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ORIGINAL ARTICLE

TONGUE FUNCTION, SALIVARY FLOW RATE AND IgA, IgM AND IgG TOTAL SALIVARY LEVELS IN CHRONIC CHAGASIC PATIENTS

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ABSTRACT

Although microscopic alterations have been detected in tongues and salivary glands of chagasic patients and the identification of biomarkers in saliva has proved advantageous, there are no studies evaluating tongue function and total salivary IgA, IgG and IgM levels in chronic chagasic patients. The aim of this study was to evaluate tongue function, salivary flow rate, and total salivary IgA, IgG and IgM levels comparing chronic and non-infected individuals. 37 patients were selected: chronic cardiac chagasic patients (n=6), chronic chagasic patients with the associated form of the disease (cardiopathy and megaesophagus) (n=11), and non-chagasic individuals (n=20). The tongue function underwent a phonoaudiological evaluation. The salivary flow rate was measured by sialometry. The total salivary IgA, IgG and IgM levels were evaluated by sandwich ELISA assay. Chagasic patients with the associated form of the disease presented higher salivary flow rate and lower salivary protein levels. No significant differences were noted in the lingual function or in the total salivary immunoglobulin levels among the groups. Although patients with chagasic megaesophagus presented higher levels of salivary flow and lower salivary protein, the fact that there were no significant differences in lingual function and total salivary immunoglobulin levels among the groups led to the conclusion that chronic chagas disease does not modify the lingual function or the total IgA, IgG and IgM salivary levels. The present study was the first to evaluate the function of the tongue and salivary total immunoglobulin levels in Chagas disease.

KEY WORDS: Chagas disease; immunoglobulin; saliva; tongue function.

INTRODUTION

Chagas disease (CD) or American trypanosomiasis is a parasitic systemic infection caused by the flagellate parasite *Trypanosoma cruzi* (*T.cruzi*), presenting two phases: acute and chronic (Filigheddu et al., 2017;

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Pérez-Molina & Molina, 2018). The chronic phase can present four forms: undetermined, cardiac, digestive or associated form (Prado et al., 2012); the digestive or associated form may cause alterations in the esophagus causing difficulty in swallowing (dos Santos et al., 2011).

The tongue plays an essential role in deglutition (Felton et al., 2007). Previous studies have shown that in chronic chagasic patients the tongue presents several microscopic morphological alterations, such as increased vascular diameter, increased vascular wall area, increased blood vessel density, increased thickening of the basal capillary membrane (Pereira et al., 2009), as well as a higher percentage of collagen and higher density of mast cells (Pereira et al., 2007). Parasitism of the skeletal muscle in the chronic phase also triggers repair mechanisms through collagen deposition resulting in alterations in muscle contractility (Novaes et al., 2016).

In addition to the microscopic modifications observed in the connective tissue of the tongue, some studies, performed on patients with chagasic megaesophagus, have shown an enlargement of the ducts in the von Ebner salivary glands (Pereira et al., 2006), parotid, submandibular and sublingual glands (Martini et al., 1990), as well as an increase in salivary flow rate observed in 90% of chronic chagasic patients presenting megaesophagus (Araújo et al., 2000).

Saliva is an important biofluid with several functions in the first line of defense due to the presence of immunoglobulins, cytokines and antimicrobial peptides (Nurkka et al., 2001). Most of the immunoglobulin (Ig) concentration present in the saliva belongs to the IgA subclass, followed by IgM (Borges et al., 2015). Concentration levels of IgG in the saliva are low, derived from the crevicular fluid and the mucosal transudate (Malamud, 1997). One study demonstrated that transient reductions in IgA levels detected in saliva are associated with an increased susceptibility to gastrointestinal tract infections (Gleeson et al., 1995). Saliva is, therefore, a "body mirror" in regard to health and disease, and its use as a diagnostic source through the identification of biomarkers in the immune response is fast and non-invasive, presenting several advantages in relation to blood samples.

Although microscopic changes in the chagasic tongue have been described (Pereira et al., 2006; Pereira et al., 2007; Pereira et al. 2009) and IgG antibodies anti-*T. cruzi* IgG have already been detected in infected patients (Barros et al., 1999), there are no studies evaluating lingual function and total antibody levels in the saliva of chagasic patients. Thus, the purpose of the present study was to evaluate the tongue function, salivary flow rate and total immunoglobulin A (IgA), immunoglobulin M (IgM) and immunoglobulin G (IgG) levels in the saliva of chronic chagasic patients in comparison with non-infected individuals.

MATERIAL AND METHODS

Ethical aspects

After approval by the Research Ethics Committee of the Federal University of Triangulo Mineiro (UFTM), under protocol number 2256, patients signed the Informed Consent Form (ICF). All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Study Design: Patient selection

The individuals were selected consecutively during follow-up visits in a period from March 2017 to February 2018 in the Integrated Clinics of Uberaba University, Minas Gerais, Brazil.

All patients over 18 years of age, who fulfilled the inclusion criteria and agreed to participate in the study, were included (n=37) and homogenized in accordance to gender, age and ethnicity. Next, the patients were subdivided into three groups according to the following clinical criteria: chronic cardiac chagasic patients (n=6), chronic chagasic patients with the associated form of the disease (cardiopathy and megaesophagus) (n=11), and non-chagasic patients (n=20).

In the chagasic group, patients were included on presenting at least two positive serological tests to *T. cruzi*, such as indirect immunofluorescence, hemagglutination and Enzyme-Linked Immunosorbent Assay (ELISA) (Zymed®, USA), and at least one morphological finding suggestive of chronic CD, such as cardiomegaly and/or megaesophagus demonstrated by imaging exams.

The following patients were excluded from the study: patients on medication; with other systemic infectious diseases; craniofacial malformation history; smoking and alcoholism history; temporomandibular dysfunction, or presenting neoplasm. Patients with caries, gingivitis, periodontitis; disorders that cause stress or chronic pain in the head or neck; mouth breathers; patients with allergic rhinitis and patients who suffered a cerebrovascular accident or any type of facial paralysis were also excluded.

Tongue function examination

After anamnesis, clinical tongue examination was conducted using a protocol similar to that applied in a previous study (Clark et al., 2003). The following parameters were evaluated: muscle tone, mobility, muscle strength with counter-resistance, muscle strength without counter-resistance and contraction of the lingual apex. Sterile gloves and spatulas were used to perform this evaluation (Figure 1).

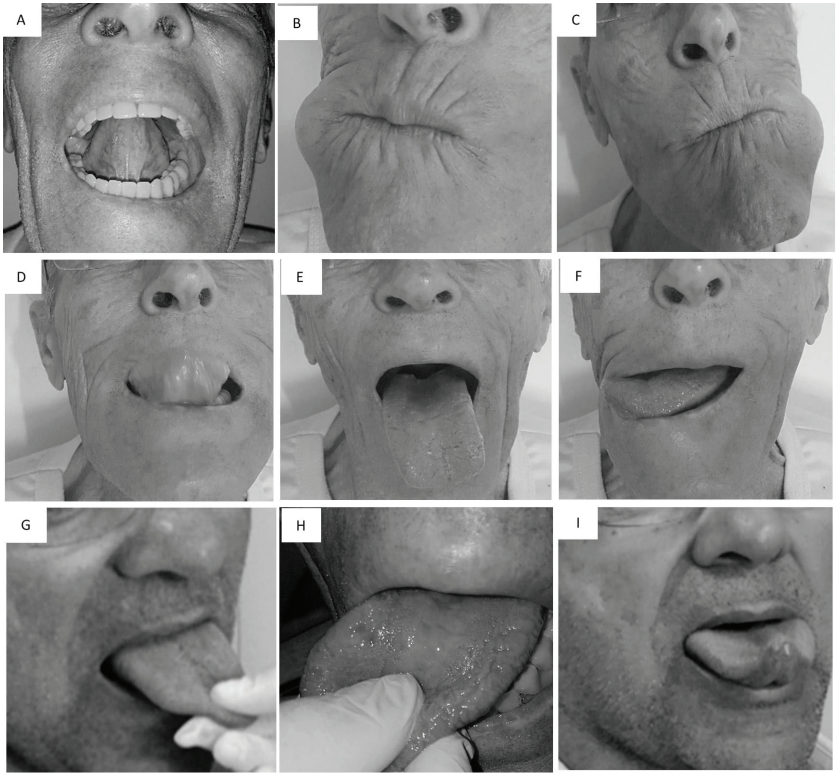

Figure 1. Analysis of tongue function: Muscle tone (A), mobility (B, C), muscle strength without counter-resistance (D, E, F), muscle strength with counter-resistance (G, H) and contraction of the lingual apex (I).

For muscle tone evaluation, the patients were asked to open their mouths and move their tongues up in order to slightly touch the palate. Hence, their ability to maintain the tongue vertically tensioned was evaluated, and the tongue considered normal or flaccid (Figure 1A). In order to evaluate normal or altered mobility, the patients were asked to move the tongue laterally with the mouth closed (Figures 1B and C). Muscle strength without counter resistance was analyzed by protruding the tongue out, followed by free up and down movement and from side to side to test whether the patients had sufficient strength to perform the movement (Figure 1D, E and F). The counter-resistance muscle strength was assessed by asking the patients to point the tongue out and make free movements up and down and from side to side while the examiner applied an opposing force to the movement (Figure 1G and H). To assess the contraction of the lingual apex, patients were asked to put their tongue out and move it to elevate the lingual apex (Figure 1I).

Saliva collection

Once the groups were formed, saliva collection was performed in a quiet environment between 1 p.m. and 3 p.m. Each patient salivated, in an unstimulated way, for five minutes into a sterile container two hours after lunch. The patients themselves held the container in the lower area of the lower lip with maximal mouth opening, so that the saliva would be collected passively into the container, without induction. Subsequently, the saliva was removed from the container with a 5mL graduated disposable vacuum syringe in order to determine the exact volume.

Analysis of total salivary IgA, IgM and IgG levels

The total salivary IgA, IgM and IgG levels were determined by Enzyme-Linked Immuno Sorbent Assay (ELISA) (Zymed®, USA), in 96-well polystyrene plates (Costar®, USA). Plates were sensitized with affinity purified goat anti-human IgA or IgM or IgG antibody solution (Zymed®, USA). Next, the plates were washed twice and blocked with bovine serum albumin (BSA). The saliva samples were added in duplicate and incubated for two hours at room temperature with shaking. The plates were incubated with mouse anti-human IgA or IgM or IgG antibody followed by goat anti-mouse IgG biotinylated antibody (Sigma- Aldrich®, St. Louis, Missouri, EUA). The plates were subsequently washed with streptavidin-alkaline phosphatase conjugate (Sigma®) and incubated with p-nitrophenyl phosphate substrate (Sigma®). Substrate reaction was performed at room temperature for 30 minutes and the intensity of the reactions was measured at a wavelength of 405 nm using ELISA reader (VersaMax®, Molecular Devices, Sunnyvale, CA, USA). Non-sensitized wells of the same plates and sensitized wells, which had not been incubated with saliva, were used as negative controls. Serial dilutions of purified human IgA, IgM and IgG (Zymed®) were made at concentrations of 2.0, 1.0, 0.5, 0.25 and 0.125 µg/mL.

Quantification of total protein using the Bradford method

Total protein concentration in saliva was determined by the Bradford method (Sigma®) (Hildebrandt et al., 2008). Bradford reagent was applied to diluted saliva and incubated for 15 minutes at room temperature and then read in a spectrophotometer (Synergy HT, BioTek, USA) at 595 nm with results expressed in µg/mL.

Statistical analysis

GraphPad Prism 8.0 statistical software was used for statistical analyses. The Shapiro-Wilk normality test was performed in all groups. For group homogenization Fisher's exact test was applied to compare genders and ethnic groups, and the ANOVA test was used to compare ages. For tongue function analysis, the following scores were utilized: 0= normal; and 1= altered; and then the Kruskal-Wallis test with Dunn's post-test was applied. ANOVA and Tukey's post-test were used to compare salivary flow rate and protein between groups. The Kruskal-Wallis test was used to compare the total salivary levels of immunoglobulins between cardiac chagasic, associated and non infected individuals. The differences were considered significant when the probability of rejecting the null hypothesis was lower than 0.05 (5%).

RESULTS

The number of cardiac chagasic patients; patients with the associated form with megaesophagus; and non-chagasic patients were, respectively: Caucasian (5/11/19); non-Caucasian (1/0/1); male (4/7/8); and female (2/4/12). There was no significant difference regarding gender, ethnicity and age among the groups, hence showing a homogeneous distribution of variables among the three groups (Table 1).

Table 1. Demographic data of the different groups: infected with the cardiac form of the disease, associated (cardiac plus megaesophagus) and non-infected.

	Cardiac chagasic (n =6)	Chagasic associated form (with megaesophagus) (n=11)	Non-chagasic (n = 20)
Ethnicity * (C:NC)	5:1	11:0	19:1
Gender** (M:F)	4:2	7:4	8:12
Age***	69.2±4.4	65.7± 10.7	66.4± 10.7

C= Caucasians; NC=Non-Caucasians; M= Male; F= Female; *Exact Fisher Test, p=0.6419; **p=0.4256; *** ANOVA Test, p=0.7837; age (mean ± standard deviation).

The alteration of muscle tone with lingual flaccidity was the most frequent alteration, detected in 83.3% of the cardiac chagasic patients. However, there were no statistical differences among the groups in relation to any of the evaluated parameters: muscle tone, mobility, muscle strength with counter-resistance, muscle strength without counter-resistance and contraction of the lingual apex (Table 2).

Table 2. Analyses of tongue function in the groups chagasics cardiac form, chagasics mixed form with megaesophagus and non-chagasics

	Cardiac chagasic (n =6)	Chagasic associated form (with megaesophagus) (n=11)	Non-chagasic (n = 20)
Muscle tone (flaccid)*	5 (83,3%)	7 (63.6%)	12 (60%)
Mobility (altered)**	3 (50%)	4 (36.4%)	7 (35%)
Muscle strength with counter-resistance (no)***	4 (66.7%)	8 (72.7%)	13 (65%)
Muscle strength without counter-resistance (no)****	1 (16.7%)	0 (0%)	1 (5%)
Contraction of the lingual apex (no)*****	1 (16.7%)	1 (9.1%)	4 (20%)

*Kruskal Wallis test, p=0.5819; ** Kruskal Wallis test. p=0.4436; *** Kruskal Wallis test, p=0.9090; ****Kruskal Wallis test, p=0.3560; ***** Kruskal Wallis test, p=0.7386.

Chagasic patients with the associated form of the disease presented a higher salivary flow rate than non-chagasic patients with a significant difference (Figure 2).

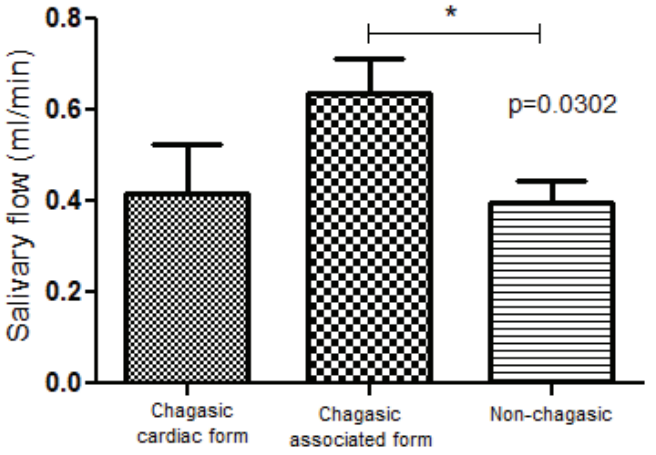

Figure 2. Comparison of salivary flow rate among the different groups: infected with the cardiac form of the disease, associated (cardiac plus megaesophagus) and non-infected individuals. ANOVA, post-test (Tukey's test), p = 0.0302.

The mean level of salivary proteins in patients with the associated form of the disease (494.6±179.5 µg/mL) was significantly lower than in cardiac chagasic patients (825.2±324.4 µg/mL) (Figure 3). In addition, there was no statistical difference between the groups with regard to total IgA, IgM, IgG levels (Table 3).

Table 3. Total salivary levels of IgA, IgM, and IgG in µg/mL of groups: chagasics cardiac form, chagasics mixed form with megaesophagus and non-chagasics.

	Chagasic cardiac form (n =6)	Chagasics associated form (with megaesophagus) (n=11)	Non-chagasic (n = 20)
IgA*	19.6 ± 4.0	15.9± 5.4	15.3± 6.4
IgM**	11.2± 4.6	11.8± 5.1	9.4± 6.5
IgG***	7.9± 5.1	4.2± 2.5	8.0± 5.7

*Kruskal Wallis test, p>0.126; ** Kruskal Wallis test, p>0.183; *** Kruskal Wallis test, p>0.083.

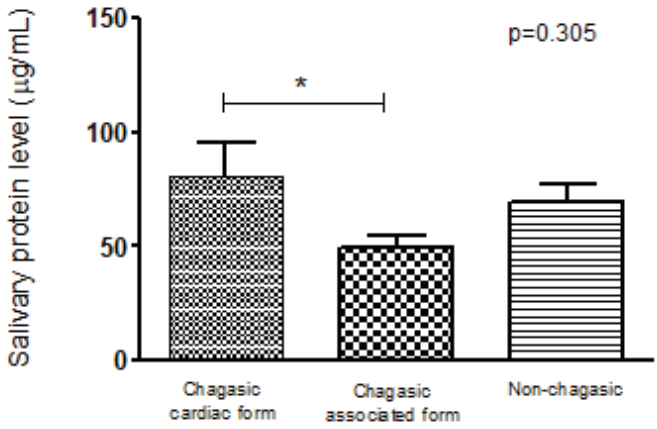

Figure 3. Comparison of protein salivary levels between cardiac chagasic individuals, chagasic individuals with the associated form of the disease with the presence of megaesophagus, and non-chagasic individuals. *ANOVA, post-test (Tukey's test), p = 0.0305.

DISCUSSION

Chagasic patients with megaesophagus presented a higher salivary flow rate compared to non-chagasic patients, which corroborates another study (Martini et al., 1990). The larger salivary flow in chagasic individuals with megaesophagus probably occurs due to dilation of the salivary gland ducts caused by the neuronal destruction typical of CD (Martini et al., 1990; Rassi et al., 2010). In addition, increased capillary filtration due to the increased number and diameter of blood vessels (Pereira et al., 2006) and slow pharyngeal transit in individuals with chagasic megaesophagus (Martini et al., 1990, dos Santos et al., 2011) might cause increased salivary flow in these individuals. The increase in salivary flow would trigger a decrease in salivary protein levels due to dilution (Kugler et al., 1992), as found in the individuals with megaesophagus in the present study.

During the chronic phase of CD, *T. cruzi* may invade and parasitize different cell types, including tongue muscle cells (Sica et al., 1995). The destruction of tongue muscle cells by *T. cruzi* results in fibrosis by means of a repair mechanism similar to that described in the heart of chagasic individuals (dos Reis et al. 2005). A study on rats infected with *T. cruzi* showed decreased muscle contractility during infection (Ramirez-Archila et al., 2011). Damaged myofibrils were observed in biceps brachial muscle biopsies of humans chronically infected with *T. cruzi* (Rosestolato et al., 2002), thus

compromising the skeletal muscle contractile function. This is due to damage to the neuromuscular junction of skeletal muscle cells, which causes loss of sensitivity and contractile structure stretching (de Araújo et al., 2000). In the present study, no significant differences were noted between the groups in relation to any functional parameter evaluated in the tongue, such as muscle tone, mobility, muscular strength with resistance, muscular strength without resistance and contraction of the lingual apex. However, although significant differences were not detected among the functional parameters evaluated, muscle tone alteration was the most frequent finding detected in 83.3% of the cardiac chagasic patients. Thus, we believe that in patients with the cardiac form, *T. cruzi* would also present tropism through the striated musculature of the tongue triggering tissue lesions and a decrease in lingual tonus. However, since this was the first study to evaluate tongue function in chagasic patients, further studies should be carried out with a larger number of patients in order to corroborate these results.

IgA is an immunoglobulin present primarily on mucosal surfaces and is the first line of defense against infectious diseases. Reduced levels of IgA have been associated with increased episodes of gastrointestinal tract infection (Gleeson et al., 1995). One study demonstrated higher levels of anti-amastigote IgA in the blood of individuals with the chronic digestive form of CD, especially in patients with severe esophageal involvement (Primavera et al., 1990). The increase of IgA levels in the blood of individuals with megaesophagus occurs due to the lesion of the mucosa by retention of the esophageal content associated to the decrease in motility, which would allow the entrance of bacterial antigens into the circulation. These antigens would stimulate the precursors of IgA-producing plasma cells located in the lamina propria of the gastrointestinal tract (Shroff et al., 1995). However, as in the present study there was no significant difference in total salivary IgA levels among the groups, we believe that total IgA levels would be more diluted in saliva when compared to blood levels.

In the acute phase of CD there is an increase in the production of specific IgM, but when CD becomes chronic there is a disease of specific IgM to early production of IgG (Coura-Vital et al., 2008). Specific subclasses of immunoglobulin G (IgG) antibodies have been shown to be associated with human CD, although not correlated with clinical forms (Cordeiro et al., 2001), which corroborates our findings since no significant difference was found in total salivary IgM levels between the groups.

Thus, the fact that the tongue does not present significant functional alterations answers a series of questions that have arisen in the development of several studies carried out by this team (Pereira et al., 2006; Pereira et al., 2007; Pereira et al. 2009). However, it is clear that the present study presents limitations, such as the relatively small number of patients, since many of the patients that attended the clinic did not present CD or did not agree to

participate in the study. Therefore, new studies should be performed with a greater number of patients to better understand the oral alterations in this parasitic disease.

Although patients with chagasic megaesophagus presented higher salivary flow and lower salivary protein levels, the fact that there were no significant differences in lingual function and total salivary immunoglobulin levels among the groups leads to the conclusion that chronic CD would not modify the lingual function and total IgA, IgG and IgM salivary levels. The present study was the first to evaluate the tongue function and salivary total immunoglobulin levels in CD.

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