Delay in maturation of the submandibular gland in Chagas disease correlates with lower DNA synthesis

José B Alves/+, Marcelo H Napimoga, Marcelo S Araujo, Gerluza AB Silva[1]

Laboratório de Biologia Molecular, Universidade de Uberaba, Av. Nenê Sabino 1801, 38055-500 Uberaba, MG, Brasil [1]Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

It has been demonstrated that the acute phase of Trypanosoma cruzi *infection promotes several changes in the oral glands. The present study examined whether* T. cruzi *modulates the expression of host cell apoptotic or mitotic pathway genes. Rats were infected with* T. cruzi *then sacrificed after 18, 32, 64 or 97 days, after which the submandibular glands were analyzed by immunohistochemistry. Immunohistochemical analyses using an anti-bromodeoxyuridine antibody showed that, during acute* T. cruzi *infection, DNA synthesizing cells in rat submandibular glands were lower than in non-infected animals ($p < 0.05$). However, after 64 days of infection (chronic phase), the number of immunolabeled cells are similar in both groups. However, immunohistochemical analysis of Fas and Bcl-2 expression did not find any difference between infected and non-infected animals in both the acute and chronic stages. These findings suggest that the delay in ductal maturation observed at the acute phase of Chagas disease is correlated with lower expression of DNA synthesis genes, but not apoptotic genes.*

Key words: submandibular gland - *Trypanosoma cruzi* - Chagas disease - immunohistochemistry

Chagas disease (South American trypanosomiasis), caused by the hemoflagellate *Trypanosoma cruzi* (Chagas 1909), is one of the most common and dangerous diseases in South America. The disease is characterized by an initial acute phase with trypomastigote forms in the blood and acute myocarditis (Andrade 1999, de Souza et al. 2003), followed by a silent indeterminate period with subpatent levels of parasitemia. Approximately 30% of infected individuals progress to a severe, diffuse and fatal chronic myocardiopathy (Rossi 1995). Also, several another organs such as digestive system (Köberle 1968) and the salivary glands (Vieira 1961) are affected by Chagas disease complications. The parotid is most often involved, but enlargement of the submandibular and even sublingual glands may often be observed (Alves et al. 1995). Patients with enlarged salivary glands frequently show amylasemia and increased amylase content in the parotid tissue (Vieira 1961).

The observation that acute infection with *T. cruzi* can induce an enlargement of the submandibular gland was first reported by Alves and Machado (1980). In addition, during the acute phase of experimentally induced Chagas disease, despite of the absence of an appreciable inflammatory process in the submandibular glands of *T. cruzi*-infected rats, these glands undergo a severe reduction in sympathetic (Machado et al. 1984) and parasympathetic innervation (Alves & Machado 1984). In addition, animals infected with *T. cruzi* show lower levels of testosterone and therefore a delay in maturation of granular convoluted tubules, which positively correlates with decreased epidermal growth factor production by submandibular glands cells (Moreira et al. 2008). In this context, we evaluated the bromodeoxyuridine (BrdU) incorporation as well as Fas and Bcl-2 expression in submandibular glands cells from infected and non-infected animals to determine if delayed ductal maturation on *T. cruzi* infection may correlate with induction of apoptosis or inhibition of DNA synthesis.

+ Corresponding author: propg.pr@uniube.br
Received 4 June 2008
Accepted 16 September 2008

MATERIALS AND METHODS

Animal inoculation - Forty male Holtzman rats, aged 27-29 days and weighing 90-94 g were inoculated intraperitoneally with 0.15 mL of mouse blood containing ~300.000 trypomastigotes of Y strain *T. cruzi*. This strain was isolated from a patient with Chagas disease (Silva & Nussenzweig 1953) and has been maintained in mice by repeated blood passages every seven days. Infection was confirmed by the presence of living trypomastigotes in the blood of all inoculated animals 10 days after inoculation, as previously described by Brener (1962).

This animal study was designed according to the Brazilian Guidelines (Resolution 196 of the National Health Council, 1996) and the protocol was approved by the Research Ethical Committee of Universidade Federal de Minas Gerais (protocol 141/07).

BrdU incorporation in submandibular gland cells - *T. cruzi*-infected and non-infected rats were euthanized under deep anesthesia after 18, 32, 64 or 97 days of inoculation. The rats were injected intraperitoneally with 1mL of BrdU per 100 g of body weight [concentration of 10 mmol/L in phosphate buffer saline solution (PBS), pH 7.4] 5 h prior to euthanization. The right and left submandibular glands were dissected out, fixed in Bouin's fluid for 7 h, briefly washed in running tap water, dehydrated, embedded in paraffin wax and sectioned at 6 μm. Serial sections were mounted on glass slides and prepared for immunostaining for BrdU. DNA synthesizing cells were detected by peroxidase-anti-peroxidase immunostaining with anti-BrdU antibodies using the Cell Prolifera-

tion Kit, RPN 20 (Amersham Bioscience, Little Chalfont, UK). Randomized sections of tissue were used as a negative control by omitting the anti-BrdU antibodies. After dehydration in an ethanol series, the sections were etched with xylene (15 min, twice). Specimens were then washed three times with PBS, drained of excess wash solution and the slides were wiped around the specimen. Next, sufficient reconstituted nuclease/anti-5-bromo-2"deoxyuridine was added (Cell Proliferation Kit, RPN 20, Amersham Bioscience, Little Chalfont, UK) to cover specimen and specimens were incubated for 20 h at rt. The sections were washed three times with PBS and the area around the specimens was wiped. Sufficient peroxidase anti-mouse IgG2a (Cell Proliferation Kit, RPN 20, Amersham Bioscience, Little Chalfont, UK) as add to cover the sections and sections were incubated for 2 h at rt. The slides were washed three times with PBS. Finally, the sections were immersed in DAB solution (Cell Proliferation Kit, RPN 20, Amersham Bioscience, Little Chalfont, UK) for 30 min. The slides were washed three times in distilled water. The immunostained sections were counterstained with Harris hematoxylin. Images were obtained at 400X magnification using a Zeiss capture plate on a microscope (Olympus BX 50) interfaced with a computer and software imaging tools. The immunolabeled cells were counted (immunolabeled cells located on grid line intersections were counted).

Histological and immunohistochemical study - Another set of animals were infected as previously described and euthanized under deep anesthesia 18, 32, 64 or 97 days after *T. cruzi* inoculation. The right and left submandibular glands were dissected, fixed in 10% buffered neutral formalin for 48 h, briefly washed in running tap water, dehydrated and embedded in paraffin wax. Each sample was sliced into 6 μm sagittal sections. Sections were mounted on glass slides and stained with hematoxylin and eosin to analyze the tissue organization of the gland.

Additional sections were mounted on glass slides and used for immunohistochemical analysis to examine Bcl-2 and Fas (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100). Slides were pre-treated with 3-aminopropyltriethoxy-silane (Sigma, St. Louis, MO, USA) immersed for 3 min in xylene to eliminate the paraffin, dehydrated in absolute alcohol and rehydrated with Tris-buffered saline (TBS). The sections were rinsed in TBS and immersed in 3% hydrogen peroxide in methanol for 10 min to block the endogenous peroxidase activity and incubated for 30 min at 90°C for antigen recovery. The slides were then incubated with monoclonal anti-Bcl-2 or anti-Fas antibody for 2 h at 37°C and then rinsed with TBS three times for 3 min.

Sections were then incubated with the appropriated secondary biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min and rinsed with TBS three times for 3 min. The streptavidin-biotin-peroxidase complex (Vector) was then incubated on the slides for 30 min, rinsed in TBS, counterstained with Mayer's hematoxylin, dehydrated and mounted. Staining specificity was tested by omission of primary antisera or substitution of primary antisera with non-immunized normal rabbit serum.

Statistical analysis - Data were expressed as mean ± standard deviation. Statistical comparisons between groups were made using analysis of variance followed by Tukey's test. Significance was accepted when the p value was ≤ 0.05.

RESULTS

Sections stained with hematoxylin and eosin showed similar histological features, as previously described by Alves and Machado (1980). In this study, it was observed that glands from non-infected animals displayed normal histological development after 18 (Fig. 1A) and 97 days (Fig. 1C). Also it was observed acinar enlargement and delayed granular duct maturation in rats with acute Chagas disease. Normal histological patterns returned after the disease entered the chronic stage, as observed in Figs 1B, D. The absence of inflammation of submandibular gland cells was confirmed by immunohistochemistry, in which the expression of tumor necrose factor-α and interferon-γ were not observed in infected animals (data not shown).

We next investigated if the delayed granular duct maturation of the submandibular gland from animals infected with *T. cruzi* occurred due to induction of apoptosis. There were no significant differences found in the number of Fas-immunostained cells between uninfected (Figs 2A, C) and infected animals (Figs 2B, D) analyzed during the course of infection. These results indicated that apoptosis was not occurring, even during the later stages of *T. cruzi* infection (after 97 days of Chagas disease).

In light of this, we examined the possibility that *T. cruzi* might be inhibiting apoptosis by stimulating host cell production of Bcl-2. Bcl-2 is a mitochondrial protein that functions to inhibit apoptosis in mammalian cells and thus prolongs cell survival. Using immunostaining, we did not find an increased level of Bcl-2 in infected submandibular gland cells (Figs 3A, D). Therefore, apoptosis does not appear to be inhibited in host cells by a parasite-induced increase in Bcl-2.

Next, we evaluated if the delayed granular duct maturation was associated with DNA synthesis using a BrdU uptake assay. We examined the ability of submandibular gland cells from *T. cruzi*-infected or uninfected animals to induce DNA synthesis. Immunostaining of the submandibular gland cells revealed a dramatic decrease upon BrdDu uptake in *T.cruzi*-infected animals (Figs 4A, D). As seen in Fig. 5, there is a significant difference ($p < 0.05$) in the number of BrdU-immunolabeled cells between non-infected and infected animals at the beginning of the infection. However after 64 days of infection (chronic phase), the numbers of immunolabeled cells are similar in both groups. Negative control staining was carried out by omitting exposure to the primary antibodies. Tissue staining specificity was confirmed by a lack of immunostaining for all experimental groups (data not shown).

DISCUSSION

We previously demonstrated that, in *T. cruzi*-infected rats, there occurs a morphological change in the granular convoluted tubule cells of the submandibular glands, along with acinar enlargement and delayed ductal maturation at the developing granular ducts (Alves & Macha-

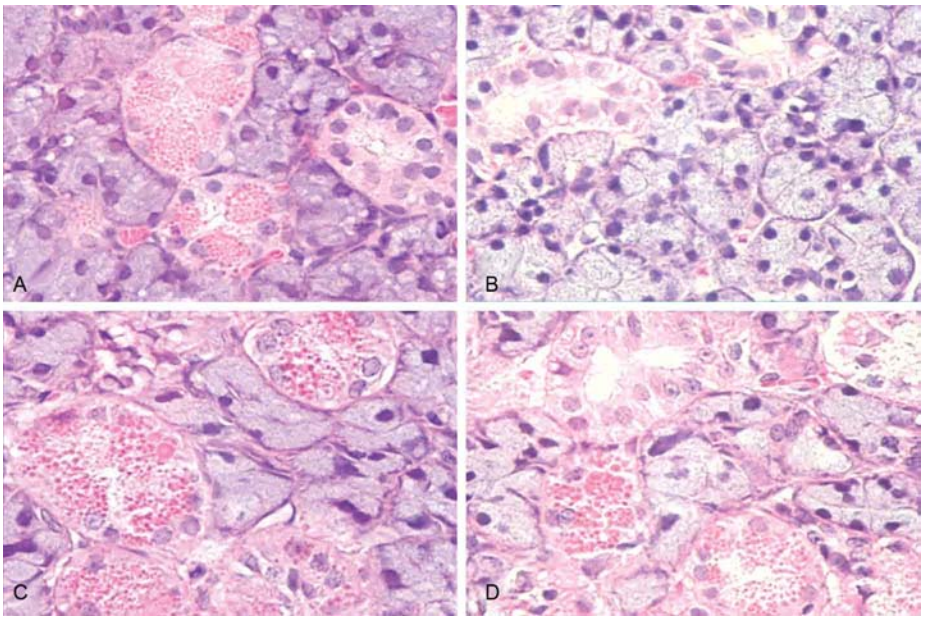


Fig. 1: morphological aspect of the submandibular glands. A: submandibular glands of non-infected rats after 18 days; B: submandibular glands after 18 days post-infection. It is possible to observe that the infected animals demonstrated a increased acinar development and a delayed ductal maturation; C: submandibular glands of non-infected rats after 97 days; D: submandibular glands after 97 days post-infection. No differences were observed between control and infected rats after 97 days of infection. H & E staining; magnification 400X.

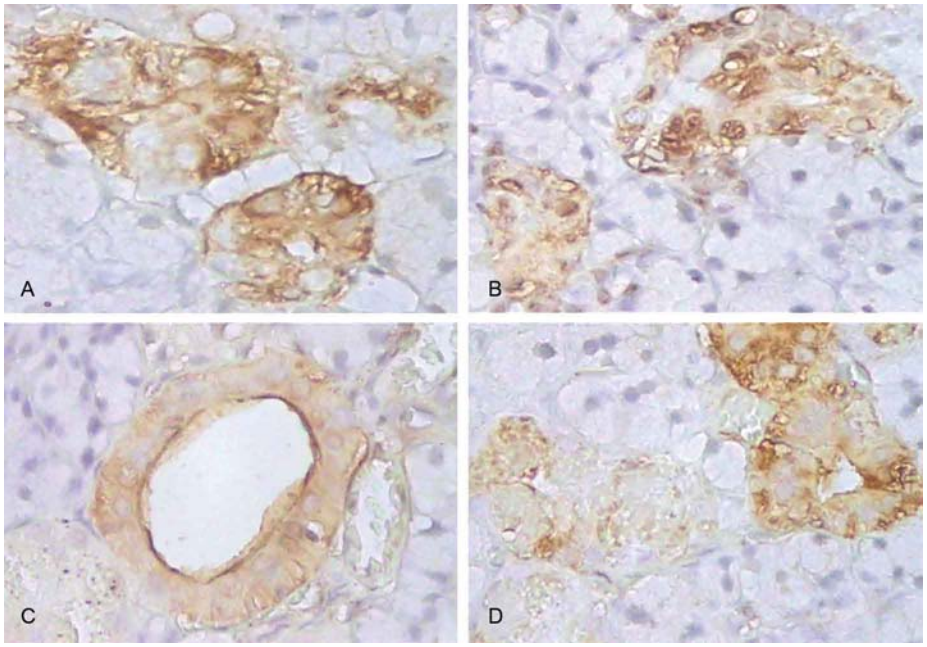


Fig. 2: immunohistochemical photomicrographs expression of Fas from submandibular glands of infected and non-infected rats after 18 and 97 days. Note Fas immunolabelled cells at the submandibular glands of non-infected (A) and infected animals (B) after 18 days was similar, and the same pattern was observed in both groups after 97 days in non-infected (C) or infected animals (D); magnification 400X.

do 1980). The results presented here show that the ductal delay may be due to inhibition of DNA synthesis during the acute phase of Chagas disease and in unrelated to apoptotic pathways.

Intracellular pathogens are known to modulate their host's response to infection to facilitate their own survival. Apoptosis is used as a defense strategy for the elimination of virus, bacteria and parasite-infected cells by the immune system, as well as in cell selection during the development and maintenance of tissue homeostasis (Vaux et al. 1994). Pathogen-infected apoptotic cells are recognized and phagocytosed by macrophages and the pathogen and host cells are eliminated in an immunologically silent manner (Ren & Savill 1998). In con-

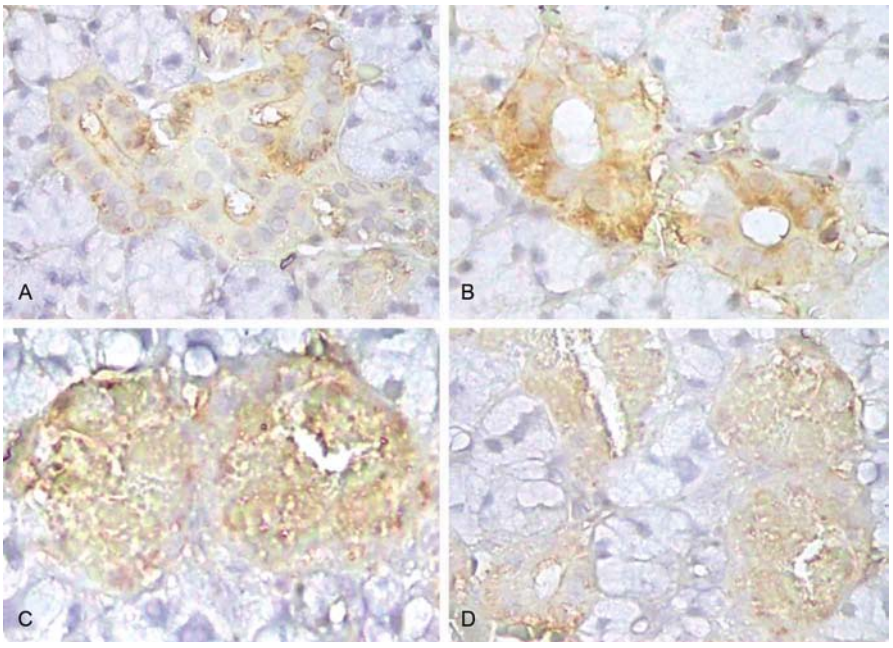


Fig. 3: immunohistochemical photomicrographs expression of Bcl-2 from submandibular glands of infected and non-infected rats after 18 and 97 days. Note Bcl-2 immunolabelled cells at the submandibular glands of non-infected (A) and infected animals (B) after 18 days was similar, and the same pattern was observed in both groups after 97 days in non-infected (C) or infected animals (D); magnification 400X.

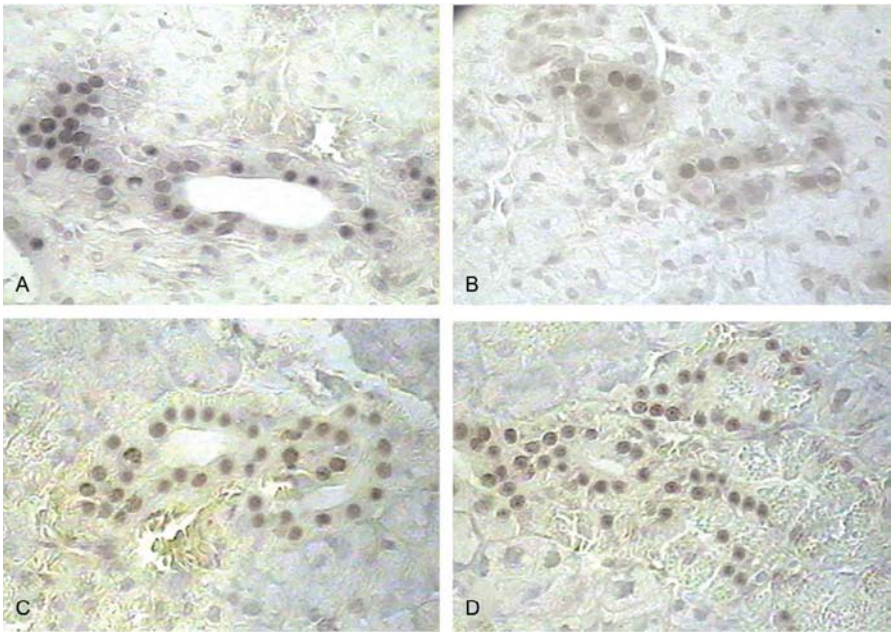


Fig. 4: immunohistochemical photomicrographs expression of BrdU from submandibular glands of infected and non-infected rats after 18 and 97 days. Note bromodeoxyuridine (BrdU) immunolabelled cells at the submandibular glands of non-infected after 18 days (A), whereas submandibular of infected-rats glands showed lower BrdU immunolabelled cells (B), immunolabelling of BrdU in non-infected (C) and infected animals (D) after 97 days was similar; magnification 400X.

trast, pathogens have been found to antagonize apoptotic death of invaded host cells, prolonging their survival and allowing them more time to replicate (Moss et al. 1999).

T. cruzi has also been shown to modulate induction of apoptotic pathways. Clark and Kuhn (1999) attempted to determine whether *T. cruzi* infection of murine fibroblasts induced apoptosis, however after five days there was no indication of the induction of apoptotic pathways. Another study showed that microarray analysis of uninfected and *T. cruzi*-infected BALB/c fibroblasts suggested that

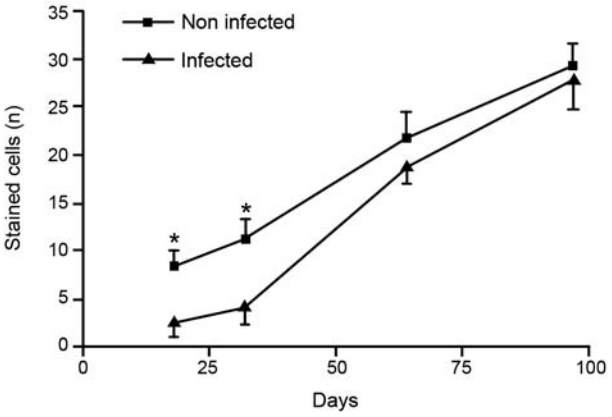


Fig. 5**:** sections of the submandibular glands were immunostained with anti-bromodeoxyuridine (BrdU) and the numbers of stained cells were counted. The results are expressed as mean ± SD. Asterisks indicate $p < 0.05$ compared with non-infected animals at the same period.

expression of genes in the TNFRI apoptotic pathway is downregulated in *T. cruzi*-infected cells. This indicated that, in BALB/c fibroblasts, the parasite decreases the expression of genes that lead to host cell apoptosis (Moore-Lai & Rowland 2004). It has been also shown that *T. cruzi* inhibit Fas-mediated apoptosis in HeLa cells through inhibition of caspase 8 activation (Nakajima-Shimada et al. 2000). In our study, we did not observe any Fas-mediated transduction pathways induced by *T. cruzi*, which could explain the delay in ductal cells maturation observed in the acute stage of Chagas disease.

Another potential effect of *T. cruzi* infection on the host cell may be inhibition of apoptosis. A mammalian protein, Bcl-2, functions as an inhibitor of apoptosis in many cell types. When overexpressed, Bcl-2 prolongs the life of a cell that would normally undergo apoptosis (Zamzami et al. 1996). However, we were unable to demonstrate by immunohistochemistry that Bcl-2 was overexpressed in *T. cruzi*-infected submandibular gland cells. Therefore, if parasites do inhibit apoptosis of host cells, it is not due to an increase in the intracellular level of Bcl-2. It has been reported that *T. cruzi* uses the host's cellular FLICE inhibitory protein (c-FLIP), the only known inhibitor specific for death receptor-mediated apoptosis in mammals (Thome & Tschopp 2001), for inhibition of Fas-mediated apoptosis by posttranscriptional up-regulation. This finding indicates that *T. cruzi* modulates and exploits a host molecule to counteract death receptor signaling (Hashimoto et al. 2005).

At the histological level, the main changes observed were accelerated acinar development and delayed ductal maturation, characteristics that are more evident in the developing granular ducts (Alves & Machado 1980). The enlargement induced by *T. cruzi* infection during the acute phase is mediated through the β-receptor since it is blocked by propranolol (Alves & Machado 1986). This enlargement is similar to that observed in isoproterenol-treated animals, which is believed to involve both hypertrophy, reflecting the excessive activation of protein synthesis and the enlargement of acinar cells and hyperplasia associated with cell proliferation (Matsuura & Suzuki 1997).

The enlargement of the acinar cells observed during the acute phase of experimentally induced Chagas disease is mainly due to hypertrophy rather than to proliferation (hyperplasia), since there was no BrdU incorporation in these cells. In fact, the observed enlargement of acinar cells may reflect the increased number and size of secretory granules, as well as altered expression of the secretory protein cystatin (Alves et al. 1994, Silva et al. 1995). We observed increased BrdU incorporation in the granular ducts in both groups, although, in the acute phase of Chagas disease, a higher incorporation can be observed in non-infected rats. We previously demonstrated that rats infected with *T. cruzi* may exhibit a delay in testes development with a reduction in testosterone levels (Moreira et al. 2008).

In conclusion, the alterations observed at the acute phase of experimental Chagas disease, leads to retardation of the differentiation of granular cells due to decreased DNA synthesis, however, there is no correlation with alterations in the apoptotic pathway.

REFERENCES

Alves JB, Alves MS, Naito Y 1994. Induction of synthesis of the rat cystatin S protein by the submandibular gland during the acute phase of experimental Chagas disease. *Mem Inst Oswaldo Cruz 89*: 81-85.

Alves JB, Machado CR 1980. Histological and histoquantitative study of the rat submandibular gland in Chagas disease. *Arch Oral Biol 25*: 437-443.

Alves JB, Machado CR 1984. Changes in acetylcholinesterase-positive nerves of the submandibular salivary gland during experimental infection with a protozoon, *Trypanosoma cruzi*, in rats. *Arch Oral Biol 29*: 647-651.

Alves JB, Machado CR 1986. Effect of adrenoceptor blockers on the acinar enlargement induced by Chagas' disease in the rat submandibular glands. *Braz J Med Biol Res 19*: 69-73.

Alves JB, Ulrich-Karine MC, Alves MS 1995. Morphology of rat submandibular gland acinar secretory granules and their alteration during the acute phase of experimental Chagas' disease. *Braz J Med Biol Res 28*: 761-766.

Andrade ZA 1999. Immunopathology of Chagas disease. *Mem Inst Oswaldo Cruz 94*: 71-80.

Brener Z 1962. Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*. *Rev Inst Med Trop São Paulo 4*: 389-396.

Chagas CRJ 1909. Nova tripanosomíase humana. Estudos sobre a morphologia e o ciclo evolutivo do *Schizotrypanum cruzi* n. gen. n. esp., agente da nova entidade mórbida do homem. *Mem Inst Oswaldo Cruz 1*: 159-218.

Clark RK, Kuhn RE 1999. *Trypanosoma cruzi* does not induce apoptosis in murine fibroblasts. *Parasitology 118*: 167-175.

de Souza E, Araujo-Jorge TC, Bailly C, Lansiaux A, Batista MM, Oliveira GM, Soeiro MN 2003. Host and parasite apoptosis following *Trypanosoma cruzi* infection in *in vitro* and *in vivo* models. *Cell Tissue Res 314*: 223-235.

Hashimoto M, Nakajima-Shimada J, Aoki T 2005. *Trypanosoma cruzi* posttranscriptionally up-regulates and exploits cellular FLIP for inhibition of death-inducing signal. *Mol Biol Cell 16*: 3521-3528.

Köberle F 1968. Chagas' disease and Chagas' syndromes: the pathology of American trypanosomiasis. *Adv Parasitol 6*: 63-116.

Machado CR, Alves JB, Machado AB 1984. Adrenergic innervation and noradrenaline content of the rat submandibular gland during the experimental *Trypanosoma cruzi* infection. *J Neural Transm 59*: 289-297.

Matsuura S, Suzuki K 1997. Immunohistochemical analysis of DNA synthesis during chronic stimulation with isoproterenol in mouse submandibular gland. *J Histochem Cytochem 45*: 1137-1145.

Moore-Lai D, Rowland E 2004. Microarray data demonstrate that *Trypanosoma cruzi* downregulates the expression of apoptotic genes in BALB/c fibroblasts. *J Parasitol 90*: 893-895.

Moreira A, Napimoga MH, Benatti BB, Silva GAB, Rocha-Rodrigues DB, Clemente-Napimoga JT, Vieira GA, Alves JB 2008. Morphological changes and EGF expression in the granular convoluted tubule cells of submandibular glands of *Trypanosoma cruzi* infected rats. *Tissue Cell 40*: 293-298.

Moss JE, Aliprantis AO, Zychlinsky A 1999. The regulation of apoptosis by microbial pathogens. *Int Rev Cytol 187*: 203-259.

Nakajima-Shimada J, Zou C, Takagi M, Umeda M, Nara T, Aoki T 2000. Inhibition of Fas-mediated apoptosis by *Trypanosoma cruzi* infection. *Biochim Biophys Acta 1475*: 175-183.

Ren Y, Savill J 1998. Apoptosis: the importance of being eaten. *Cell Death Differ 5*: 563-568.

Rossi MA 1995. Pathogenesis of chronic Chagas' myocarditis. *São Paulo Med J 113*: 750-756.

Silva GA, Alves JB, Alves MS 1995. Cystatin S in secretory granules fractions isolated from submandibular gland of infected rats by *Trypanosoma cruzi*. *Tissue Cell 27*: 167-172.

Silva LHP, Nussenzweig V 1953. Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folia Clin Biol 20*: 191-208.

Thome M, Tschopp J 2001. Regulation of lymphocyte proliferation and death by FLIP. *Nat Rev Immunol 1*: 50-58.

Vaux DL, Haecker G, Strasser A 1994. An evolutionary perspective on apoptosis. *Cell 76*: 777-779.

Vieira CB 1961. Hyperamylasemia and hyperactivity of salivary glands associated with megaesophagus. *Am J Dig Dis 6*: 727-741.

Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G 1996. Mitochondrial control of nuclear apoptosis. *J Exp Med 183*: 1533-1544.