Lingual salivary gland hypertrophy and decreased acinar density in chagasic patients without megaesophagus

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ABSTRACT

Although the salivary glands present several functions, there are few studies evaluating these glands in Chagas disease (CD). This study aimed to compare the percentage of collagen, the presence of inflammation, the density of chimase and tryptase mast cells, the area and density of lingual salivary gland acini in autopsied individuals with and without (CD). We analyzed 400 autopsy reports performed in a tertiary public hospital from 1999 to 2015 and selected all the cases in which tongue fragments were collected (27 cases), 12 with chronic CD without megaesophagus (CH) and 15 without CD (non-chagasic - NC). The histological sections of the tongue were stained by Picrosirius red for collagen evaluation and Hematoxylin-eosin for morphometric evaluation of salivary gland acini and inflammation. Anti-chimase and anti-tryptase antibodies were used for the immunohistochemical evaluation of mast cells. The chagasic patients presented higher volume and lower density of salivary glands acini. There was no difference in the collagen percentage, inflammation and density of mast cell chymase and tryptase between the groups. Although we did not observe a significant difference between the groups regarding the collagen percentage, inflammatory process and mast cell density, our results suggest that even without megaesophagus, chagasic patients present hypertrophy of the lingual salivary glands and lower acinar density probably due to mechanisms independent of the esophagus-glandular stimulus.

KEYWORDS: Acinar density. Chagas disease. Megaesophagus. Morphometry. Salivary glands. Tongue.

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Received: 14 August 2019

Accepted: 13 November 2019

INTRODUCTION

Chagas disease (CD), described by Carlos Chagas (1909), is caused by the monophlagetic protozoan *Trypanosoma cruzi* and is transmitted to man by the triatomine insect (*Triatoma infestans*)[1]. Approximately 6 to 7 million people are estimated to be infected worldwide, most of them in Latin America. CD is responsible for the high morbidity and mortality rate in endemic countries, including Brazil[2].

It has been demonstrated, in the acute phase of CD, that the lingual salivary glands present disorganized parenchyma with alterations in the acini and presence of amastigotes in the acinar and ductular cells, in the interacinar connective tissue and in the acinar lumen[3]. On the other hand, it has already been demonstrated, in the chronic phase of CD, dilation of parotid glands[4], submandibular glands and sublingual glands[5]. Similarly, a study by our group showed that the lingual salivary glands of patients with chronic CD present a higher number of alterations when

compared to non-chagasic patients, such as ductal dilation, inflammatory infiltrate, perineuritis and myositis[6].

Patients with chronic CD may have megaesophagus due to the destruction of Meissner's and Auerbach's intramural nerve plexuses of the esophagus with consequent decrease in peristalsis and failure of the lower sphincter in response to swallowing. Consequently, esophageal stasis, ataxia, dilation and decreased esophageal contraction capacity occur[7]. In chagasic patients with megaesophagus, there is an increase in the sensitivity of the salivary glands to all secretory stimuli, from chewing to pharmacological stimulation by pilocarpine. This hypersensitivity was attributed to the impairment of innervation in these glands, triggering hypersalivation and consequent salivary gland hypertrophy, which are common manifestations of all obstructive esophagopathies due to the difficulty of food transit in the esophagus[3,8].

It is known that saliva plays an essential role in the maintenance of health, such as lubrication of the oral tissues, antimicrobial action, maintenance of mucosal integrity, cleaning, dental remineralization, digestion and phonation[9], besides contributing to the formation of film acquired through the adsorption of salivary glycoproteins that protect teeth from chemical and mechanical aggressions[10]. A study performed on parotid gland biopsies of individuals with chronic CD and megaesophagus showed glandular hypertrophy, fibrosis, inflammatory infiltrate and dilation of salivary gland ducts[4].

However, there have been few studies evaluating the salivary glands in CD, and our team was the pioneer in the investigation of lingual salivary glands in response to *T. cruzi* infection[6]. However, the involvement of the salivary glands in CD has not yet been fully clarified. In the present study, we hypothesized that the salivary glands of patients with CD have a higher presence of inflammation, a greater density of chymase and tryptase mast cells, and a larger acini area and lower acinar density.

Therefore, the aim of the present study was to compare the percentage of collagen, the inflamatory response, the density of chymase and tryptase mast cells and the area and density of lingual salivary glands acini in autopsied individuals with or without CD.

MATERIALS AND METHODS

This study was approved by the Human Research Ethics Committee of the University of Uberaba (UNIUBE), protocol Nº CAAE 64945517.1.0000.5145. This is a cross-sectional study with the analysis of 400 autopsy reports performed at the Clinical Hospital of the Federal University of Triangulo Mineiro (UFTM) from 1999 to 2015. During

this analysis, all CD patients without megaesophagus that had tongues collected during autopsy (CH) (n = 12) and 15 patients without CD (NC), totaling 27 cases were selected.

Inclusion criteria for the CD group were three positive serology tests in pericardial fluid collected during autopsy (complement fixation, indirect immunofluorescence and hemagglutination). CD patients with megaesophagus were excluded to rule out the possibility of mechanical interference in salivation. In the NC group, the exclusion criterion was the diagnosis of infectious diseases or any esophageal disease.

Tongue fragments in the region of the lingual salivary glands were evaluated. Paraffin blocks containing tongue fragments were serially cut into four sections measuring about 5 μm thick. Subsequently, they were placed on glass slides and stained by the hematoxylin-eosin method to evaluate the presence of inflammation and to perform the morphometric analysis of acini. Then, sections were also stained by the Picrosirius red for collagen evaluation. The other two slides were salinized for immunohistochemical analysis of mast cells chimase and tryptase.

Morphometric analysis of collagen

For the morphometric evaluation of collagen, an Axio 4.1 common light microscope (Zeiss, Berlin, Germany), 20 X objective, polarizing filter, AxioCam image capture camera (Zeiss, Berlin, Germany) as well as a computer and Axiovision 4.8 software (Zeiss, Berlin, Germany) were used. This analysis was performed in all the fields in which lingual salivary glands were observed, both in the interacinar and submucosal regions. The images were transmitted to the computer monitor. In the polarized image, the collagen presented birefringence with yellowish, reddish or greenish coloration, being semi-automatically quantified.

Morphometric evaluation of salivary gland acini

For the morphometric evaluation of the salivary gland acini area, an Axio 4.1 common light microscope with a 20 X objective and an AxioCam color video camera connected to a video capture card in a computer with ImajeJ software (National Institutes of Health, Bethesda, USA) were used. To determine the area occupied by each gland, the contour of each acinus was determined with the aid of a cursor. This analysis was performed in all fields in which lingual salivary glands were observed.

To assess the acinar density, initially, acini counts were performed in all of the fields of the fragment. Then the total area evaluated was calculated by multiplying the area of each field (0.31 mm²) by the number of evaluated fields.

Table 1 - Demographic characteristics of individuals with and without Chagas disease

	CH Group (n = 12)	NC Group (n=15)
Ethnicity[a] (Caucasian: Non-Caucasian)	11:1 (91.6%: 8.3%)	13:2 (86.6%:16.6%)
Gender[b] (Male: Female)	7:5 (58.3%: 41.6%)	10:5 (66.6%:33.3%)
Age[c] (Years: Mean ± SD)	68.23 ± 2.87	59.36 ± 3.36

CH Group: Chagas disease patients; NC Group: patients without Chagas disease; SD: Standart desviation; [a]Fisher exact test (p = 1.0); [b]Fisher exact test (p = 0.70); [c]Student t test (p = 0.79).

Considering the number of acini and total area, the acinar density was determined and expressed in mm^2.

Immunohistochemical analysis

For immunohistochemical evaluation of mast cells anti-tryptase antibodies (1:20; R&D, Minesota, USA) and anti-chimase antibodies (1: 2000; R&D, Minesota, USA) were employed in peroxidase-conjugated Avidin-Biotin techniques (DAKO, Carpinteria, USA).

As some cases presented fragments with only 10 evaluable fields, the evaluation was standardized considering 10 high-magnification fields for all cases. Immunolabeled mast cells were counted using a 40 X objective and a common light microscope. In the slides where there were more than 10 evaluable high-magnification fields, the 10 fields with the highest number of immune-labeled mast cells were selected.

Inflammatory infiltrate assessment

Regarding inflammatory infiltrates, an Axio 4.1 common light microscope was used to evaluate all the fields in which salivary glands were present. The inflammatory infiltrate was initially classified as: a) present or absent; b) superficial: when present in the submucosa; deep: when it was located among salivary acini. The inflammatory infiltrate was classified as absent (0), mild (1), moderate (2) and intense (3) according to the amount of inflammatory cells present in the tissue[11,12]. Due to the low frequencies, during the statistical analysis, we have arbitrarily chosen to group the mild (1) and absent (0) cases, which were then classified as 0 (mild + absent = 0), aside from the moderate (2) and intense (3) cases, that were also grouped and classified as 1 (moderate + intense = 1).

Statistical analysis

Data were entered into a Microsoft Excel spreadsheet and analyzed using GraphPad Prism 7 statistical software (GraphPad, San Diego, California, USA). To compare the variables with non-normal distributions between two groups, the Mann Whitney test was performed. For qualitative variables (male/female; Caucasian/non-Caucasian) the Fisher exact test was used. To compare the ages in both groups, the Student's "t" test was used and the results were expressed as mean and standard deviation. The assumed significance level was 5% (p <0.05).

RESULTS

Demographic data of the groups of individuals with Chagas disease (CH) and without Chagas disease (NC) are shown in Table 1.

The acini area of lingual salivary glands was significantly larger in the CH group when compared to the NC group (p < 0.0001) (Figure 1A).

The individuals in the CH group had lower acini densities compared to the NC group (p = 0.0016) (Figure 1B).
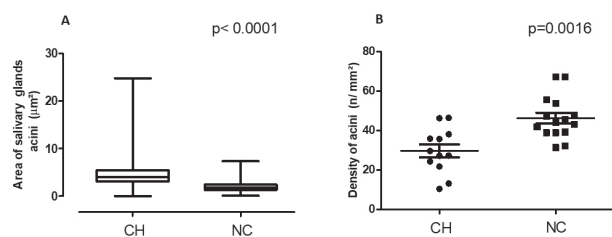

Figure 1 - Area and density of lingual salivary glands of individuals with Chagas disease (CH) and without Chagas disease (NC). A) Acini area, Mann-Whitney test; p <0.0001; B) Acini density, Mann-Whitney test; p = 0.0016 (B).

No significant difference in collagen percentages were observed between CH and NC groups (p = 0.4987) (Figures 2A, 2B, 2C and 2D).

There was no significant difference between the groups regarding the immunostaining for chimase and tryptase mast cells (Figures 3A, 3B, 3C and 3D).

Chronic inflammatory infiltrates were present in both, the interacinar and submucosal regions, with no
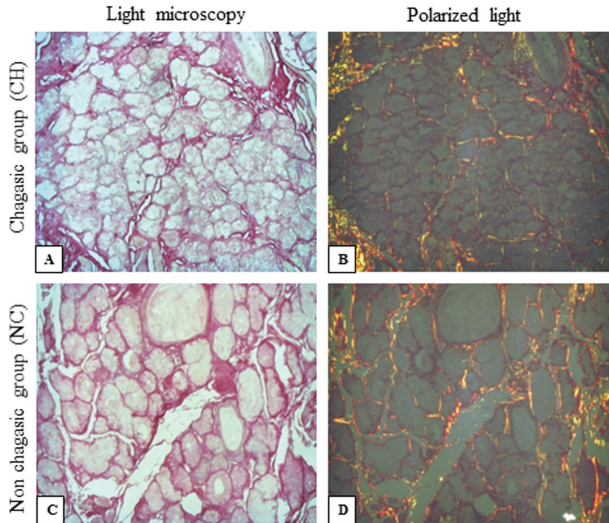
Figure 2 - Presence of collagen in the lingual salivary glands of individuals with Chagas disease (CH) and without Chagas disease (NC). A) and B): CH group: in A) common light microscopy; in B) polarized light (yellowish-orange refringent collagen); C) and D): NC group: in C) common light microscopy; in D) polarized light (yellowish-orange refringent collagen) (Hematoxylin and Eosin and Picrosirius red, 400 X).
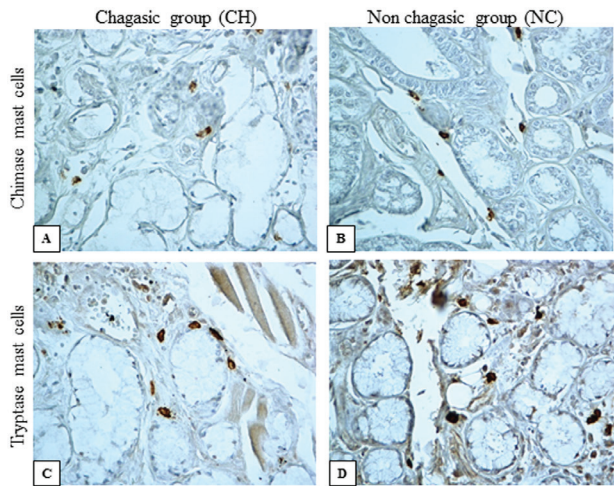

Figure 3 - Chimase mast cells and tryptase mast cells in the lingual salivary glands of individuals with Chagas disease (CH) and without Chagas disease (NC). Positive immunostaining for chimase in a CH group (A) and in a NC group (B); positive tryptase immunostaining in a CH group (C) and in a NC group (D) (Immunohistochemistry, 400 X).

significant difference between the groups regarding location (p = 0.7152) and presence of infiltrates (p = 1.0) (Figures 4A, 4B, 4C and 4D).

DISCUSSION

Although an increase in the acini area of rat submandibular glands has been demonstrated in the acute phase of CD[13], glandular hypertrophy is more common in the chronic
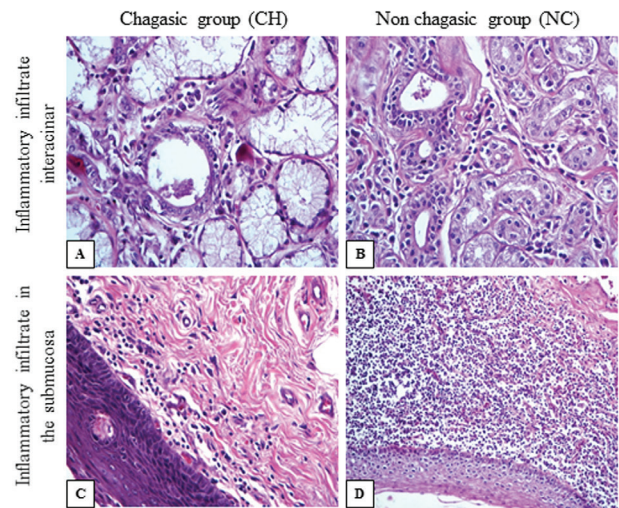

Figure 4 - Mononuclear inflammatory infiltrate permeating acini from lingual salivary glands and in the submucosal region of the tongue in individuals with Chagas disease (CH) and without Chagas disease (NC). Inflammatory infiltrate permeating the acini in an individual from a CH group (A) and from a NC group (B); mononuclear inflammatory infiltrate in the submucosal region from a CH group (C) and a NC group (D) (Hematoxylin and Eosin, 400 X).

phase of the disease. Studies performed on parotid glands have demonstrated the presence of hypertrophy in chronic chagasic patients with megaesophagus[14,15]. It is known that in patients with megaesophagus, the difficulty of food passage may cause reflex stimulation in the lingual salivary glands with consequent increase of saliva production and glandular hypertrophy[3].

Although we did not find in the literature a description of increased volumes of lingual salivary gland acini in patients with non-digestive form of CD, in the present study we observed a higher volume of lingual salivary gland acini in chagasic patients when compared to non-chagasic patients. Chagasic patients with megaesophagus have already been shown to present increased sensitivity of the salivary glands to all secretory stimuli, from chewing to pharmacological stimulation by pilocarpine, which occurs due to impaired innervation of these glands[8]. Thus, we believe that this hypersensitivity occurs in all chagasic patients, even those without megaesophagus, probably due to stimuli triggered by *T. cruzi* antigens, which would justify the acinar hypertrophy observed in the lingual salivary glands of chagasic patients without megaesophagus in the present study.

We have also observed a lower density of lingual salivary gland acini in chagasic patients compared to non-chagasic patients. Although a reduction in acinar density of salivary glands has already been described in other diseases such as diabetes[16] and Sjogren's syndrome[17], we did not find studies that evaluated the acin density of salivary glands in

chagasic patients. However, we believe that the decrease in acinar density in chagasic patients was due to glandular acini hypertrophy that occupied a larger area of the tissue.

Increased collagen deposition is common in chronic diseases, which is stimulated by fibroblast growth factor (FGF) and secreted extracellular matrix, contributing to healing. Under the influence of transforming growth factor-β1 (TGF-β1), fibroblasts differentiate into myofibroblasts, expressing the smooth muscle actin. Thus, in addition to collagen synthesis, fibroblasts are able to contract, shrinking scar tissues, because they have actin filaments associated with myosin[18]. In chronic disease, inflammation is intense resulting in more extensive scarring.

Since CD is a chronic disease, we hypothesized that there would be higher collagen deposition in the lingual salivary glands as already shown in other organs, such as the myocardium[1], the esophagus[19], the colon[20] and in the tongue[21]. However, in the present study there was no significant difference in the percentage of collagen between groups. Thus, we believe that in chagasic patients, the increment in acini volume of lingual salivary glands leads to collapse and overlaping of the inter-acinar collagen fibers, impairing the analysis. In addition, increased acinar volumes would cause vascular compression and ischemia, as has already been demonstrated with subsequent fibroblast hypotrophy. Thus, the hypotrophic fibroblasts would deposit small diameter collagen fibers.

Mast cells are hematopoietic cells involved in inflammatory response, granulation tissue formation, wound healing, angiogenesis, tissue remodeling[22], allergic reactions, innate and adaptive T cell-mediated immune responses as well as chronic inflammation[23-25]. Although several studies have already shown that in CD there is an increase in the number of mast cells and a higher intensity of inflammation in organs such as the heart[26], esophagus[27], colon[20] and in the submucosa and tongue musculature[21], we did not find significant differences regarding the density of chimase or tryptase mast cells or the presence of inflammation in the lingual salivary glands when chagasic and non-chagasic individuals were compared.

Thus, as we did not find differences regarding the intensity of inflammation, mast cell density and percentage of collagen between the groups, we believe that the inflammatory response to *T. cruzi* in the lingual salivary glands behaves differently than already shown in other organs. Furthermore, we suggest that the same stimulus that cause hypertrophy of lingual salivary glands in chagasic patients without megaesophagus could also be acting on other glands with consequent hormonal overproduction and dysregulation of chagasic patients homeostasis.

Therefore, although a significant difference between groups regarding collagen percentage, inflammatory process and mast cell density was not observed, our results suggest that, even without megaesophagus, chagasic patients present hypertrophy of lingual salivary glands and lower acinar density probably due to mechanisms independent of esophageal-glandular stimulation. However, further studies are needed to better understand the pathogenesis of chronic CD in lingual salivary glands.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design, data acquisition and interpretation, writing of the paper, critical review of intellectual content, reading of the paper and approval of the final version. Barbara Bellocchio Bertoldo attended and selected the patients, elaborated the figures, performed the statistical analysis and conducted the review of the paper; Renata Margarida Etchebehere conducted the histochemical and immunohistochemical analysis; Taíssa Cássia de Souza Furtado and Juliana Barbosa de Faria performed the submission and modification of the paper; Camilla Beatriz Silva performed the histochemical processing; Márcia Fernandes de Araújo performed the immunohistochemical technique; Denise Bertulucci Rocha Rodrigues contributed to the review of the paper; Sanivia Aparecida de Lima Pereira contributed to the conception and planning of the study, writing, submission and review of the paper.

FUNDING

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