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Instituto de Biociências de Botucatu
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Título: Distúrbios na motilidade gastrintestinal associados a alterações morfométricas de neurônios entéricos em modelos experimentais de *Diabetes mellitus*

Juliana Fernandes de Matos

Orientador: **Prof. Dr. José Ricardo de Arruda Miranda**

Coorientadora: **Prof. Dra. Madileine Francely Américo**

Tese apresentada ao Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu para obtenção do título de Doutora no Programa de Pós-Graduação em Farmacologia e Biotecnologia, Área de concentração *Biologia Celular Estrutural e Funcional.*

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Palavras-chave: Biosusceptometria AC; Estômago; Motilidade; Neurônios; Ratos.

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(Albert Einstein)

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Resumo

O estado hiperglicêmico está relacionado com alterações fisiológicas em todo o organismo no diabete. É conhecido a recorrência de distúrbios gastrintestinais em indivíduos diabéticos. Estudos sobre alterações da motilidade gastrintestinal em diabéticos mostram danos em neurônios entéricos e na parede gástrica. Há uma lacuna no conhecimento sobre a relação entre níveis glicêmicos, motilidade gastrintestinal e neurônios entéricos. Portanto o objetivo foi analisar a atividade de contração, esvaziamento gástrico e trânsito oro-cecal em ratos com modelos de diabete grave e moderado e avaliar a integridade da parede gástrica e dos neurônios entéricos. Os animais foram induzidos ao diabete com Streptozotocin (STZ) (diabete moderado: 100mg/kg; glicemia de jejum 6,6-16,5 mmol/L) e (diabete grave: 50mg/kg glicemia de jejum> 16,5mmol/L). A Biosusceptometria AC (BAC) aferiu a contratilidade gástrica, quantificados por frequência e meia-largura da banda em ciclos por minuto. Assim como o esvaziamento gástrico (EG) e o trânsito oro-cecal (TOC), analisados por tempo médio de EG e tempo médio de chegada ao ceco, respectivamente. A atividade elétrica gástrica foi aferida por eletrogastrograma (EGG). A histologia da parede gástrica foi realizada por hematoxilina-eosina e mensurada a espessura da mucosa e das musculares longitudinal e circular, do corpo do estômago. Os neurônios colinérgicos (ChAT) e nitrérgicos (nNOS) do plexo mioentérico do corpo do estômago foram marcados por imuno-histoquímica, analisadas a densidade de neurônios por gânglio e perfil da área neural. Animais induzidos por STZ apresentaram glicemia de jejum elevada e perda de peso corporal. Ocorreu uma diminuição da frequência de contração e aumento da meia-largura da banda, para BAC e EGG, nos modelos de diabete comparados ao controle. O esvaziamento gástrico e o trânsito oro-cecal se tornaram lentificados em relação ao controle. A camada muscular circular e mucosa apresentaram espessamento nos grupos diabéticos. Os neurônios ChAT e nNOS não apresentaram diferença na densidade de neurônios, mas a área neuronal diminuiu nos diabéticos. Em conclusão, alterações na motilidade parecem estar associadas a mudanças na parede gástrica e à diminuição da área neuronal de neurônios nitrérgicos e colinérgicos, que foram intensificados pela exposição prolongada a condições hiperglicêmicas, mesmo que moderadas no diabetes.

Palavras-chave: Biosusceptometria AC, Estômago, Motilidade, Neurônios entéricos, Ratos.

Abstract

The hyperglycemic state is related to physiological changes throughout the body in diabetes. Recurrence of gastrointestinal disorders in diabetic individuals is known. Studies on changes in gastrointestinal motility in diabetics show damage to enteric neurons and the gastric wall. Few are the knowledge about the relationship among glycemic levels, gastrointestinal motility and enteric neurons. Therefore, the aim was to analyze the activity of contraction, gastric emptying and oro-cecal transit in rats with mild and severe diabetes models and to evaluate the integrity of the gastric wall and the enteric neurons. The animals were diabetic with Streptozotocin (STZ) (moderate diabetes: 100mg/kg; fasting blood glucose 6.6-16.5 mmol/L) and (severe diabetes: 50mg/kg fasting blood glucose>16.5mmol/L). AC biosusceptometry (ACB) measured gastric contractility, quantified per frequency and half-band width in cycles per minute. As well as gastric emptying (EG) and oro-cecal transit, analyzed by mean time of EG and mean time of arrival to the cecum, respectively. Gastric electrical activity was measured by electrogastrogram (EGG). The histology of the gastric wall was performed by hematoxylin-eosin and measured the thickness of the mucosa and the longitudinal and circular muscles of the stomach body. The cholinergic (ChAT) and nitrergic (nNOS) neurons of the myenteric plexus of the stomach body were marked by immunohistochemistry, the density of neurons per ganglion and neural area profile were analyzed. STZ-induced animals had elevated fasting blood glucose and loss of body weight. There was a decrease in the contraction frequency and increase of the band half-width, for BAC and EGG, in the diabetes models compared to control. Gastric emptying and oro-cecal transit became slowed in relation to control. The circular muscular layer and mucosa presented thickening in the diabetic groups. ChAT and nNOS neurons showed no difference in neuron density, but neuronal area decreased in diabetics. In conclusion, alterations in motility seems to be associated with changes in the gastric wall and the decrease in the neuronal area of nitrergic and cholinergic neurons that were intensified by long term exposure to hyperglycemic conditions, even that moderate in the diabetes.

Keywords: Biosusceptometry AC, Stomach, Motility, Enteric neurons, Rats.

Lista de Abreviaturas

DM - *Diabetes mellitus*

DM1 - *Diabetes mellitus* tipo 1

DM2 - *Diabetes mellitus* tipo 2

DM1W - *Diabetes mellitus* 1 semana

DM1M - Diabetes mellitus 1 Mês

DM mild - Diabetes mellitus moderado/mild

CT - Controle

BAC/ACB - Biousceptometria de Corrente Alternada

EGG - Eletrogastrograma

STZ - Streptozotocin

ChAT – Neurotransmissor Acetiltransferase colina

NOS – Enzima óxido nítrico sintetase

nNOS – Neurônio óxido nítrico sintetase

NO - Óxido nítrico

HuC/D - Anticorpo pan-neuronal

DAPI - 4,6 diamino 2-phenylindole dihydrochride

TRIS- hidroximetil aminometano

EDTA - ácido etilenodiaminotetracético

PBS - Tampão fosfato salino

TGI/GIT - Trato gastrintestinal

CPM - Ciclos por minuto

AG/GA - Atividade gástrica

MGET - Tempo médio de esvaziamento gástrico

EG/GE - Esvaziamento gástrico

OCT - Trânsito oro-cecal

MCAT - Tempo médio de chegada ao ceco

HE - Hematoxilina e Eosina

SNE/ENS - Sistema nervoso entérico

SNC/CNS - Sistema nervoso central

FFT - Transformada rápida de Fourrier

AUC - Área sob a curva

AGEs - Prdutos finais da glicação avançada

ROS/EROs - Espécies reativas do oxigênio

Sumário

Lista de Ilustrações

Introdução

1. Introdução

A epidemia global de diabete afeta atualmente mais de 440 milhões de pessoas no mundo (CHAN, 2014; MA, 2018). O *Diabetes mellitus* (DM) compreende um grupo de distúrbios metabólicos caracterizados por hiperglicemia crônica resultante de defeitos na ação da insulina e/ou na sua secreção (DORSEY; BECKER; AL., 2018; RIDDLE et al., 2018). Existem principalmente 2 tipos de DM: tipo 1 e o 2. O DM1 está relacionado à destruição de células beta pancreáticas, usualmente levando a uma dependência absoluta de insulina. Enquanto o DM2 decorre de um defeito progressivo da secreção de insulina levando a uma resistência insulínica (RIDDLE et al., 2018). Algumas formas de DM1 não têm etiologias conhecidas, mas é definido que há pelo menos um marcador celular autoimune indicando a destruição de células beta pancreáticas. Os pacientes apresentam insulinopenia permanente e são propensos a cetoacidose (RIDDLE et al., 2018). Indivíduos com DM2 tem resistência à insulina e normalmente uma relativa deficiência desse hormônio, porém a maioria dos pacientes não necessita de tratamento com insulina para sobreviver. A etiologia do DM2 é desconhecida, mas sabe-se que o risco de a desenvolver aumenta com a obesidade, avanço da idade e a falta de atividade física (RIDDLE et al., 2018).

Em indivíduos saudáveis, a concentração de glicose plasmática é fortemente regulada e mantida dentro de uma faixa estreita (KELLEY et al., 1988). Em contrapartida, pacientes diabéticos são expostos a longos períodos de hiperglicemia e apresentam disfunções em todo o organism (DORSEY; BECKER; AL., 2018).

Anormalidades na motilidade gástrica ocorrem em 30-50 % dos pacientes com longo histórico de diabete (DE BLOCK et al., 2006; NILSSON, 1996). Estudos de frequência de sintomas gastrintestinais em pacientes com DM1 e DM2 mostraram que 68% apresentam pelo menos um sintoma (JEBBINK et al., 1994; SPÅNGÉUS et al., 1999). O DM1 afeta todo o trato gastrintestinal (TGI) e isso acarreta uma maior preocupação na investigação dessas alterações e na busca de uma melhora na qualidade de vida dos pacientes, visando reduzir a morbidade causada pelo diabete. As alterações mais comuns encontradas no diabete são no esvaziamento gástrico, como lentificação (MARD et al., 2016; SAMSOM et al., 1997; WOERLE et al., 2008) ou esvaziamento acelerado (BHARUCHA et al., 2015; CHOI et al., 2007; HAUSCHILDT et al., 2018). Presença de arritmias gástricas e anormalidade nas atividades motora e elétrica gástrica são reportadas em modelos experimentais de diabete a longo termo (HAUSCHILDT et al., 2018; MARQUES et al., 2014).

Indivíduos com DM1 apresentam uma retenção gástrica significativa dos conteúdos ingeridos em resposta à hiperglicemia quando comparados a indivíduos saudáveis, sugerindo uma adaptação inadequada ao aumento da glicemia (MARATHE et al., 2013). Consequentemente, com o esvaziamento gástrico atrasado há uma dificuldade em manter o controle glicêmico, sendo esse um mecanismo de retroalimentação (WOERLE et al., 2008). O retardo no esvaziamento gástrico em momentos de hiperglicemia é uma resposta fisiológica pós-prandial, mecanismo este que auxilia na absorção dos nutrientes ingeridos. Porém em pacientes com DM1, esse mecanismo favorece a manutenção de altos índices glicêmicos e distúrbios na motilidade gastrointestinal (WOERLE et al., 2008).

Há trabalhos que enfatizam a importância do controle glicêmico no diabete, e enfatiza as alterações provocadas pela glicemia na motilidade gastrointestinal (MARATHE et al., 2013; SOGABE et al., 2005). Um estudo ressalta que a hiperglicemia induz um relaxamento na região fúndica do estômago causando um fluxo pilórico retrógrado, que por sua vez, pode levar a uma retenção do bolo alimentar (EBERT, 2005).

Com relação aos indivíduos com DM2 os estudos são inconclusivos, em razão de diferenças nas metodologias adotadas e pouco controle das variáveis ambientais sobre o esvaziamento gástrico. Foi demonstrado que indivíduos com DM2 sem controle glicêmico quando tratados com insulina, não apresentaram diferença no esvaziamento gástrico, e que há pacientes com DM2 que tem esvaziamento gástrico acelerado e outros retardado (BHARUCHA et al., 2015).

A motilidade gastrintestinal é a contração e relaxamento do musculo liso necessário para transportar e processar os alimentos, sob estrito controle (CUMMINGS; OVERDUIN, 2007; JOHNSON, 2014). A contração do estômago é necessária para triturar o alimento, misturar com o suco gástrico e propelir em direção ao piloro. O controle da frequência e amplitude dessas contrações são essenciais para absorção dos nutrients (JANSSEN et al., 2011). Assim como o esvaziamento gástrico é controlado por peptídeos gastrintestinais (GLP-1, GIP, VIP), pela taxa glicêmica, insulina, composição do bolo alimentar e controle neural. Todos esses controles são necessário para regular a taxa de esvaziamento e promover a absorção correta pelo intestino. Alterações em qualquer uma dessas vias desencadeia diversos distúrbios gastrintestinais, como dores e desconfortos abdominais (BOECKXSTAENS et al., 2016; CAMILLERI; BROWN; MALAGELADA, 1986; CUMMINGS; OVERDUIN, 2007;

MARATHE et al., 2013; WU et al., 2013).

O diabete afeta o TGI comprometendo a motilidade, além de possivelmente alterar a parede gastrintestinal, assim como os neurônios entéricos responsáveis pelo controle das funções do TGI. A inter-relação entre biomecânica gastrintestinal e neurônios entéricos é relevante e deve ser melhor esclarecida. Essas alterações morfofuncionais são fatores que podem contribuir com a dor abdominal, saciedade precoce, náuseas, vômitos, e constipação em pacientes com DM (EBERT, 2005; FURNESS, 2012; MARATHE et al., 2013; ZHAO; NAKAGUCHI; GREGERSEN, 2009).

O sistema nervoso entérico (SNE) está presente em todo o TGI e é responsável por regular funções motoras, sensoriais e secretoras, além de integrar e ser modulado pela sinalização do sistema nervoso central (SNC) e periférico (FURNESS, 2000). É dividido em plexo submucoso que está presente na camada submucosa e apresenta funções secretoras. O plexo mioentérico está presente entre as camadas musculares circular e longitudinal, e tem funções sobre a motilidade do TGI. Os principais neurônios envolvidos no controle da motilidade são os excitatórios colina acetiltransferase (ChAT) e os inibitórios óxido nítrico sintetase (nNOS) (FURNESS, 2012).

A neuropatia diabética é um evento presente em pacientes com longo histórico de diabete e pode estar correlacionada com gastroparesia e distúrbios gastrintestinais (CAMILLERI, 2007; CAMILLERI; BHARUCHA; FARRUGIA, 2011; CHANDRASEKHARAN; SRINIVASAN, 2007; NESHATIAN; GIBBONS; FARRUGIA, 2015). A perda de células de Cajal e diminuição do neurotransmissor óxido nítrico (NO) podem estar diretamente relacionados com disfunções na motilidade gástrica nesse tipo de pacientes diabéticos (CELLEK et

al., 2004; DU et al., 2009; WANG et al., 2009; WATKINS et al., 2000; WOOD, 2016). Trabalhos evidenciam fases da degeneração neuronal em indivíduos diabéticos, apresentando eventos reversíveis com o controle glicêmico, em momentos precoces da doença (CELLEK et al., 2004; CELLEK; FOXWELL; MONCADA, 2003). Isso evidencia a necessidade de um diagnóstico preciso e precoce para se evitar danos irreversíveis aos indivíduos com DM.

Além disso, indivíduos com DM1 são mais susceptíveis a lesões gástricas malignas, primárias de gastrite autoimune, presente em 15-20% desses pacientes (DE BLOCK et al., 2003). Ou seja, quanto mais conhecimento sobre os mecanismos desses distúrbios gastrintestinais mais rápido seria o diagnóstico, aperfeiçoando os esquemas terapêuticos e a qualidade de vida de indivíduos diabéticos.

Como ferramenta experimental, utilizam-se modelos animais portadores de diabete para um melhor entendimento da fisiopatologia dessa síndrome. Mimetizando a hiperglicemia do DM1, há o modelo experimental de diabete grave (glicemia>300mg/dL), induzida por uma dose de Streptozotocin-STZ (droga com efeito citotóxico sobre as células beta pancreáticas) intraperitoneal na vida adulta (FREGONESI et al., 2001). Para mimetizar a glicemia do DM2, utiliza-se o modelo experimental de diabete moderado (glicemia entre 120 e 300mg/dL), induzida por administração subcutânea de STZ no período neonatal (DAMASCENO et al., 2013, 2014; I.L. et al., 2010).

A Biosusceptometria de Corrente Alternada (BAC) é uma técnica simples, barata e sensível, reproduzível em diversas áreas da pesquisa. Com a utilização dessa técnica é possível registrar a atividade mecânica e funcional de órgãos contráteis. Essa técnica já foi validada para várias áreas, como para avaliação

do esvaziamento gástrico e trânsito oro-cecal em ratos(QUINI et al., 2012). Estudos *in vivo* e *in vitro* validaram a BAC como uma técnica biomagnética capaz de aferir atividade de contração gástrica (AMÉRICO et al., 2010). Em modelo de diabete a longo tempo induzido por Aloxana, os sinais de contratilidade e esvaziamento gastrintestinal, aferidos por BAC, estavam comprometidos (MARQUES et al., 2014). Estudos recentes com a BAC, em modelos de hiperglicemia moderada a longo tempo, revelam distúrbios gástricos como esvaziamento acelerado e amplitudes da contração gástrica mais vigorosas (HAUSCHILDT et al., 2018).

Em estudos que analisam características motoras do TGI, a aquisição da atividade elétrica é fundamental para o entendimento da eletrofisiologia do tecido estudado. O eletrogastrograma (EGG) é uma técnica bastante simples, minimamente invasiva e utilizada principalmente em pesquisas da atividade elétrica gástrica. O entendimento das funções motoras e elétricas do TGI são essenciais nas pesquisas sobre comorbidades relacionadas aos distúrbios gastrintestinais (CHEN et al., 1996).

A relação entre distúrbios gastrintestinais no diabete e o tempo de instalação das condições hiperglicêmicas ainda é pouco compreendido. Além disto, uma avaliação das atividades elétricas, mecânicas e funcionais do TGI contribuirão fortemente para um melhor entendimento de mecanismos deletérios da instalação de doenças metabólicas. Portanto o objetivo foi analisar a atividade de contração, esvaziamento gástrico e trânsito oro-cecal em ratos com modelos de diabete grave e moderado e avaliar a integridade da parede gástrica e dos neurônios entéricos.

Capítulo 1

2. Capítulo 1

Manuscript Type: ORIGINAL ARTICLE

Basic Study

Disorders in gastrointestinal motility associated with morphometric alterations of enteric neurons in experimental models of *Diabetes mellitus*

Matos J F *et al.* Changes in gastrointestinal motility in diabetic rats.

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Abstract

BACKGROUND

The hyperglycemic state is related to physiological changes throughout the body in diabetes. Recurrence of gastrointestinal disorders in diabetic individuals is known. Studies on changes in gastrointestinal motility in diabetics show damage to enteric neurons and the gastric wall. Few are the knowledge about the relationship among glycemic levels, gastrointestinal motility and enteric neurons.

AIM

To analyze the activity of contraction, gastric emptying and oro-cecal transit in rats with mild and severe diabetes models and to evaluate the integrity of the gastric wall and the enteric neurons.

METHODS

The animals were diabetic with Streptozotocin (STZ) (moderate diabetes: 100mg/kg; fasting blood glucose 6.6-16.5 mmol/L) and (severe diabetes: 50mg/kg fasting blood glucose>16.5mmol/L). AC biosusceptometry (ACB) measured gastric contractility, quantified per frequency and half-band width in cycles per minute. As well as gastric emptying (EG) and oro-cecal transit, analyzed by mean time of EG and mean time of arrival to the cecum, respectively. Gastric electrical activity was measured by electrogastrogram (EGG). The histology of the gastric wall was performed by hematoxylin-eosin and measured the thickness of the mucosa and the longitudinal and circular muscles of the stomach body. The cholinergic (ChAT) and nitrergic (nNOS) neurons of the myenteric plexus of the stomach body were marked by immunohistochemistry, the density of neurons per ganglion and neural area profile were analyzed.

RESULTS

STZ-induced animals had elevated fasting blood glucose and loss of body weight. There was a decrease in the contraction frequency and increase of the band half-width, for BAC and EGG, in the diabetes models compared to control. Gastric emptying and oro-cecal transit became slowed in relation to control. The circular muscular layer and mucosa presented thickening in the diabetic groups.

ChAT and nNOS neurons showed no difference in neuron density, but neuronal area decreased in diabetics.

CONCLUSIONS

In conclusion, alterations in motility seems to be associated with changes in the gastric wall and the decrease in the neuronal area of nitrergic and cholinergic neurons that were intensified by long term exposure to hyperglycemic conditions, even that moderate in the diabetes.

Key Words

Gastrointestinal motility, Gastric wall, Enteric neurons, Hyperglycemia, Experimental diabetes.

INTRODUCTION

The global epidemic of *Diabetes mellitus* (DM) currently affects more than 440 million people worldwide[1,2]. It is known that the hyperglycemic state is related to physiological changes throughout the body in diabetes.

There is a high recurrence of gastrointestinal (GI) disorders in diabetic individuals. Abnormalities in gastric motility occur in 30-50% of patients with a long history of diabetes[3,4]. Studies of the frequency of gastrointestinal symptoms in patients with type 1 DM (DM1) and type 2 DM (DM2) showed that 68% had at least one symptom [5,6]. DM1 affects the entire gastrointestinal tract (GIT), and this leads to more significant concern in the investigation of these alterations and the search for an improvement in patient's quality of life. The most common changes found in diabetes are in gastric emptyings, such as slowing down[7–9] or accelerated emptying [10–12]. Presence of gastric arrhythmias and abnormalities in motor and gastric electrical activities are reported in experimental models of long-term diabetes [12,13].

Individuals with DM1 present significant gastric retention of the ingested contents in response to hyperglycemia when compared to healthy individuals, suggesting an inadequate adaptation to the increase of glycemia[14]. Consequently, with delayed gastric emptying, there is a difficulty in maintaining glycemic control, which is a feedback mechanism[9]. The delay in gastric emptying at times of hyperglycemia is a postprandial physiological response, a mechanism that helps in the absorption of ingested nutrients. However, in patients with DM1, this mechanism favors the maintenance of high glycemic indexes and disturbances in gastrointestinal motility[9].

Diabetes affects GIT by compromising motility, possibly altering the gastrointestinal wall, as well as the enteric neurons responsible for the control of GIT functions. The interrelationship between gastrointestinal biomechanics and enteric neurons is relevant and should be further clarified. These morphofunctional changes are factors that may contribute to abdominal pain, early satiety, nausea, vomiting, and constipation in patients with DM[14–17].

The enteric nervous system (ENS) is present throughout the GIT and is responsible for regulating motor, sensory and secretory functions, as well as integrating and being modulated by the central nervous system (CNS) and peripheral[18]. It is divided into a submucosal plexus that is present in the submucosal layer and presents secretory functions. The myenteric plexus is present between the circular and longitudinal muscular layers and has functions on the motility of the GIT. The principal neurons involved in motile control are the excitatory choline acetyltransferase (ChAT) and the inhibitory nitric oxide synthetase (nNOS)[16].

Diabetic neuropathy is an event present in patients with a long history of diabetes and may be correlated with gastroparesis and gastrointestinal disorders[19–22]. The loss of Cajal cells and decreased neurotransmitter nitric oxide (NO) may be directly related to dysfunctions in gastric motility in this type of diabetic patients[23–27]. Studies evidenced phases of neuronal degeneration in diabetic subjects, presenting reversible events with glycemic control, at early stages of the disease[25,28]. This evidences the need for an accurate and early diagnosis to avoid irreversible damage to individuals with DM. That is, the more knowledge about the mechanisms of these gastrointestinal disorders, the faster the diagnosis, improving the therapeutic schemes and the quality of life of diabetic individuals.

There are many contradictions about the application of different experimental models of diabetes and their findings on gastrointestinal motility. In animal models of long-term diabetes induced by *Aloxan*, gastric contractility presented arrhythmias, and gastrointestinal emptying became slow[13]. Recent studies in models of mild hyperglycemia over a long time reveal gastric disorders such as accelerated emptying and more vigorous gastric contraction amplitudes[12].

The relationship between gastrointestinal disorders in diabetes and the time of setting of hyperglycaemic conditions is still poorly understood. Also, a temporal evaluation of the electrical, mechanical and functional activities of GIT will actively contribute to a better understanding of deleterious mechanisms of the installation of metabolic diseases. Therefore, the aim was to analyze the activity of contraction, gastric emptying, and oro-cecal transit in rats with mild and severe diabetes models and to evaluate the integrity of the gastric wall and the enteric neurons.

MATERIALS AND METHODS

All the experiments were performed according to the protocol of animal ethics of the Institute of Biosciences of Botucatu (CEUA 752).

Animal models

Male Sprague-Dawley rats from the ANILAB Laboratory (Paulínea-SP/Brazil) with 90 days of age and 300-350 g were used. The animals were kept under controlled conditions of temperature (22 ± 2 ° C), humidity (50 ± 10%), and light/dark cycle (12h), with water and feed ad libitum. Before the experiments, the body weight determined. After completion of the experiments, the animals were killed with 5% Isoflurane (Cristália®) overdose followed by decapitation.

Induction of Diabetes mellitus models

- Control animals (CT, N=8 rats) received an intraperitoneal injection of vehicle and presented fasting blood glucose between 4.95-5.5 mmol/L.
- Mild diabetes (DM mild, N=12 rats) was induced on the first day of life of the animals by subcutaneous administration of Streptozotocin (STZ-SIGMA Chemical Company, St. Louis, MO) at a dose of 100 mg/kg body weight diluted in 0.1 mol/L of (pH 4.5)[29–31]. The animals kept with the mother until weaning. In the adult phase (90 days of age) the fasting glucose was performed to confirm the diabetic state, using the glucometer (ACCU-CHEK Performa, Roche, Germany). The rats remained fasted overnight before collecting the blood sample, which was performed by retro-orbital puncture. The inclusion criterion established for the mild diabetic group consisted of rats that presented glycemic values between 6.6 and 16.5 mmol/L[32]. This model resembles the hyperglycemic conditions of DM2.
- Severe diabetes-induced on the 90th day of life by intraperitoneal administration of STZ at the dose of 50 mg/kg body weight diluted in citrate buffer. The inclusion criterion established to compose the severe diabetic group consisted of rats that presented glycemic values higher than 16.5 mmol/L [29,33]. This model resembles the hyperglycemic conditions of DM1.

The animals were submitted to the experiments at two different times: one week (DM1W, N=12 rats) and one month (DM1M, N=12 rats) after induction [34].

Gastrointestinal experiments were performed in all animals in the adult phase (around 90 days of life).

Gastric activity (GA)

In all experiments using ACB, the rats were fed 2 g of ferromagnetic material used as a marker (0.5 g of powdered ferrite and 1.5 g of feed) after a 12 hour fast.

At the end of the feeding, gastric activity was measured by ACB (mechanical activity) and Electrogastrography (EGG-electrical activity), acquiring frequency of contractions of the stomach in cycles per minute (CPM). The animals were anesthetized with Isoflurane® (5% induction, 2% maintenance) and placed in dorsal decubitus for the acquisition of the records. The magnetic sensor positioned over the abdomen in the anatomical region of the stomach. Due to gastric contractions, the magnetic marker is moved away and approached, respectively, from the sensor. That movement generates an oscillation in the magnetic field, which is converted into a digital signal and recorded in real time by a multi-channel recorder (MP150 System; BIOPAC®, CA-United States). For the EGG acquisition, the bipolar arrangement of needle electrodes (the positive entry in the left hypochondrium, the negative one in the right hypochondrium) and the ground in the hind paw (0.35 x 12 mm Ethicon®), and connected to the electrical signal acquisition system (Biopac EGG150C). The records lasted for 30 min and were performed simultaneously with a rate of 20 Hz.

The signal irregularity was measured through the Half-bandwidth, extracted from the contraction frequency signals of ACB and EGG.

Mean Gastric Emptying Time (MGET) and Mean Cecum Arrival Time (MCAT)

At the end of the feeding, gastric emptying (EG) and oro-cecal transit (OCT) performed at two points: stomach and cecum (anatomical references)[35]. Measurements of EG and OCT by ACB consist of assaying the amount of magnetic

material in the stomach and cecum with the magnetic sensor after ingestion of the marked feed for 6 hours (measurements every 15 min) in an awake animal. Two parameters, Mean Gastric Emptying Time (MGET) and Mean Cecum Arrival Time (MCAT) was obtained[35,36].

Histological analysis

After the experiments, the animals were anesthetized and killed; the stomach was collected and opened by the larger mesenteric border. A portion of the body region of the stomach was collected. The samples washed with Phosphate Buffered saline (PBS) and fixed with 4% Paraformaldehyde in 0.1M Phosphate Buffer overnight. Then the samples were dehydrated in a graduated ethanol series (70, 80, 90 and 100%) and embedded in paraffin. The materials were slice perpendicular to the sample, with 0.5µm thickness using microtome. Sections were rehydrated in 100, 90, 80 and 70% ethanol, followed by a wash in tap water and stained with Hematoxylin and Eosin (HE). The images were obtained using the LEICA DM LS microscope with a 10x magnification, and image analysis performed by *Image-Pro Plus software*.

Immunohistochemistry

Immunohistochemistry performed on the histological sections of the stomach body of all groups. The slides were dewaxed with xylol washes and hydrated with graduated ethanol series (100, 90, 80 and 70%). Antigen retrieval was then performed to expose the epitopes and prepare for the immunohistochemical labeling reaction. For antigen retrieval, the slides were immersed in TRIS-EDTA pH 9.0 buffers (Labsynth Ltda) and placed in a 95 ° C water bath for 45 min. After the slides cooled to room temperature, the sections incubated in 10% normal horse serum in PBS containing 1.5% Triton X-100 for 45 min at room temperature. Double labeling was performed using the nNOS (host-sheep, dilution 1: 2000, Millipore) antiserum combinations with pan-neuronal HuC/D (host-mouse, dilution 1: 200, Molecular Probers) and ChAT-goat, 1: 200 dilution, Chemicon) with HuC/D. After incubation in primary antisera for one hour, the sections were washed three times

for 10 min in PBS and incubated in the respective secondary antibodies (Donkey anti-sheep IgG 488 1: 400 and Donkey anti-mouse IgG 594 1: 200; Molecular Probes). The sections were washed three times for 10 min in PBS and incubated for 5 min in 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) to label all nuclei. After the last wash in PBS, the slides mounted on 0.5 M sodium carbonate buffered glycerol. The slides were examined using epifluorescence from the Nikon 80i microscope, and the images were captured using a digital camera, NIS Elements software (Nikon) and analyzed with *Image-Pro Plus software.*

Data analysis

For gastric contractility parameters, magnetic signals acquired in real time by Acknowledge® 4.0 software were analyzed by MatLab (Mathworks, Natick, MA, USA) by visual inspection. Subsequently, the use of Butterworth bi-directional bandpass filters by Fast Fourier Transform (FFT), with a cutoff frequency of 2-7 CPM. The highest peak frequency for each FFT determined as the dominant gastric frequency, and the lowest peak represented as signal noise. Half-bandwidth was quantized using the two points of the curve, where the intensity was half the maximum intensity (y-axis). In these two points, the corresponding frequencies (x-axis) indicate the aspect of the spectral distribution. It is performed a Gaussian adjustment of the spectral distribution to measure the half-width of the band at half height. This methodology was developed to quantify the irregularity of AG signals[13].

For the analysis of gastric emptying and oro-cecal transit, we used the statistical moment, obtained by the temporal average normalized by the area under the curve (AUC) of gastric emptying and total transit. The MGET and MCAT (in minutes) characterized by the amount of test meal emptied at a time t.

For the morphometry of the muscular layers (longitudinal and circular) and mucosa of the stomach body, it was performed ten measurements of the perimeter (μm) of each layer of all animals. In the Immunohistochemistry was calculate the number of neurons per ganglion. For each animal examined using each of the 3 markers, around ten ganglia were identified.

The number of neurons per ganglion was counted under 40X magnification. To calculate the neuronal profile area (μm²) of the cell bodies (ChAT, nNOS e HuC/D), 50 neurons from all animals for each of the 3 markers were photographed. The measurements were performed using the Image-Pro Plus.

Statistical analysis

The variables normality evaluated by the Kolmogorov-Smirnov test. The parametric data were analyzed using Student's t-test. The Mann-Whitney test used in non-parametric data. Values of $p < 0.05$ were considered statistically significant. Data expressed as mean ± standard error.

RESULTS

Fasting blood glucose determination and body weight measurement

In the figure 2A, animals induced by STZ showed increased fast blood glucose (FBG) and body weight (BW) loss, with DM1W (FBG 18.03±0.78; BW 288±7.1; P≤0.001), DM1M (FBG 23.79±1.58; BW 257.5±10.77; P≤0.001) and DMmild (FBG 10.84±0.71, P≤0.001; BW 312±8.85, P≤0.05), all groups compared to CT.

Gastric activity (GA)

Figure 1 exemplifies the signals of contractility obtained from the groups studied. In that signals, it is possible to observe how the signal of the gastric activity of diabetic animals presents irregularities. Figure 2B shows the quantification in the frequency of gastric contraction and the half-bandwidth (CPM) measured by ACB and EGG. There is no significant difference between the frequencies of electrical (EGG) and mechanical activity (BAC) for the same animal. There is a decrease in the frequency of contraction of the diabetes models DM1W (ACB 3.85±0.093, P≤0.001; EGG 3.99±0.11, P=0.007), DM1M (ACB 3.69±0.10, P≤0.001; EGG 3.61±0.11, P≤0.001) and DMmild (ACB 3.68±0.17, P=0.006.; EGG 3.94±0.16, P=0.006) compared to CT (ACB 4.41±0.074; EGG 4.43±0.077).

There was no significant difference in the frequency of contraction (BAC and EGG) among the diabetes models. In figure 2B we have the quantification of the half-bandwidth. The signal from diabetic animals has a spectrum with greater dispersion, with DM1W (ACB 0.31±0.021, P≤0.001; EGG 0.33±0.041, P≤0.001), DM1M (ACB 0.37±0.030, P=0.001; EGG 0.38±0.040, P=0.001) and DMmild (ACB 0.59±0.067, P≤0.001; EGG 0.56±0.067, P≤0.001) compared to CT (ACB 0.14±0.018; EGG 0.15±0.021). There was a significant increase of half-bandwidth in DMmild compared to DM1W (BAC and EGG P<0.001) and DM1M (BAC and EGG P<0.001).

Mean Gastric Emptying Time (MGET) and Mean Cecum Arrival Time (MCAT)

Figure 2C represents the mean values of MGET and MCAT for all groups. Regarding MGET, the diabetes models showed an increase in gastric emptying time (min), (DM1W 109.85±1.79, P=0.003), (DM1M 110.68±1.34, P<0.001) and (DMmild 115.35±1.45, P<0.001) compared to CT (102.45±1.47). There was no significant difference in the MGET among the diabetes models. The MCAT was higher in diabetic rats, with (DM1W 288.45±1.54, P<0.001), (DM1M 277±1.64, P<0.001) and (DMmild 275.45±1.19, P<0.001) compared to CT (261.05±1.50). There was a significant increase of MCAT in DM1W compared to DM1M (P<0.001) and DMmild (P<0.001).

Histological analysis

Morphometry results shown in figure 2D. The circular muscle layer showed a significant thickening in the diabetic groups (DM1W 231,14±14,60, P<0.001), (DM1M 254,25±18,16, P<0.001) and (DMmild 268,64±16,57, P<0.001) compared to CT (160,68±10,22). Diabetic groups showed a significant thickening of mucosa (DM1W 638,35±28,09, P<0.001), (DM1M 675,09±30,54, P<0.001) and (DMmild 619,48±28,27, P=0.009) compared to the CT (488,49±10,37). There was no difference among the models of diabetes. The longitudinal layer showed no significant difference among the studied groups.

Immunohistochemistry

Figure 2E quantified the number of neurons per ganglion, and there is no difference among the markers and studied groups. In the figure 2F, the ChAT neurons of the diabetic animals (DM1M 432.93±10.92, P<0.001) and (DMmild 369.20±17.44, P<0.001) presented decreased of profile neuronal area compared to CT (635.44±18.85). DM1W (600,34±15,61) animals showed no significant difference compared to CT, but showed to have a larger area than DM1M and DMmild (P<0,001). The nNOS neurons of the diabetic animals (DM1M 430.68±13.67, P<0.001) and (DMmild 414.59±18.03, P<0.001) presented decreased of profile neuronal area compared to CT (703.25±25.72). DM1W (660,53±24,04) animals showed no significant difference compared to CT, but exhibit to have a larger area than DM1M and DM mild (P<0,001). The HuC/D neurons of the diabetic animals (DM1M 431.47±11.99, P<0.001) and (DMmild 387.02±14.15, P<0.001) presented decreased of profile neuronal area compared to CT (622.56±15.40). DM1W (579,25±16,21) animals showed no significant difference compared to CT, but present to have a larger area than DM1M and DM mild (P<0,001).

Figure 4 shows the images of the double labeling of ChAT and HuC/D antibodies and the nuclei by DAPI in the myenteric plexus for all groups. Figure 5 shows the images of the double labeling of the nNOS, HuC/D, and DAPI antibodies.

DISCUSSION

In our results we found disturbances in the frequency of gastric contraction, slowing of gastric emptying and oro-cecal transit in different experimental models of diabetes. The data showed a decrease in the profile of the neuronal area of nitrergic and cholinergic neurons, suggesting that in diabetic rats morphometric changes of these neurons occur due to the time of exposure to different hyperglycemic conditions. These changes in neurons may indicate future degeneration and loss of function of the innervated tissue[33,37], but it has already been possible to diagnose disorders of gastrointestinal motility, as well as structural changes, occurred in the gastric wall of diabetic groups, such as thickening of the mucosa and the circular muscular layer of the stomach. This event may be

associated with remodeling after tissue damage[17], and have consequences on gastric contractility.

Damage caused by diabetes in the GIT may be related to glycemic levels and the time of exposure to hyperglycemia[12]. The influence of glycemia was evidenced in the results of the one-week severe (DM1W) induced diabetes group, which presented decreased contraction frequency, slowing of emptying and gastrointestinal transit, and thickening of the gastric wall, but there were no morphometric changes in ChAT and nNOS neurons. Thus, disorders of gastrointestinal motility found in diabetes may occur before damage to enteric neurons, perhaps due to the influence of hyperglycemia on GIT[33,37]. On the other hand, in the one-month-induced animals (DM1M) and the DMmild group, morphometric alterations were already present in the enteric neurons, as well as changes in the motility and morphometry of the gastric wall.

The results of DM1M and DMmild groups for GI motility, gastric wall morphometry, and enteric neurons were similar, even though mild diabetes had lower glycemic levels. The hypothesis is that DMmild animals were exposed to hyperglycemic conditions for a longer time because they were induced to diabetes on the first day of life. However, this model of moderate diabetes is very controversial, with different methodologies in the animal age literature and the induction form[30,38,39].

AC Biosusceptometry (ACB) is a simple, inexpensive and sensitive technique, reproducible in several areas of research. This technique has already validated as capable of measuring gastric contraction activity[40]. As well as for evaluation of gastric emptying and oro-cecal transit in rats [35,41]. For studies on gastrointestinal motility, it is essential to evaluate the mechanical and electrical activities of the smooth muscle. Electrogastrogram (EGG) is a rather simple, minimally invasive technique used mainly in researches on gastric electrical activity. Understanding the motor and electrical functions of GIT are essential in research on comorbidities related to gastrointestinal disorders[42,43]. In our data, there was no difference between the electrical activity measured by EGG and the mechanical activity measured by the ACB in all the studied groups. However, it has reported a decrease

in the electrical signal coupling about the mechanical signal in long-term diabetic rats[13].

Many studies have shown alterations in gastric motility in diabetes[3,42,44], such as impairment of electrical activity, reduction of basal tonus and contraction[45]. Our results indicate a decrease in the frequency of gastric contraction and increase of the half bandwidth in the DM1W, DM1M and DMmild diabetes models compared to CT. Our data suggest a decrease in contractility and the presence of gastric arrhythmias. The literature reports increased gastric arrhythmias and Abnormal rhythmic index (ARI) in models of severe and moderate diabetes with more than three months of induction[12,13]. In these studies, no changes were reported in the frequency of gastric contraction, besides studying long-term experimental models of diabetes[12,13].

There is concordance in most of the results regarding the slowing of gastric emptying (GE) in diabetes[44,46–49]. This changes in GE corroborates with the results of our DM models used. There are also studies showing accelerated gastric emptying in diabetes[12,50]. The use of obese diabetic mouse models showed that hyperglycemia causes an increase in *Cajal* cells in the stomach, triggered by the activation of proliferation pathways and cell survival, leading to increased gastric emptying[52].

Disturbances in oro-cecal transit were also found in diabetes[45]. In our results, the DM1W, DM1M, and DMmild groups showed an increase in MCAT time about to CT. Studies with long-term experimental models of diabetes report structural changes in the small intestine such as circular muscle thickening and longer organ length in diabetic rats. However, these structural alterations do not affect the time of intestinal transit by mechanisms of adaptation to the damages caused by diabetes[45]. There is little knowledge about the changes in intestinal transit and if there is any relation with other disorders of the GIT [53] in diabetes.

Abnormalities in smooth muscle GI may also play a relevant role in the gastrointestinal disorders of diabetics[54]. Morphofunctional changes of the gastrointestinal wall, such as thickening of the colon wall, and intestinal wall stiffness have been reported in diabetic rats[17].

A 3D distribution of the volume and complacency of the stomach regions showed that there is a decrease in compliance in the stomach fund of diabetic rats[55]. The hypothesis is that the thickening of the layers of the gastrointestinal wall occurs because the mucosa is injured continuously by the high concentration of glucose and the formation of oxidative stress. Mucosal damage can make the outer wall tenser and is stimulated to thicken by biomechanical processes. This process was called remodeling[56,57]. Our data corroborate with the literature, finding an expressive thickening of the mucosa and circular muscular layer of the diabetic models. However, there is still little knowledge of the molecular mechanisms of these morphometric changes, and how they are related to disorders of gastrointestinal motility[17,37,45].

The morphometry of enteric neurons was already evaluated in the duodenum, jejunum, ileum, and colon of STZ-induced rats at 1 to 9 weeks. There is a conflict between results of neuronal area morphometry, and show a possible increase[33,58] or decreased cell body area[37], but the mechanisms are unknown[37,58].

It is known that diabetes can trigger cell death and degeneration of enteric neurons[25,28,59,60]. It has been demonstrated that hyperglycemia can lead to the loss of inhibitory (nNOS) and excitatory enteric neurons (ChAT)[59,60]. However, there is little knowledge of the temporal relationship of neuronal damage to glycemic levels in experimental models of diabetes.

Most of the mechanisms proposed to explain tissue and neuronal damage of GIT in diabetes are related to oxidative stress[61,62]. Hyperglycemia in DM can increase levels of oxidative stress, which is related to the production of advanced glycation endproducts (AGEs) and reactive oxygen species (ROS)[47,63]. The formation of AGEs in cells and tissues leads to changes in intracellular signaling related to cell death[64]. The accumulation of these molecules and their deleterious effects are related to the duration of diabetes and the degree of glycemic control[25,65]. Some studies suggest that nNOS neurons are more susceptible to damage caused by hyperglycemia. The NOS enzyme is Ca^{2+} dependent, and the increase in cytoplasmic Ca^{2+} may produce excessive levels of nitric oxide (NO). In the presence of ROS, NO can react with O^{2-} to produce peroxynitrite ($ONOO^{-}$), which

nitrosylates tyrosine residues in proteins, damaging protein function and possibly compromising DNA[63].

Studies on ChAT neurons in diabetes are contradictory. Some studies indicate an increase in the density of ChAT neurons in the myenteric plexus in the ileum and duodenum and may be related to the increase in contractility and arrhythmias[28]. Another work indicates a decrease in both ChAT and nNOS neurons in the colon of rats induced STZ at 12 weeks[26].

Our results indicate a decrease in the neuronal area of nitrergic and cholinergic neurons in the stomach, suggesting that in the DM1M and DMmild models, there is a decrease in the cell body area of these neurons. In conclusion, changes in motility appear to associated with changes in the gastric wall and a decrease in the neuronal area of nitrergic and cholinergic neurons, which were intensified by prolonged exposure to hyperglycemic conditions, even if moderate in diabetes.

Future work should be applied in the discovery of possible molecular mechanisms responsible for the decrease of the profile of the enteric neuron area, and how this process is related to the pathophysiology of the disorders in the gastrointestinal motility found in diabetes.

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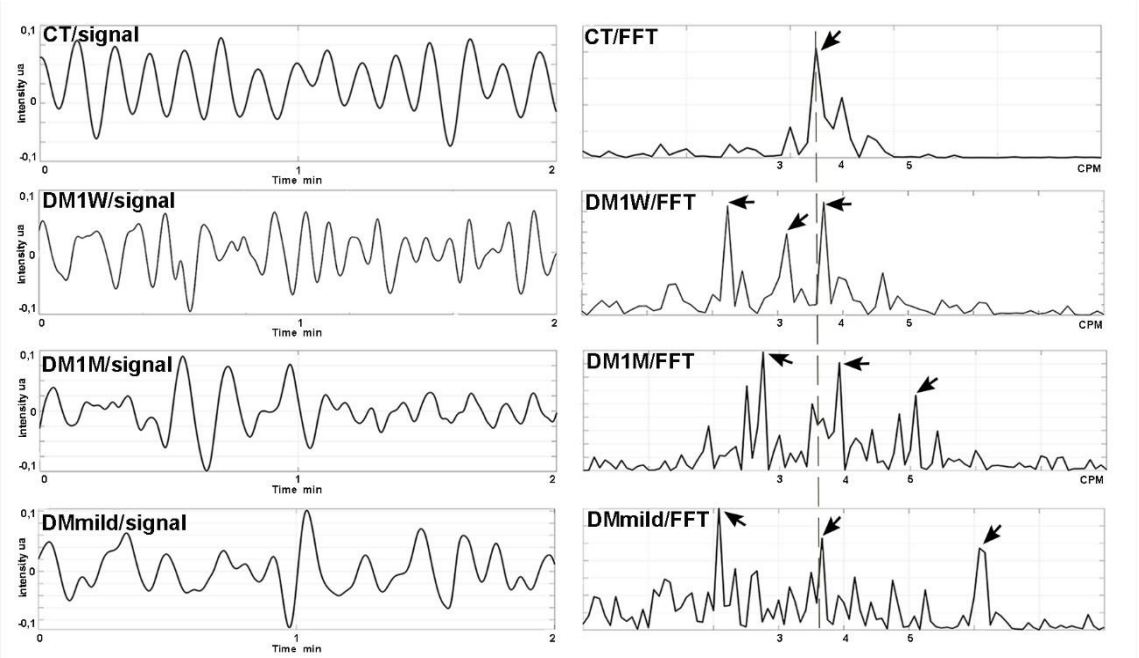

Figura 1 Profile of gastric motility signals measured by ACB. The left column shows the profile of the signal acquired and the right column shows the dominant frequencies quantified by the FFT for all animals analyzed. The arrows indicate the dominant frequency of each profile around the dotted line indicate average frequency of 4 CPM.

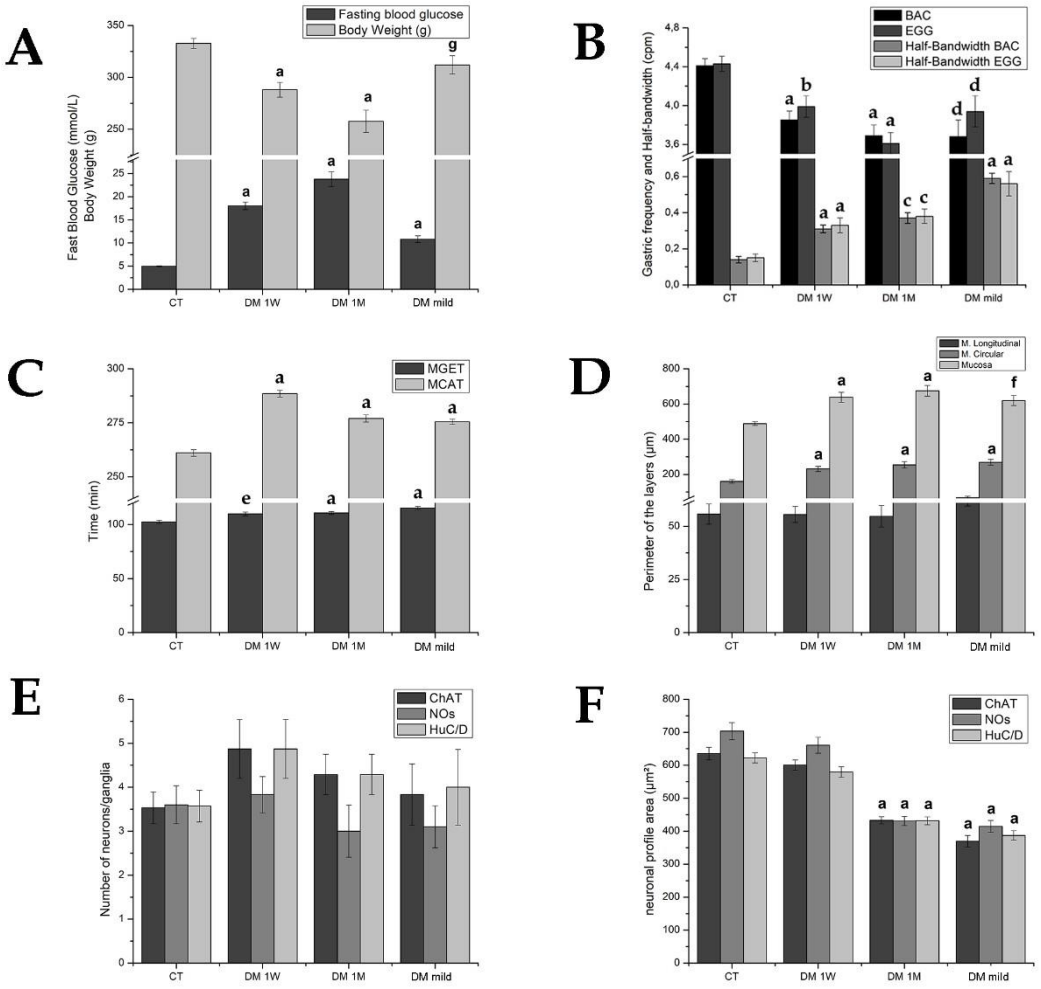

Figura 2 Analysis of gastrointestinal motility and morphometry of the gatric wall and enteric neurons of diabetic and control rats. A: glycemic values and body weight of the all animals studied ($^{a}P \leq 0{,}001$ for DM1W and DM1M; $P \leq 0{,}05$ for DMmild, all groups compared to CT). B: Frequency gastric and half-bandwidth mensured for BAC e EGG in the groups ($^{a}P \leq 0{,}001$ and $^{b}P = 0{,}007$ for DM1W; $^{a}P \leq 0{,}001$ and $^{c}P = 0{,}001$ for DM1M; $^{a}P \leq 0{,}001$ and $^{d}P = 0{,}006$ for DM mild; all compared to CT), only in the half-bandwidth difference between the models of diabetes occurred, DMmild compared to DM1W (BAC and EGG $^{a}P < 0.001$) and DM1M (BAC and EGG $^{a}P < 0.001$). C: MGET and MCAT results ($^{e}P = 0{,}003$ and $^{a}P \leq 0{,}001$ for DM1W; $^{a}P \leq 0{,}001$ for DM1M and DMmild; all compared to CT), only in the MACT difference between the models of diabetes occurred, DM1W compared to DM1M ($P < 0.001$) and DMmild ($P < 0.001$). D: perimeter of the muscular layers and mucosa of the stomach ($^{a}P \leq 0{,}001$ for DM1W and DM1M;

[f]P=0,009 for DM mild; all compared to CT). E: quantification of the number of neurons (ChAT, nNOS and HuC/D) per ganglia labeled with the respective antibodies. F: neuronal profile area (µm²) of the cell bodies (ChAT, nNOS and HuC/D) labeled with the respective antibodies ([a]P≤0,001 for DM1M and DMmild compared to CT), DM1M and DMild showed smaller area than DM1W (P<0,001).

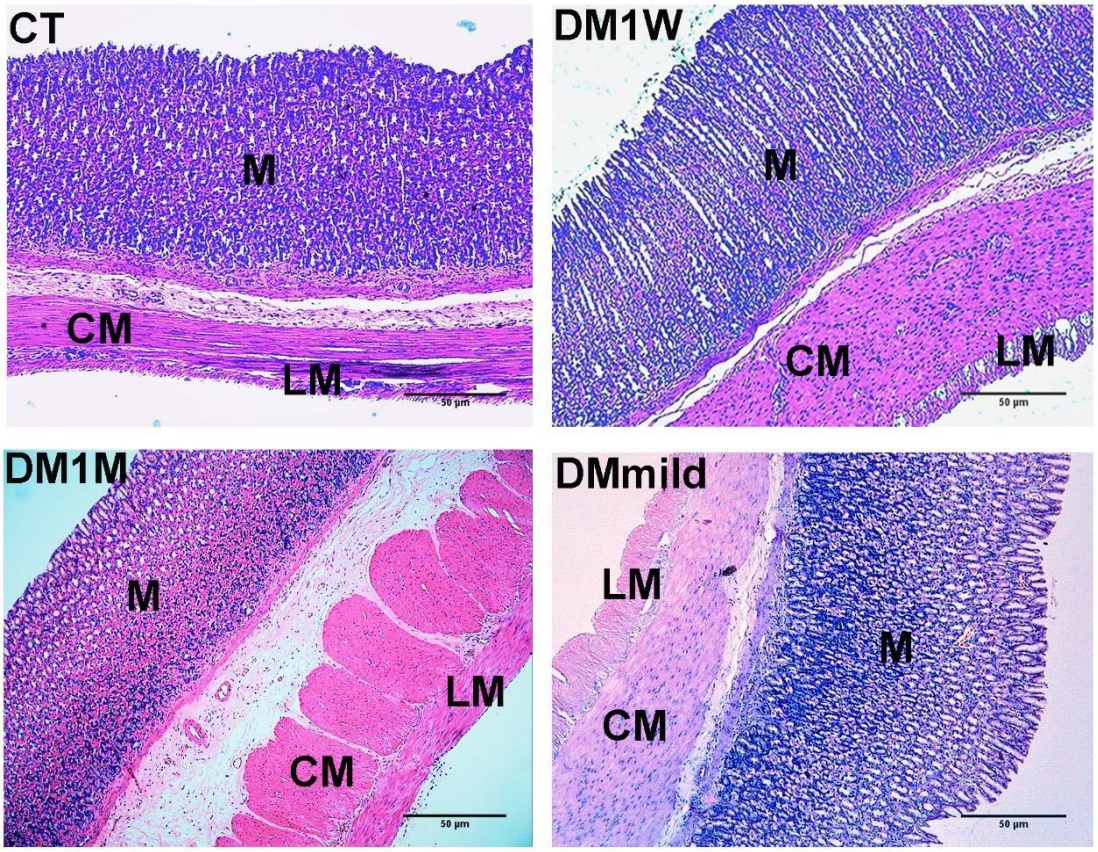

Figura 3 Analysis of the muscular layers of the stomach of all groups using HE staining. M = musosa, CM = circular muscle, LM = longitudinal muscle. Scale bar= Scale bars: 50 µm.

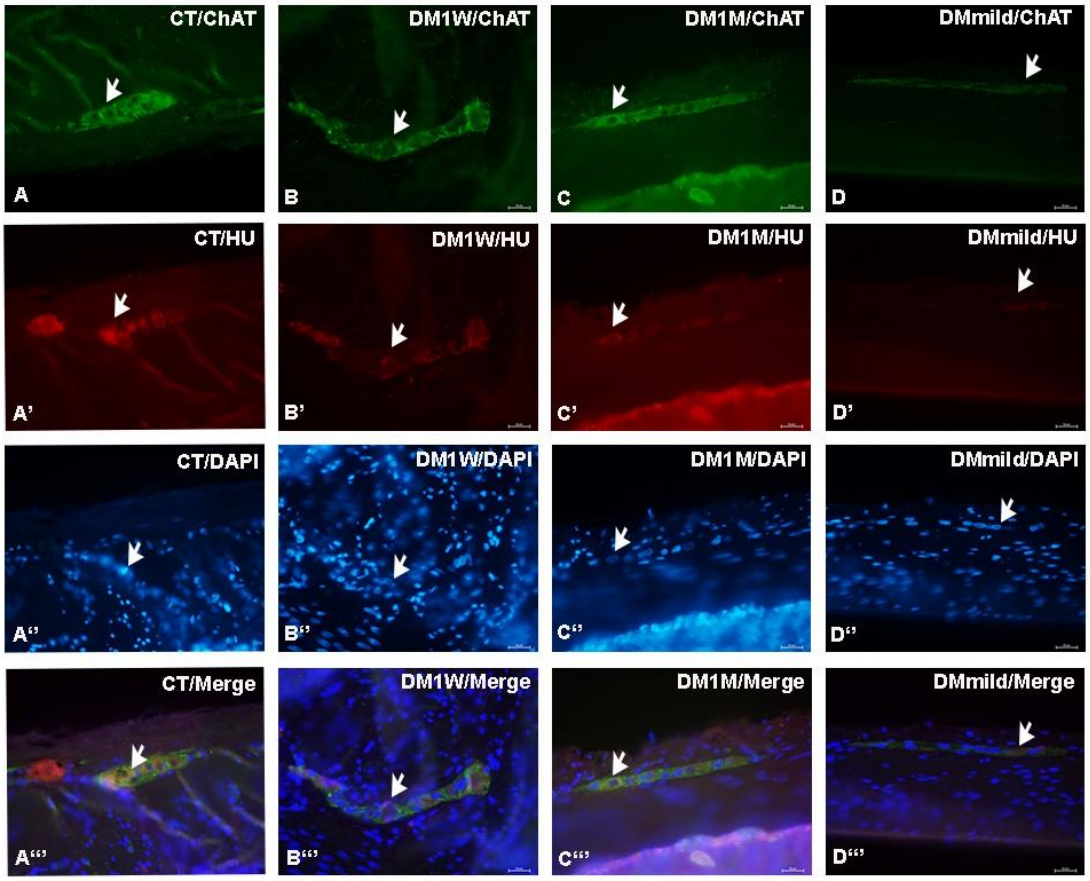

Figura 4 Colocalization of HuC/D immunoreactivity with ChAT immunoreactivity in neurons of the stomach myenteric plexus in the diabetic and control animals. ChAT (A, B, C e D), HuC/D (‘A,’B,’C e ‘D), DAPI (’’A,’’B,’’C e ’’D) and Merge (’’’A,’’’B,’’’C e ’’’D). The arrows indicate ChAT that colocalized with HuC/D (’’’A,’’’B,’’’C e ’’’D). Scale bars: 50 µm.

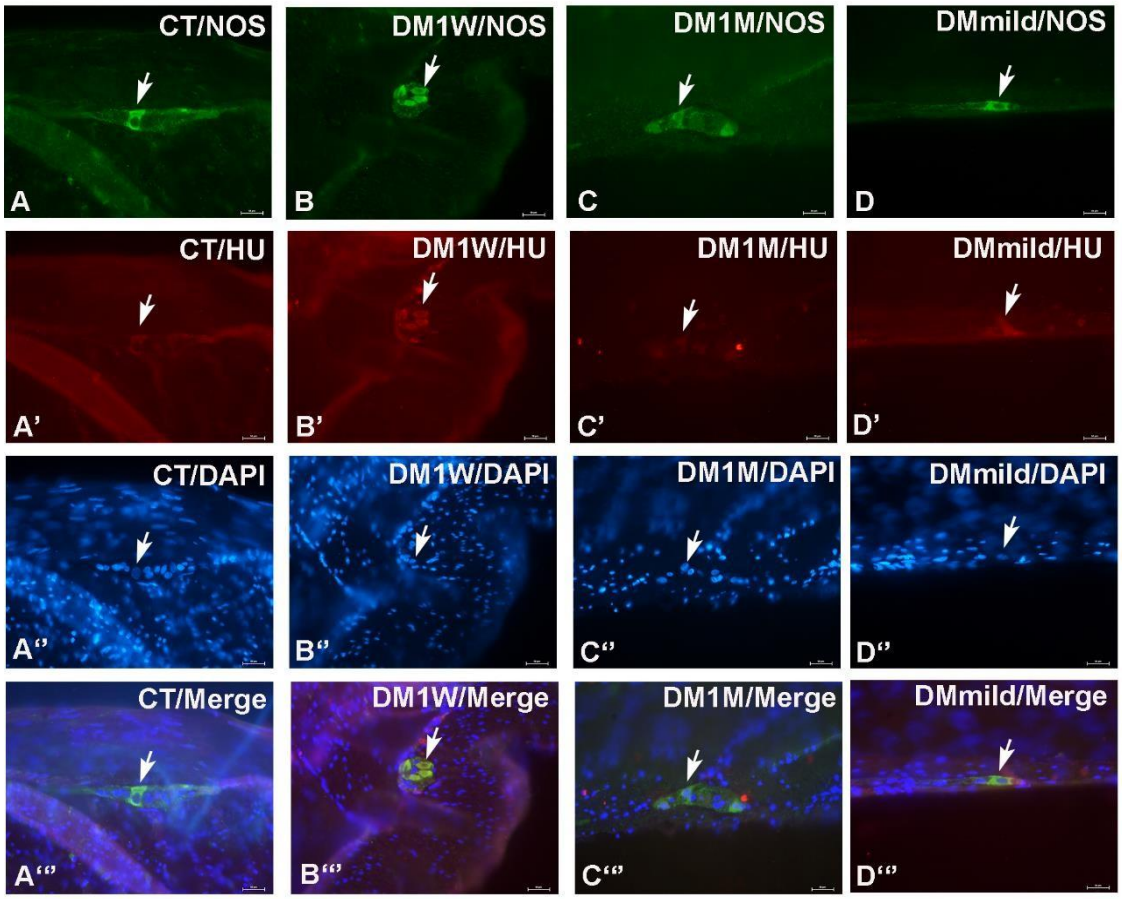

Figura 5 Colocalization of HuC/D immunoreactivity with NOS immunoreactivity in neurons of the stomach myenteric plexus in the diabetic and control animals. NOS (A, B, C e D), HuC/D ('A,'B,'C e 'D), DAPI (''A,''B,''C e ''D) and Merge ('''A,'''B,'''C e '''D). The arrows indicate NOS that colocalized with HuC/D ('''A,'''B,'''C e '''D). Scale bars: 50 µm

Conclusões

3. Conclusões

Nossos resultados apresentaram diminuição da contratilidade gástrica, aumento do índice de arritmias, lentificação do esvaziamento e trânsito oro-cecal. Assim como foi encontrado espessamento das camadas da parede gástrica, diminuição da área (μm^2) do corpo celular de neurônios nitrérgicos e colinérgicos. Sugerindo que em modelos de diabete grave e moderado ocorrem mudanças morfométricas relacionadas ao tempo de exposição a condições hiperglicêmicas. Essas mudanças neuronais e histológicas podem indicar uma futura degeneração, morte celular e perda de função do tecido inervado, porém já foi possível diagnosticar distúrbios da motilidade gastrintestinal. Em conclusão, alterações na motilidade parecem estar associadas a mudanças na parede gástrica e à diminuição da área neuronal de neurônios nitrérgicos e colinérgicos, que foram intensificados pela exposição prolongada a condições hiperglicêmicas, mesmo que moderadas no diabetes.

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4. Referências

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Apêndice

5. Apêndice A

Name of Journal: NEUROGASTROENTEROLOGY AND MOTILITY

Manuscript Type: ABSTRACT

Basic Study

Morphological changes and disorders in gastrointestinal motility in *Diabetes Mellitus*

J. Fernandes de Matos[1]; M. F. Américo[2]; Y. K. Sinzato[3]; D. C. Damasceno[3]; M. Dal-Pai[4]; J. R. de Arruda Miranda[1]

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DOI: 10.1111/nmo.13443
JCR (2019): 3.842
FNM 2018 – 3rd Meeting of the Federation of Neurogastroenterology and Motility, which took place from 29 August to 1 September 2018 in Amsterdam, Netherlands.

Objetive: The aim is to characterize the profile of gastric motility in rats with severe diabetes model and evaluate the influence of glycemic variations in TGI.
Methods: Biosusceptometry of Alternating Current (BAC) is a fairly simple technique, low cost and versatile in research related to the human TGI. BAC is composed of magnetic sensors based in inductions coil. An electrode was inserted for measurement of electrogastrography (EGG), and a ferrite bead to measure the BAC in the rats. Histological sections of the stomach were stained with hematoxylin and eosin, analyzed at a 10X magnification. Morphometry was performed to analyze the thickness of the muscular layers of the stomach. Severe diabetes was induced by a beta-cytotoxic agent (streptozotocin – 40 mg/kg, ip) at adult rats. They were evaluated contractility and gastric emptying of severe diabetic and control animals. $P<0.05$ as statistical significance limit.
Results: There was a significant decrease in contraction frequency and increase in gastric emptying time of the diabetic animals compared with control. There was a thickening of the circular muscle and the mucosa layer of the stomach of diabetic animals. There is an influence of glycemic status on the GI tract and may have alterations of frequency and gastric emptying.
Conclusion: It is concluded that diabetes caused disorders in the gastric electrophysiology in the form of dysrhythmia, as well as delay in emptying and morphological changes.
Acknowledgement: FAPESP 2015/05045-8
Key Words: Diabetes, Stomach, AC Biosusceptometry

6. Apêndice B

Certificado de ética animal (CEUA 752)

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o projeto intitulado "Caracterização da motilidade gastrintestinal em modelos experimentais de hiperglicemia", Protocolo nº 752-CEUA, sob a responsabilidade de José Ricardo de Arruda Miranda, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado "*Ad referendum*" da *COMISSÃO DE ÉTICA NO USO DE ANIMAIS* (CEUA), nesta data.

Vigência do Projeto:	*Início: 01/11/2015*	*Término: 02/03/2019*
Espécie/linhagem:	*Rato Wistar*	
Nº de animais:	*50*	
Peso:	*250g*	*Idade: 90 dias*
Sexo:	*Macho*	
Origem	*Anilab Animais de Laboratório Criação e Comércio Ltda. – Paulínia/SP*	

Botucatu, 6 de agosto de 2015.

Prof. Adj. Wellerson Rodrigo Scarano
Presidente da CEUA

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