Fig. 3F).

The quantity of IL-6 by the J774 cells exposed to **SGE4** for 24 h was significantly lower at the concentration of 1 µg/mL (Fig. 4C) when compared with the control group. All concentrations of **SGE4** also significantly decreased TNF-α secretion when compared with the control group (Fig. 4E).

The quantity of IL-2 secreted by the J774 cells exposed to **SGE4** for 48 h showed a significant increase at the concentrations of 2 µg/mL in comparison with the control group (J774 + LPS) (Fig. 4B). The concentrations of 1, 0.5 and 0.1 µg/mL caused significantly lower secretion of IL-6 in comparison with the control group (Fig. 4D). In addition, all the concentrations of the extracts induced a significant reduction in the secretion of TNF-α, in comparison with the control group (Fig. 4F).

3.4. Morphological analysis

The control group was comprised of J774 cells not exposed to the extracts, and, when stained with HE, demonstrated morphophysiological characteristics similar to those described in the literature for histiocytic sarcoma neoplastic cells (Affolter and Moore, 2002), i.e.: a) pleomorphism (cells with different shapes and sizes), although these cells are frequently fusiform in *in vitro* cultures (Figs. 5 and 6 **A1-2**); b) presence of multinucleated giant cells, round or star shaped (Fig. 5 **A2**); c) vacuolated cytoplasm (Figs. 5 and 6 **A1-2**); d) presence of cytoplasmic extensions (Figs. 5 and 6 **A1-2**); and e) nuclei with different shapes and sizes, with one or several visible nucleoli and, in some cases, condensed chromatin (Figs. 5 and 6 **A1-2**).

The control group exposed to the inflammatory stimulus LPS, when stained with HE, showed cells with similar morphology to the negative control (only cells), but with some intensified characteristics (either after 24 or 48 h), such as: a) dispersed cytoplasm (Figs. 5 and 6 **B1-2**); b) irregular nuclei (in some cells the nuclei were large and with visible nucleoli while others presented highly condensed chromatin) (Figs. 5 and 6 **B1-2**); c) stellar cells with large and numerous cytoplasmic extensions (Figs. 5 and 6 **B1-2**); and d) multinucleated giant cells (Figs. 5 **B1 and** 6 **B1**).

The cells exposed to the concentrations of 2 and 1 µg/mL of **SGE2** for 24 and 48 h and stained with HE presented the most significant alterations, such as: highly dispersed cytoplasm and poorly defined nuclei (Fig. 5 **D and E1-2**); hyperchromatic nuclei (Fig. 5 **D and E1-2**); and numerous cytoplasmic extensions (Fig. 5 **D and E1-2**), in addition to more frequent multinucleated giant cells (Fig. 5 **E1-2**). At the other concentrations of the extract, these characteristics were also present, but they were less evident: less dispersed cytoplasm and fewer

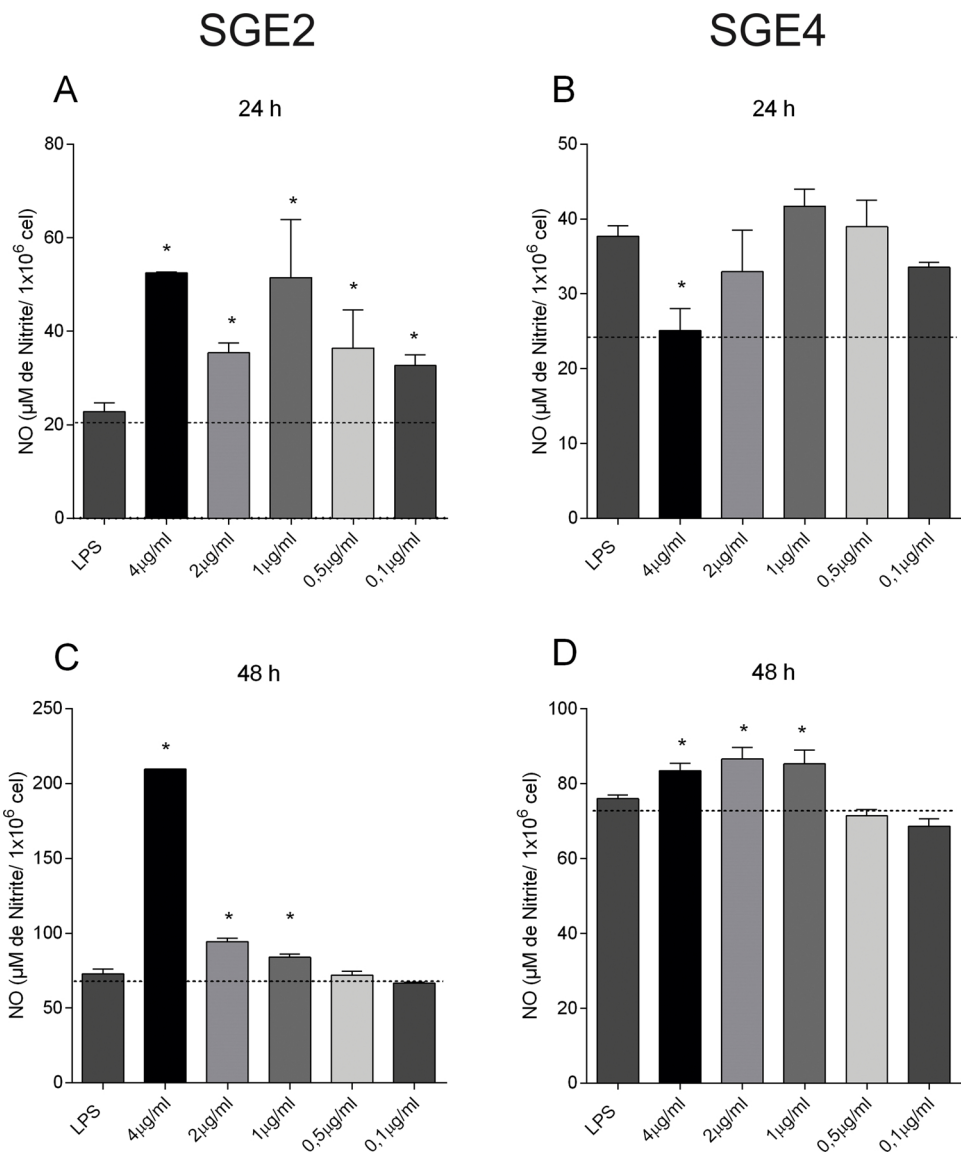

Fig. 2. (A–D): Nitric oxide production by J774 cells exposed to different concentrations of the salivary gland extracts (**SGE2 and SGE4**) for 24 and 48 h. The columns represent the means ± SEM (standard error of the mean) of the data obtained through quadruplicate testing. (*) indicates statistically significant differences ($p < 0.05$) in comparison with the control group (J774 + LPS).

extensions (Fig. 5 **C, F and G1-2**); elongated or fusiform cells with intensely stained cytoplasm, others with large and occasionally hypertrophic nuclei with one or two nucleoli (Fig. 5 **C, F and G1-2**); and multinucleated giant cells (Fig. 5 **F2**).

The exposure to **SGE4** caused the most significant alterations at the concentrations of 4, 2 and 1 µg/mL (after 24 and 48 h): highly dispersed cytoplasm, moderately vacuolated and with abundant cytoplasmic vacuolation (Fig. 6 **C1-E2**); hypertrophic nuclei (Fig. 6 **C1-E2**) with two or three nucleoli (Fig. 6 **C1-E1**); and presence of multinucleated giant cells (Fig. 6 **C1 and E2**). At lower concentrations of the extract (0.5 and 0.1 µg/mL), either after 24 or 48 h, multinucleated giant cells (with up to 5 nuclei/cell), and irregular nuclei, in some cases intensely stained due to chromatin condensation, and dispersed cytoplasm with extensions were observed (Fig. 6 **F1-G2**).

4. Discussion

Tick saliva contains a pharmacological arsenal, comprised of bioactive substances with vasodilatory, anti-inflammatory, anti-hemostatic and even anti-tumor activities (Abreu et al., 2014; Francischetti et al., 2009; Camargo-Mathias et al., 2011; Mor, 2006; Ribeiro et al., 1985; Xiang et al., 2009). According to Furquim et al. (2014), the secretory behavior of *R. sanguineus* s. l. salivary glands during the feeding period changes according to the host resistance, i.e., the production of saliva components (proteins, lipids, polysaccharides, acid phosphatase and calcium) is different depending on the host's immunological response (Kotál et al., 2015).

Despite knowledge about the immunosuppressive action of *R. sanguineus* s. l. saliva, little is known about the morphophysiological alterations in the immune system of cells when exposed to the saliva, taking into consideration the different periods of the glandular cycle: beginning (two days) and middle (four days) of the feeding process.

In this sense, we evaluated the *in vitro* immunomodulatory activity of the salivary gland extract obtained from *R. sanguineus* s. l. females fed for two and four days on host rabbits on J774 cells exposed to these stimuli for 24 and 48 h. Our aim was to detect the effects of the extracts on cell viability, NO and cytokine secretion and morphology.

The data on viability of J774 cells exposed to **SGE2** and **SGE4** demonstrated that at the concentration of 4 µg/mL, both extracts had cytotoxic effects on J774 cells, and more intensely after 48 h of

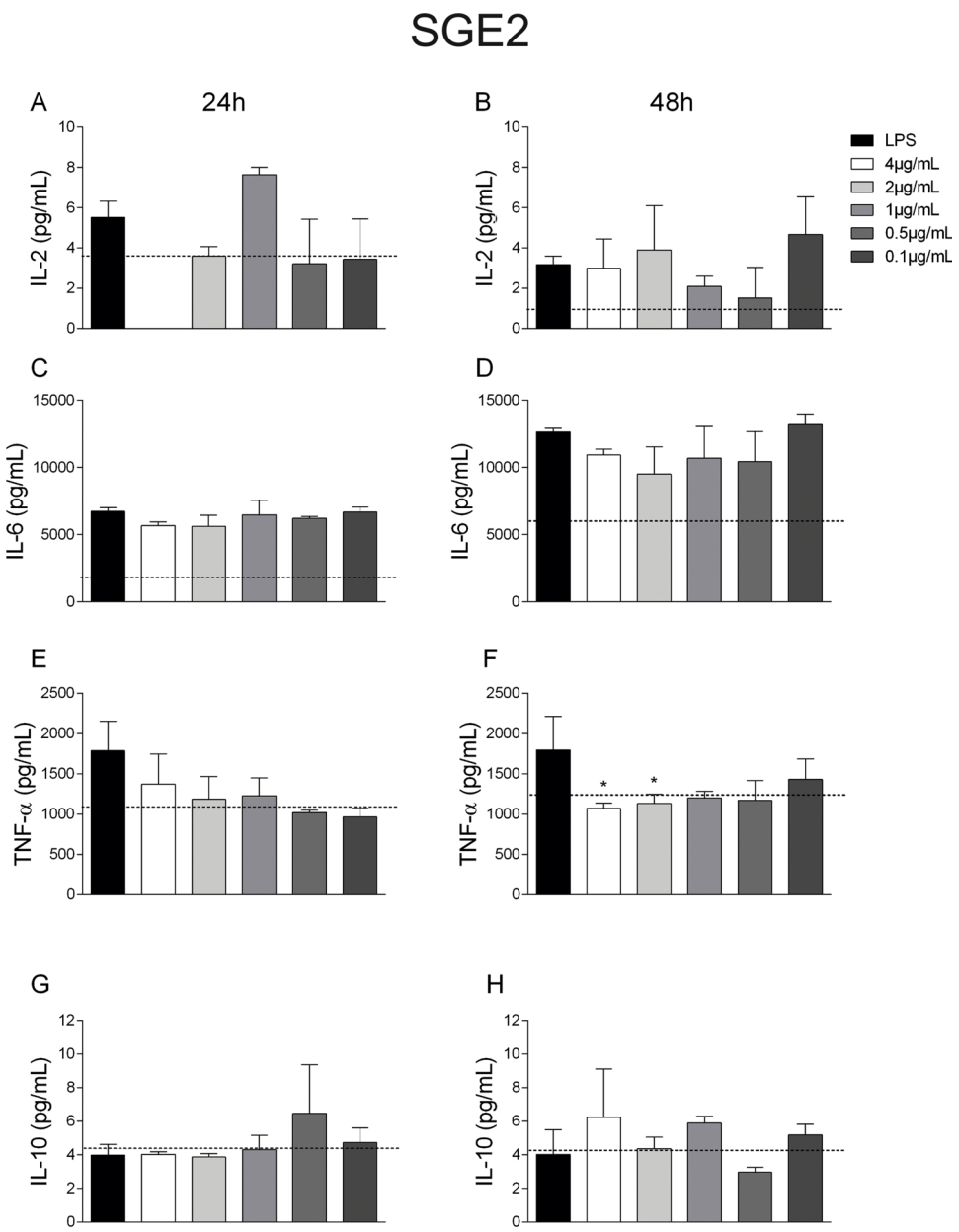

Fig. 3. (A–H): Quantification of cytokine secretion by the J774 exposed to different concentrations of **SGE2** for 24 and 48 h. The columns represent the means ± SEM (standard error of the mean) of the data obtained through quadruplicate testing. (*) indicates statistically significant differences ($p < 0.05$) in comparison with the control group (J774 + LPS).

exposure. Concomitantly, this concentration caused significant increase in NO production by both extracts (**SGE2** and **SGE4**), both after 24 and 48 h. However, the highest secretion occurred after 48 h, as observed in the cytotoxicity tests. This correlation can be explained by the fact that nitrogen reactive species, such as NO, are important cell signaling agents and cell proliferation stimulators when at low levels. However, at high levels these elements cause damage to the DNA and proteins, leading to apoptotic death (Albina et al., 1993; Justo et al., 2015; Marotta et al., 1992).

Still regarding NO production, the J774 cells exposed to 2 and 1 µg/mL of the two extracts for 48 h showed significant increase in NO production. When stimulated, macrophages can produce large amounts of NO, a molecule produced by nitric oxide-induced synthesis (iNOS) in the production of L-arginine (Abbas et al., 2015). Therefore, NO is considered an important mediator of intra and extracellular responses, with cytotoxic, antitumor and antiviral activities, playing a fundamental role in the immune response against microorganisms and tumor cells, thus consisting of an excellent indicator of macrophage activation (Bussolaro, 2006; Oliveira, 2005). The extract concentrations that stimulated the highest NO production levels in the J774 cells were the ones with proinflammatory action and able to induce macrophage activation (Kopecky et al., 1999; Oliveira et al., 2010; Oliveira, 2005; Olson and van der Vliet, 2012). Both extracts, mainly at low concentrations (0.5 and 0.1 µg/mL), either after 24 or 48 h, caused a decrease in NO production, indicating a probable anti-inflammatory effect of the extract, corroborating the literature (Ferreira et al., 2003; Ferreira and Silva, 1998; Gwakisa et al., 2001; Mor, 2006).

In addition to nitrogen reagent species, activated macrophages can phagocytize pathogenic microorganisms, present antigens to T lymphocytes and secrete cytokines responsible for mediating these activities (Morita et al., 2002). Therefore, the changes in the profile of macrophage-derived cytokines are indicators of immune response induction. IL-2, IL-6 and TNF are considered proinflammatory cytokines, responsible for the proliferation or differentiation of effector

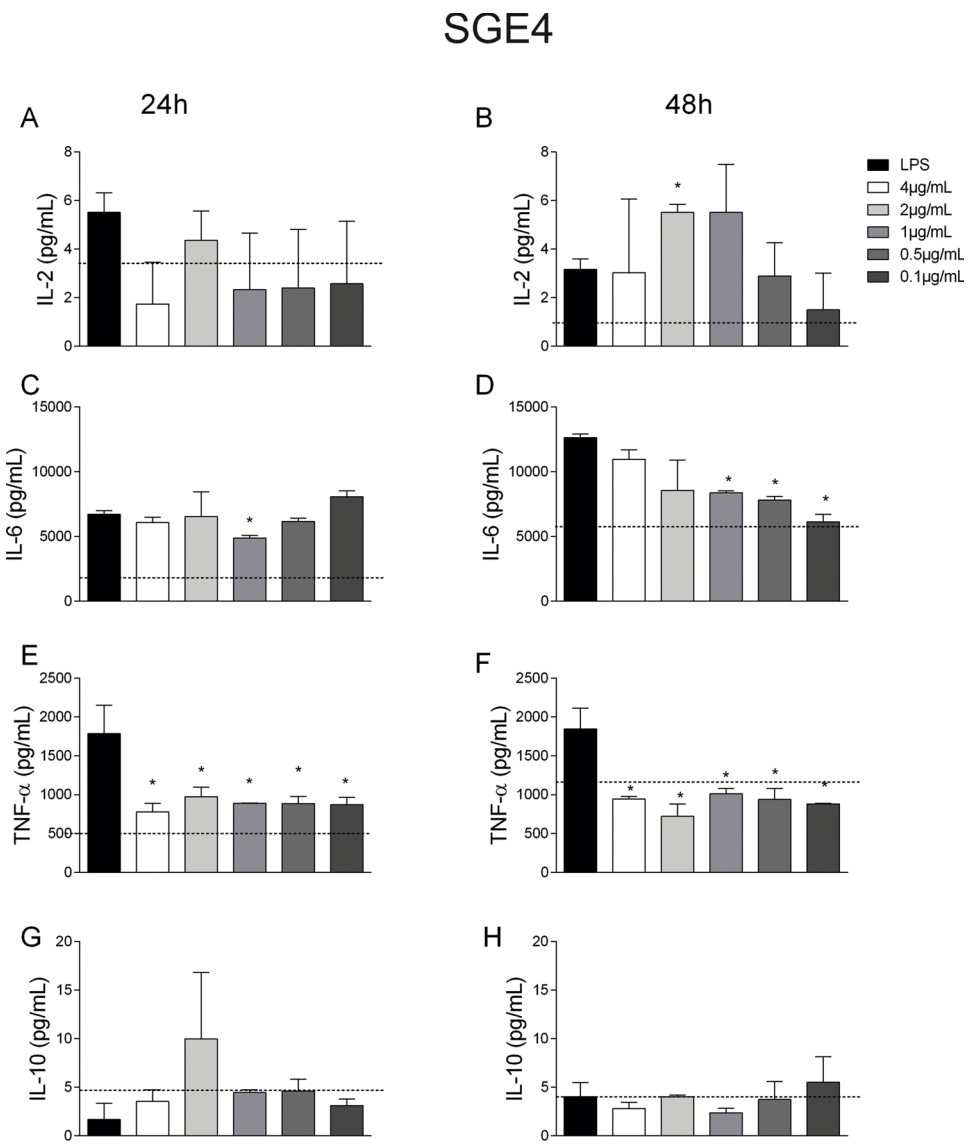

Fig. 4. (A–H): Quantification of cytokine secretion by the J774 exposed to different concentrations of **SGE4** for 24 and 48 h. The columns represent the means ± SEM (standard error of the mean) of the data obtained through quadruplicate testing (*) indicates statistically significant differences ($p < 0.05$) in comparison with the control group (J774 + LPS).

leukocytes. IL-2 is associated with T cell proliferation, maturation and growth, as well as with the proliferation and differentiation of NK cells. *in vitro*, this interleukin was able to stimulate B cell differentiation and antibody production. IL-6 is synthetized by phagocytes in response to microorganisms. Other cytokines, such as IL-1 and TNF-α, play a relevant role in the production of neutrophils, the differentiation of B lymphocytes, and the production of proinflammatory cytokines such as IL-17, inhibiting the generation of regulatory T cells. TNF-α has the function of recruiting neutrophils and monocytes to the infection site, promoting blood coagulation and tumor necrosis by causing thrombosis in the blood vessels responsible for irrigating tumor cells (Abbas et al., 2015).

The induction of proinflammatory cytokines through appropriate stimuli, such as the LPS, triggers the inflammatory response (Franzin, 2005). However, the literature has reported that the saliva obtained from different tick species has the ability to regulate and inhibit such cytokines. In studies using *Ixodes scapularis,* a salivary protein was detected (Salp15) and found able to inhibit the production of IL-2 during immune response development (Juncadella et al., 2007). Furthermore, the saliva obtained from the same species was proven to inhibit the production of TNF-α, IL-1β, IL-6, and IL-12p40 in murine macrophages following LPS stimulation (Chen et al., 2012). Salivary gland extracts from *R. microplus* decreased the production of TNF-α, IFN-γ and IL-12 by LPS-stimulated macrophages (after 24 h) (Brake and Pérez de León, 2012). Additionally, salivary gland extracts from *Dermacentor andersoni* inhibited IL-1 and TNF-α synthesis by bovine macrophages and decreased IL-2 and IFN- γ production in mice (Ramachandra and Wikel, 1992).The results of the present study regarding cytokine quantification corroborate the literature, since the concentrations of 4 and 2 μg/mL of **SGE2** caused a decrease in TNF-α production after 48 h of exposure. **SGE4** at the concentration of 1 μg/mL after 24 h, and concentrations of 1, 0.5 and 0.1 μg/mL after 48 h, significantly reduced the secretion of IL-6. In turn, all concentrations of **SGE4** caused lower secretion of TNF-α.

The cytokine quantification results showed that stimulation with 2 μg/mL of **SGE4** caused an increase of IL-2 in the period of 48 h, although there was no increase in IL-6 or TNF-α values, NO secretion increased, which suggests the occurrence of weak proinflammatory activity due to exposure to this concentration, demonstrating the importance of considering the different salivary gland secretory cycle

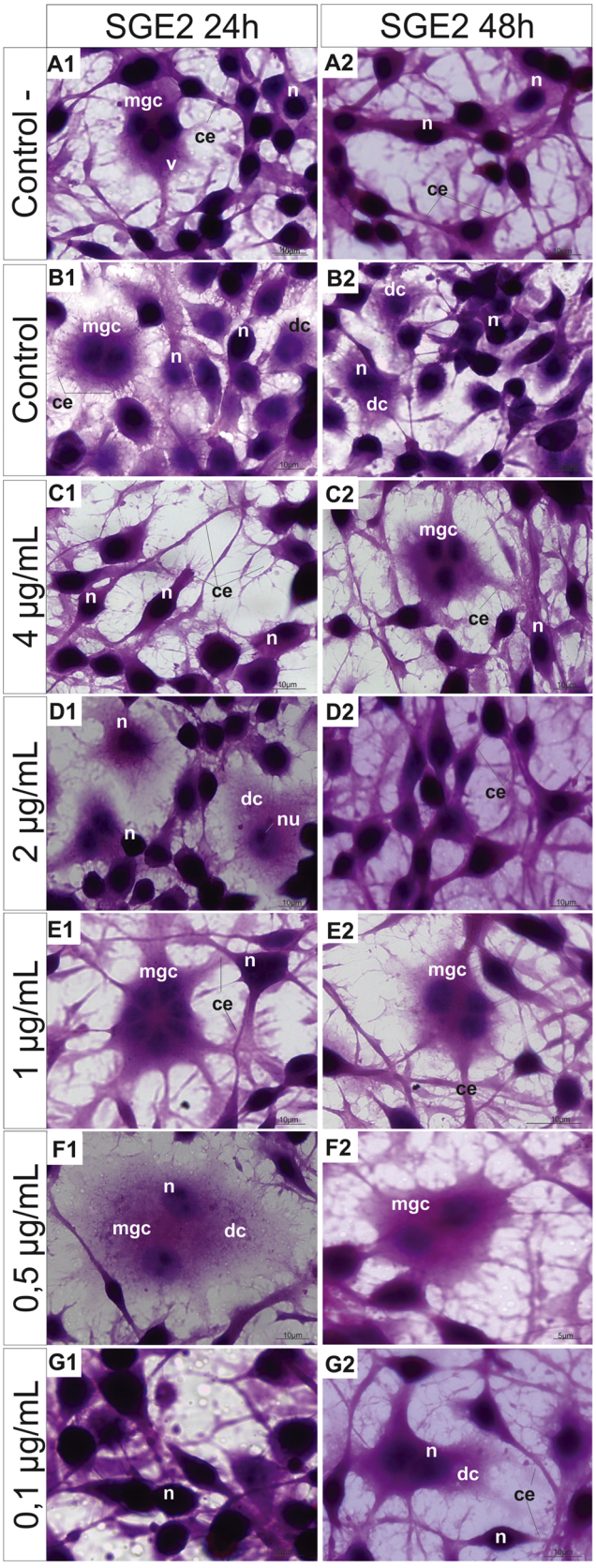

Fig. 5. Morphological analysis of J774 cells exposed to different concentrations of the extract obtained from *R. sanguineus* s. l. female ticks fed for 2 days (**SGE2**), 24 (**1**) and 48 h (**2**), stained with Harris hematoxylin and aqueous eosin (HE) and analyzed under a bright field microscope. **A1-2**: Negative control group (only cells); **B1-2**: Control group (J774 + LPS); **C1-2**: cells exposed to **SGE2** at the concentration of 4 µg/mL; **D1-2**: cells exposed to **SGE2** at the concentration of 2 µg/mL; **E1-2**: cells exposed to **SGE2** at the concentration of 1 µg/mL; **F1-2**: cells exposed to **SGE2** at the concentration of 0.5 µg/mL; **G1-2**: cells exposed to **SGE2** at the concentration of 0.1 µg/mL. **dc** = dispersed cytoplasm; **mgc** = multinucleated giant cell; **n** = nucleus; **nu** = nucleolus; **ce** = cytoplasmic extension, **v** = vacuolation.

periods, the exposure time to the extracts and concentrations of each species.

The morphological analysis of the J774 cells through light microscopy showed distinct levels of macrophage activation following exposure to different concentrations of **SGE2** and **SGE4** extracts, with slight differences between the exposure periods. The negative control group (only cells) presented typical morphology of histiocytic sarcoma neoplastic cells, i.e., with pleomorphism, presence of multinucleated giant cells, round or star shaped, with extensions, vacuolated cytoplasm with rounded or star-shaped nuclei, varying in size and in some cases with condensed chromatin, corroborating the characteristics described by Affolter and Moore (2002) for histiocytic sarcoma neoplastic cells. The control group (J774 + LPS) showed macrophages with typical activation characteristics, i.e., dispersed cytoplasm, abundant cytoplasmic extensions, cells with hypertrophic nuclei and multinucleated giant cells (North, 1978).

The morphological alterations observed in this study were caused by exposure to **SGE2** at the concentrations of 2 and 1 µg/mL. More cells with dispersed cytoplasm, higher incidence of multinucleated giant cells and cytoplasmic extensions were observed, confirming the results of cytokine quantification and NO analysis and demonstrating the proinflammatory potential (with intense macrophage activation) of these **SGE2** concentrations. The exposure to **SGE4** caused more evident alterations at the concentrations of 4, 2 and 1 µg/mL, with intense macrophage activation, i.e., scattered cells, with very dispersed cytoplasm and numerous cytoplasmic extensions, corroborating the NO analysis results (4 and 2 µg/mL). The increased IL-2 (2 and 1 µg/mL) secretion confirmed the inflammatory potential of the extracts at these concentrations. Furthermore, at the other concentrations of **SGE2** and **SGE4**, despite the presence of activated macrophages (which was expected since the cells were stimulated with LPS), the activation was less intense, and the exposure to the extracts decreased the activation characteristics, corroborating the results regarding the increase of IL-10 cytokine, mainly at lower concentrations of the extract, indicating that IL-10 would plays a role in the modulation and inactivation of previously activated macrophages (Abbas et al., 2015).

5. Conclusion

Considering the results presented here, it can be inferred that: **a)** both **SGE2** and **SGE4** at the concentration of 4 µg/mL were cytotoxic to the J774 cells exposed for 24 and 48 h; **b) SGE2**, at the concentration of 2 µg/mL for 48-hour exposure and 1 µg/mL for 24-hour exposure, and **SGE4** at the concentrations of 2 and 1 µg/mL for 48-hour exposure showed proinflammatory activity, confirmed by the increased secretion of NO and proinflammatory cytokine (IL-2), and the presence of morphological changes detected by light microscopy; **c)** the other concentrations of **SGE2** and **SGE4** presented immunomodulatory activity, demonstrated by a decrease in the secretion of NO and proinflammatory cytokines (IL2, IL-6 and TNF-α), and increase in the synthesis of IL-10, confirmed by the morphophysiological analysis.

These findings are unprecedented, and further investigation is needed about the mechanisms involved in the modulation of the host immune system when stimulated with *R. sanguineus* s. l. salivary gland extracts. However, this analysis of the action of **SGE2** and **SGE4** on J774 cells indicates this is a relevant research topic to be developed to identify the processes involved in the ectoparasite-host relationship,

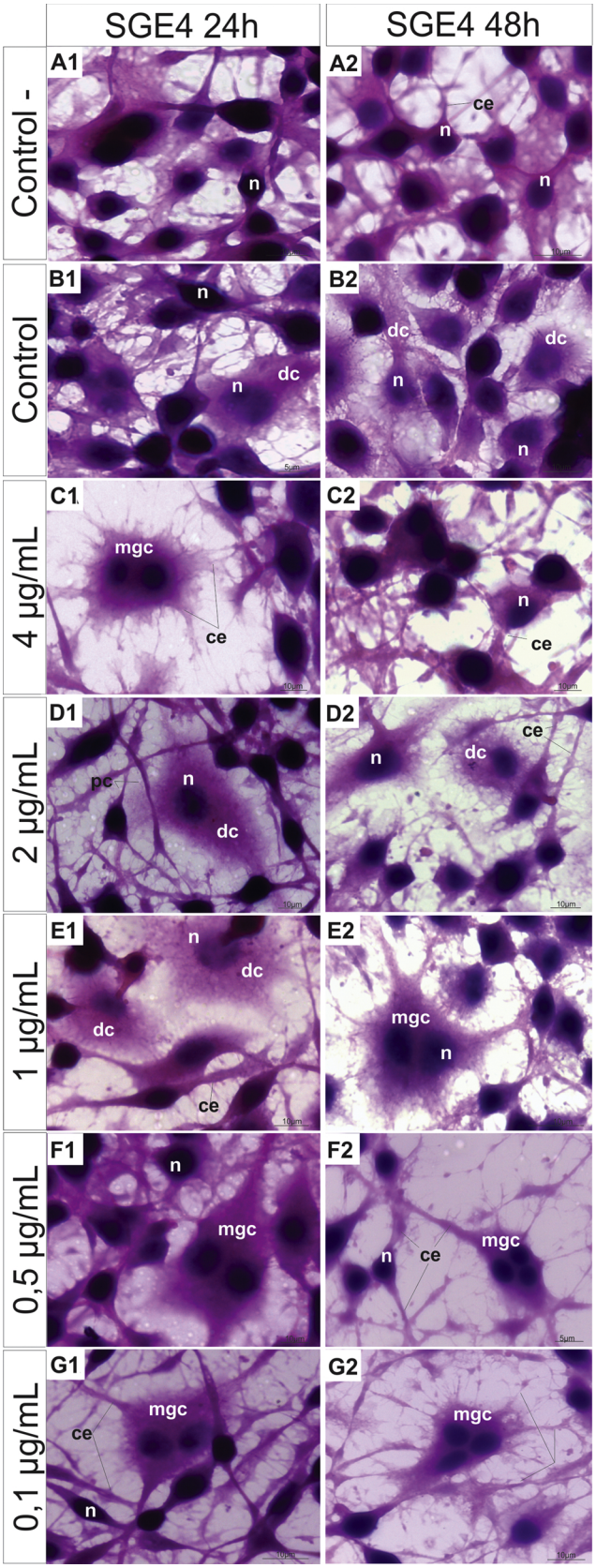

Fig. 6. Morphological analysis of J774 cells exposed to different concentrations of the extract obtained from *R. sanguineus* s. l. female ticks fed for 4 days (**SGE4**), 24 (**1**) and 48 h (**2**), stained with HE and analyzed under a bright field microscope. **A1-2**: Negative control group (only cells); **B1-2**: Control group (J774 + LPS); **C1-2**: Cells exposed to **SGE2** at the concentration of 4 µg/mL; **D1-2**: Cells exposed to **SGE2** at the concentration of 2 µg/mL; **E1-2**: Cells exposed to **SGE2** at the concentrations of 1 µg/mL; **F1-2**: Cells exposed to **SGE2** at the concentration of 0.5 µg/mL; **G1-2**: Cells exposed to **SGE2** at the concentration of 0.1 µg/mL. **dc** = dispersed cytoplasm; **mgc** = multinucleated giant cell; **n** = nucleus; **nu** = nucleolus; **ce** = cytoplasmic extension, **v** = vacuolation.

providing data for the development of more efficient tick control strategies.

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