Histological, biochemical and pharmacologycal characterization of the gastric muscular layer in Chagas disease[1]

Caracterização histológica, bioquímica e farmacológica da musculatura gástrica na doença de Chagas

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ABSTRACT
PURPOSE: To assess *in vitro* the correlation between the number of neurons and the sensitivity to cholinergic drugs and acetylcholinesterase activity in chagasic patients.
METHODS: A 3x1 cm strip of the muscle layer of the anterior part of the stomach, always close to the angular incisure, was removed from 10 chronic chagasic patients (6 men) submitted to megaesophagus or megacolon surgery and from 10 non-chagasic patients (4 men) submitted to other types of surgery (control group), aged on average 52.3 and 50.1 years, respectively, for histological and pharmacological studies. The action of cholinergic drugs was investigated in isolated preparations according to the superfusion method of Ferreira and Costa, and acetylcholinesterase activity was determined by the method of Ellman. For neuron count, the strips were cut into 8 µm sections according to the method standardized by Alcântara.
RESULTS: There was a difference in number of neurons between the chagasic (5,6) and control (7,3) groups. Acetylcholinesterase activity, in moles of hydrolyzed substrate per minute per gram tissue, was reduced in chagasic patients (4,32) compared to the controls (7,30). No hypersensitivity of the gastric musculature to cholinergic drugs was detected, with a reduced maximum response to carbachol and betanechol in the chagasic group.
CONCLUSIONS: The reduction of neurons in the myenteric plexus of the stomach of chronic chagasic patients can be demonstrated even in the absence of clinical chagasic gastropathy. The hypersensitivity of the gastric musculature to cholinergic drugs probably depends on intense denervation. The reduced acetylcholinesterase activity demonstrates the involvement of the cholinergic innervation in the stomach of chronic chagasic patients. There was no correlation between number of neurons, sensitivity to cholinergic drugs and acetylcholinesterase activity in the gastric musculature of chagasic and non-chagasic patients.
Key words: Neurons. Acetylcholinesterase. Chagas Disease.

RESUMO
OBJETIVO: Avaliar *in vitro* a correlação entre o número de neurônios e a sensibilidade a drogas colinérgicas e a atividade da acetilcolinesterase em pacientes chagásicos.
MÉTODOS: Em 10 pacientes chagásicos crônicos (6 homens) submetidos à cirurgia de megaesôfago ou de megacólon e em 10 pacientes não chagásicos (4 homens) submetidos a outros tipos de cirurgia (grupo controle), respectivamente com idade média de 52,3 e 50,1 anos, retirou-se uma tira de 3x1 cm da camada muscular da parede anterior do estômago, sempre junto á cisura angular, que serviu para os estudos histológicos e farmacológicos. A ação de drogas colinérgicas foi feita em preparação isolada de acordo com o método de superfusão de Ferreira e Costa, e a determinação da atividade da acetilcolinesterase pelo método de Ellman. Para a contagem de neurônios a tira muscular foi submetida a cortes de 8 micra segundo método padronizado por Alcântara.
RESULTADOS: Houve diferença do número de neurônios entre os grupos chagásico (5,6) e controle (7,3). A atividade da acetilcolinesterase mostrou-se diminuída nos chagásicos (4,32) expressa como número de moles do substrato hidrolisado por minuto por grama de tecido, em relação aos controles (7,30). Não se encontrou hipersensibilidade da musculatura gástrica a drogas colinérgicas, encontrando-se inclusive efeito máximo reduzido ao carbacol e betanecol no grupo chagásico.
CONCLUSÕES: A redução de neurônios no plexo mioentérico do estômago de pacientes chagásicos crônicos pode ser demonstrada mesmo na ausência de gastropatia chagásica clínica. A hipersensibilidade da musculatura gástrica a drogas colinérgicas provavelmente depende de desnervação intensa. A redução da atividade da acetilcolinesterase demonstra o comprometimento da inervação colinérgica no estômago de pacientes chagásicos crônicos. Não houve correlação entre número de neurônios, sensibilidade a drogas colinérgicas e atividade da acetilcolinesterase na musculatura gástrica de pacientes chagásicos ou não chagásicos.
Descritores: Neurônios. Acetilcolinesterase. Doença de Chagas.

Introduction

One of the first references to gastric impairment in Chagas disease was made by Amorim and Corrêa Netto[1] who observed the generalized absence of ganglion cells in the Meissner and Auerbach plexuses of the stomach associated with mucosal atrophy in a patient with megaesophagus and megacolon. The pathogenesis of the digestive form of Chagas disease was clarified in the studies of Köberle *et al*.[2-4] who established in a convincing manner the relation between infection with the trypanosome and changes in the intramural ganglia, responsible for the "megas" frequently occurring in regions endemic for Chagas disease. The role of the intrinsic denervation of the digestive tube in the pathogenesis of Chagas disease has been extensively demonstrated in the literature[1,2]. The stomach is also affected by this denervation, which may result in rare cases of megagastry[3] or of purely functional alterations[4].

Recent studies have demonstrated a high complexity of the neuronal network of the myoenteric plexus, where several types of neurons with different functions exist. Some of them are of the associative type, giving origin to intrinsic reflex arcs on the wall of the digestive tube, where the integration of complex functions can occur, such as antropyloroduodenal coordination. This integration occurs with the participation of various neurotransmitters, many of them also present in the central nervous system, and is influenced by hormonal factors and by extrinsic innervation[5,6,7].

The denervation occurring in Chagas disease does not only affect the postganglionary neurons of the parasympathetic autonomic nervous system, but also various other types of neurons present in the nervous plexuses of the stomach[6], corresponding to a type of highly variable and usually partial denervation[3].

Vieira and Godoy[8] characterized chronic chagasic gastropathy by motor hypereactivity of the stomach to metacholine, which they attributed to the denervation law of Cannon. Padovan *et al*.[9] demonstrated reduced gastric acid secretion under stimulation with histamine in chagasic patients, which was corrected with the use of betanechol, providing resources for a better understanding of the physiopathology of parasympathetic stomach denervation and for the assessment of the degree of denervation and its possible clinical implications.

One of the mechanisms suggested to explain hyperreactivity to this chemical mediator is deficiency of the enzyme that inactivates it, in this case acetylcholinesterase. Hypersensitivity to the action of acetylcholine on the isolated duodenum of chagasic rats supports the suggestion that low acetylcholinesterase levels are an importante factor in the triggering of hypersensitivity.

New investigations for a better histological, biochemical and pharmacological characterization of the stomach musculature in Chagas disease are fully justified.

Purpose

1. To evaluate the denervation of the myenteric plexus of the gastric musculature of chagasic patients without gastric symptoms by neuronal count in a small tissue fragment.

2. To evaluate *in vitro* the hypersensitivity of the gastric musculature to cholinergic drugs in Chagas disease in a manner similar to *in vivo* evaluation.

3. To evaluate the participation of cholinergic innervation in the stomach of chronic chagasic patients and in normal controls based on the action of cholinergic drugs and on the determination of acetylcholinesterase activity.

4. To determine the correlation between neuron number, sensitivity to cholinergic drugs and acetylcholinesterase activity.

Methods

The study was conducted on 20 subjects divided into two groups:

GROUP 1: ten control patients with no gastrointestinal disease with a negative Guerreiro-Machado reaction , operated upon due to calculous cholecystitis (six cases) blood diseases (two cases) or perforating abdominal wounds (two cases).

GROUP 2: ten chagasic patients with a positive Guerreiro-Machado reaction operated upon due to megaesophagus or megacolon.

Patient distribution by sex and age is presented in Table 1.

TABLE 1 - Patient distribution by sex and age

Groups	n	Sex		Age (years)	
		Male	Female	Mean	Range
1	10	4	6	50.1	28-62
2	10	6	4	52.3	41-63

1 = control 2 = chagasic patients

During laparotomy performed in chagasic patients for the treatment of megaesophagus or megacolon, or in the non-chagasic control group for the treatment of cholelithiasis or of a disease not related to the digestive tube, a gastric muscle strip was systematically removed in an extramucosal manner from the angular incisure, half way between the greater and lesser curvature. The removed fragmente measured 3 cm in the transverse direction of the organ and 1 cm in the longitudinal direction, so as to include the circular layer, which is more uniform and which best responded to to the pharmacological stimuli in preliminary tests. Immediately after removal, the fragment was immersed in ice-cold Krebs solution and oxygenated.

In the laboratory, the muscle strip was divided into two parts along its longer axis, one of them measuring 30 x 3 mm and used for the determination of acetylcholinesterase, and the other measuring 30 x 7 mm and used for the study of reaction to drugs and later for neuron count.

The action of cholinergic drugs was evaluated using an isolated preparation by the superfusion method proposed by Ferreira and Costa[10], in which muscle tissue is suspended in mineral oil in a glass container kept at a constant temperature of 37º C with water circulating on its walls. The lower extremity of the muscle was fixed to the bottom of the container and the upper extremity was tied to a polyethylene tube which was used for

continuous perfusion with Krebs solution bubbled with oxygen at a flow of 0.1 ml/minute. This same polyethylene tube was used for drug injection. The muscle contractions were captured by an isotonic transducer fixed to the tube supporting the muscle strip. Contraction was recorded with a Varian-A-25 recorder (Varian Associates, Instrument Division, USA).

The drugs to be tested, acetylcholine, carbachol and betanechol, were injected in six increasing doses each in order to obtain a dose-response curve that would permit to determine the maximum effect and the dissociation constant (Kd) of the drug-receptor complex. This constant represents the dose that produces half the maximum effect and is inversely proportional to the affinity of the drug. The doses used ranged from 0.01 to 20 µg for acetylcholine, from 0.001 to 20 µg for carbachol, and from 0.2 to 200 µg for betanechol. In this type of preparation the injected drug reaches the tissue directly, without dilution.

At the end of the pharmacological study the muscle fragment was fixed in Bouin's fluid for later neuron count. The material was embedded in paraffin and cut into 8 µm-thick sections and one of every three sections was placed on a slide for reading. This method was standardized by Alcântara[11] and its purpose is avoiding to count the same neuron twice since the mean diameter of a neuron is 24 µm. The material was stained with Masson trichrome. Twelve sections per case were counted and the result is reported as number of neurons counted in all sections. The count was always performed by the same author W.C., who was unaware of the group to which the section belonged.

Acetylcholinesterase activity was determined by the colorimetric method of Ellman[12], with and without the presence of quinidine for pseudocholinesterase inhibition. This method is based on the spectrophotometric measurement of the yellow color produced by thiocholine (product of the breakdown of acetylcholine by acetylcholinesterase) when it reacts with the dithiobisnitrobenzoate (DTNB) ion. Thus, the measurement of the rate of yellow color formation quantitates the hydrolysis process whose rapidity depends solely on acetylcholinesterase concentration , since the substrate (acetylcholine) and DTNB are used in excess amounts. This provides a full picture of the enzymatic kinetics studied and the hydrolysis process is monitored by the measurement of the reaction product, a procedure that reduces the margin of error. A BECKMAN, model 24-25, ACC spectrophotometer was used for the colorimetric measurement. The results were obtained as number of substrate moles hydrolyzed per minute, per gram tissue.

Data were analyzed statistically by the nonparametric Kruskal-Wallis test. When a significant difference was detected (≤ 0.005), multiple group comparisons were performed with the mean ranks obtained in this test (Dunn method). Parametric analysis of variance (one-way ANOVA) was also performed followed by the Newman-Keuls test for multiple comparisons of the means. Correlation between groups was determined by the Spearman method.

The procedures followed in the present study agree with the norms for non-therapeutic clinical research established by the Helsinki declaration. All patients gave written informed consent to participate in the study. None of them presented any type of complication and none suffered any losses.

Results

The neuronal count revealed a mean ($\pm$ SD) number of 19.5 ± 5.6 in the control group and of 7.3 ± 4.05 in the chagasic patients, with a statistically significant difference bewteen groups.

Acetylcholinesterase activity was decreased in the chagasic group only in the presence of quinidine (Table 2).

TABLE 2 - Acetylcholinesterase activity in the gastric musculature

Groups	Without quinidine	With quinidine
	R.10^{-7} (mean+/-s)	R.10^{-7} (mean+/-s)
1	8.78 +/- 2.48	7.30 +/- 2.24
2	5.96 +/- 3.61	4.32 +/- 2.39*

1 = control 2 = chagasic patients *lower than control for $\alpha = 0.05$
R = number of moles of hydrolyzed substrate per minute per gram tissue

In the study of sensitivity of gastric muscle to cholinergic drugs it was not possible to demonstrate hypersensitivity to any of the three drugs tested, with a reduced maximum effect of carbachol and betanechol being actually detected in the chagasic group (Tables 3 and 4).

TABLE 3 - Dissociation constant (Kd) of acetylcholine and betanechol

Group	Acetylcholine	Carbachol	Betanechol
	mean +/- s	mean +/- s	mean +/- s
1	2.65 +/- 1.78	1.08 +/- 0.83	15.03 +/- 11.11
2	3.09 +/- 1.83	3.45 +/- 2.7	16.93 +/- 8.71

1 = control 2 = chagasic patients

TABLE 4 - Maximum effect of acetylcholine carbachol and betanechol (in mm)

Group	Acetylcholine		Carbachol		Betanechol	
	mean	s	mean	s	mean	s
1	8.97	1.91	2.46	2.29	11.98	2.29
2	7.56	1.63	9.00*	1.97	6.24*	1.42

1 = control 2 = chagasic patients
*statistically significant difference for $\alpha = 0.05$ compared to Group 1

Correlation analysis

The number of neurons was not correlated with acetylcholinesterase activity or with Kd values or the maximum effect of the three drugs studied in the two groups. Similarly, there was no correlation between acetylcholinesterase activity and Kd values or the maximum effects observed.

Discussion

The chagasic and control groups were homogeneous regarding age and sex and therefore were comparable, only differing by the presence of Chagas disease in group 2.

The neuronal count by sampling in a small tissue fragment as done in the present study differs from the classical method applied to circumferential rings of the organ and to a large number of sections. However, Brandão[13] reported regular neuron distribution in counts performed on 42 8-µm section of the large intestine of rats, a fact that permits us to consider counts in 10 sections to be also representative of the degree of denervation. It should be emphasized that this method for neuron count is not suitable for the evaluation of an isolated case because of the superimposition of values. However, the method is valid for groups of individuals.

The present findings confirm the reduction of neurons in the myenteric plexus of the stomach in chagasic patients with megaesophagus or megacolon, even in the absence of gastric manifestations of the disease, as observed in the patients studied. This permits us to conclude that the neuron count in a small tissue sample may be useful for the study of denervation of the digestive tube in Chagas disease. The possibility of the existence of subclinical denervation was also demonstrated, suggesting that the clinical, morphological and even functional manifestations only occur after a certain degree of denervation.

The absence of hypersensitivity to cholinergic drugs *in vitro* observed here agrees with the observations of Vieira and Godoy *in vivo*[8], which showed that hypersensitivity was present in only 12% of the cases studied, exactly those with gastric symptoms. This led the authors to suggest the reaction to cholinergic drugs as a criterion for the diagnosis of chagasic gastropathy. The interest in elucidating the role of cholinergic innervation led us to use acetylcholine, which is hydrolyzed by cholinesterase, and betanechol and carbachol, which are resistant to this enzyme. There was no difference in the sensitivity to this drug, a fact that prevents the conclusion that inactivation of the mediator is involved in a greater or lesser action of these drugs. The reduction observed in acetylcholinesterase activity, with no correlation with the neuronal population or with the sensitivity to cholinergic drugs, points in the same direction. Thus, it is necessary to look for other mechanisms to explain Cannon's law.

The reduction of acetylcholinesterase activity observed in chagasic patients in the presence of quinidine is an original contribution to the study of the digestive form of Chagas disease. This reduction has been considered to be a possible cause of hypersensitivity in Chagas disease, since it does not occur in Hirschsprung disease, where a high concentration of the enzyme is observed in hypertrophic nerve. Thus, the present study does not support this possibility. The important presence of pseudocholinesterases in the gastric musculature was also demonstrated, since the reduced acetylcholinesterase activity was observed only in the presence of quinidine, which inhibits it. This finding should be taken into account in studies to be conducted on the activity of this enzyme.

The absence of correlation between the low acetylcholinesterase activity, the reduction of neuron numbers and the sensitivity of the gastric mucosa of chagasic patients lead us to question the real mechanism of the hypersensitivity of denervated structures to the chemical mediator and to suggest the possibility of the involvement of non-cholinergic (peptidergic) neurons whose numerical reduction has been demonstrated in studies on the pathogenesis of Chagas disease[14].

Conclusions

The results of the present study obtained under the conditions used permit us to reach the following conclusions:

1- The reduction of number of neurons in the myenteric plexus of the stomach of chronic chagasic patients was demonstrated even in the absence of clinical chagasic gastropathy.

2- The absence of hypersensitivity of the gastric mucosa to cholinergic drugs supports the idea that this hypersensitivity may occur only in association with clinical or morphological manifestations of chagasic gastropathy, a fact probably depending on more intense denervation.

3- The reduction of acetylcholinesterase demonstrates the involvement of cholinergic innervation in the stomach of chronic chagasic patients.

4- There was no correlation between number of neurons, sensitivity to cholinergic drugs and acetylcholinesterase activity in the gastric musculature of chagasic or non-chagasic patients.

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