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CARLOS VINICIUS DALTO DA ROSA

RESTRIÇÃO ALIMENTAR PROTEGE A FISIOLOGIA GERAL E A MORFOLOGIA DO INTESTINO DELGADO DOS DANOS CAUSADOS PELO DIABETES MELLITUS TIPO 2 EM RATOS WISTAR

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (área de concentração - Biologia Celular e Molecular), da Universidade Estadual de Maringá para a obtenção do grau de Doutor em Ciências Biológicas.

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Coorientador: Vilma A. Ferreira de Godoi
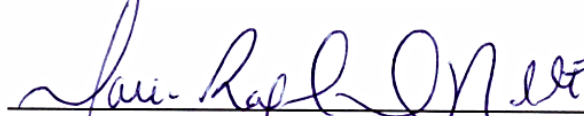
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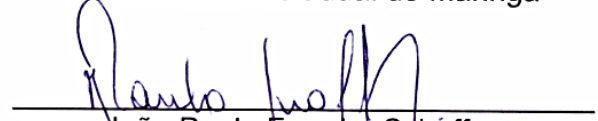
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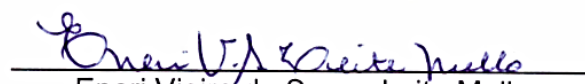
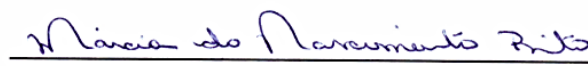Aprovado em: 28.09./2018

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AGRADECIMENTOS

APRESENTAÇÃO

Esta tese é composta de 2 artigos científicos intitulados "*Food restriction promotes damage reduction in rat models of type 2 diabetes mellitus*" e "*Food restriction protects the myenteric nervous plexus of rats with type 2 diabetes mellitus*". Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas da Universidade Estadual de Maringá, os artigos foram redigidos de acordo com as normas das revistas.

O primeiro artigo, publicado em 2018 na revista PLoS One (ISSN 1932-6203), fator de impacto 2.766 (JCR 2017), Qualis CAPES B2, descreve as características fisiológicas de dois modelos distintos de indução ao diabetes mellitus tipo 2, bem como a resposta dos ratos diabéticos a restrição alimentar que mitigou as alterações do diabetes.

Endereço do artigo:
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0199479

O segundo artigo será submetido a revista International Journal of Biological Sciences (ISSN 1449-2288), fator de impacto 4.057 (JCR 2017) Qualis CAPES A2. Este artigo apresenta os efeitos da restrição alimentar em dois modelos de diabetes mellitus tipo 2 com relação as características morfológicas dos segmentos jejuno e íleo, do intestino delgado. As normas desta revista estão anexadas logo após o artigo (http://www.ijbs.com/ms/author#submission).

RESUMO GERAL

INTRODUÇÃO – O diabetes mellitus tipo 2 (DM2) eleva o nível glicêmico gerando aumento sistêmico do estresse oxidativo. O trato gastrointestinal também é comprometido pelo diabetes, gerando disfunções, como disfagia e constipação. Há vários modelos experimentais em animais de indução do DM2, porém, não há completa similaridade com a doença humana, carecendo de melhor caracterização comparativa entre modelos de DM2. Por outro lado, a restrição alimentar gera vários benefícios ao organismo, como no retardo do processo de envelhecimento e no combate a doenças, pela redução da formação de radicais livres e, consequentemente, do estresse oxidativo. Apesar dos vários benefícios da restrição alimentar sobre indivíduos diabéticos, há uma carência de estudos que demonstrem se este modelo é capaz de recuperar a morfologia gastrointestinal.

OBJETIVOS – O objetivo deste estudo foi avaliar as diferenças de modelos de DM2, associados ou não à restrição alimentar, sobre parâmetros fisiológicos e bioquímicos do sangue, e sobre a morfologia dos segmentos jejuno e o íleo do trato gastrointestinal.

MÉTODOS – Trinta ratos Wistar machos adultos foram distribuídos inicialmente em 3 grupos (n=10): Controles (grupo C); Diabéticos tipo 2 (grupo DE) induzidos por injeção de estreptozotocina (35 mg/Kg) e dieta estilo-cafeteria (33% de ração padrão, 33% de leite condensado, 7% de açúcar cristal e água); e Diabéticos tipo 2 (grupo DN) induzidos por estreptozotocina (60 mg/Kg) e nicotinamida (80 mg/Kg), e uma semana depois nova injeção de estreptozotocina (30 mg/Kg) e nicotinamida (40 mg/Kg). Após 2 meses de instalação do quadro de DM2, cada grupo foi subdividido em outros dois grupos (n=5/grupo) no qual um dos dois grupos foi submetido a restrição alimentar (RA) e o outro passou a receber apenas a dieta padrão durante dois meses, formando os grupos: CC (controles), CR (controles submetidos a RA), DEC (diabéticos tipo 2 induzidos por estreptozotocina e dieta estilo cafeteria), DER (diabéticos tipo 2 induzidos por estreptozotocina e dieta estilo cafeteria submetidos a RA), DNC (diabéticos tipo 2 induzidos por estreptozotocina e nicotinamida) e DNR (diabéticos tipo 2 induzidos por estreptozotocina e nicotinamida submetidos a RA). A RA consistiu na oferta de 50% da ingestão diária média do grupo C. Durante os quatro meses de tratamento, avaliou-se quinzenalmente a glicemia, semanalmente a massa corporal e o consumo de água, e diariamente o consumo de ração. Ao final de cada período (pré-RA e de RA) os animais foram submetidos aos teste de tolerância a glicose e teste de tolerância a insulina, bem como amostras de sangue foram retiradas via punção cardíaca para a avaliação de parâmetros bioquímicos relacionados ao perfil lipídico, avaliação de danos hepáticos e da glicação (kits comerciais), e da dosagem da insulina (radioimunoensaio). Ao final do tratamento, o sangue coletado também foi utilizado para a avaliação do estresse oxidativo. Após eutanásia, o pâncreas e amostras do intestino delgado (jejuno e íleo) foram coletadas e processadas

histologicamente. No pâncreas avaliou-se a quantidade de células produtoras de insulina por meio de imunohistoquímica. No intestino, avaliou-se em cortes corados com Hematoxilina-Eosina a morfometria da parede total, túnica mucosa, altura dos vilos, profundidade das criptas, túnicas submucosa e muscular externa, e com Ácido Periódico de Schiff para contabilização das células caliciformes produtoras de mucinas neutras. Amostras do jejuno e íleo foram dissecadas para obtenção do preparado de membrana que foi utilizado para realização de marcação imunohistoquímica com fluorescência para evidenciação das populações neuronais que expressam as proteínas HuC/D (população geral) e nNOS (população nitrérgica), bem como das células gliais que expressam S-100. Os dados paramétricos foram submetidos a análise de variância (ANOVA) seguida de pós-teste de Tukey, e os não paramétricos ao teste de Kruskal-Wallis seguido do pós-teste de Dunns, sob nível de significância de 95%.

RESULTADOS E DISCUSSÃO – Os grupos DM2 apresentaram os sinais clássicos da doença em estágios avançados, como hipoinsulinemia, hiperglicemia, alteração da massa corporal, hiperfagia e polidipsia, e resistência à insulina. Ambos os grupos DM2 (DE e DN) apresentaram níveis elevados de frutosamina, indicando elevada glicação no sangue, além de danos hepáticos observados pelos altos níveis dos marcadores aspartato aminotransferase (AST) e alanina aminotransferase (ALT). Ressalta-se que apenas o grupo DE apresentou dislipidemia e quadros frequentes de diarreia, indicativos de neuropatia, semelhantes as características do DM2 em humanos. Ambos os grupos diabéticos também apresentaram níveis elevados de proteínas carboniladas e reduzida capacidade antioxidante no sangue, comprovando o estresse oxidativo. No intestino observou-se que tanto o jejuno quanto o íleo do grupo DEC apresentaram redução na espessura das túnicas componentes da parede. Por outro lado, no grupo DNC observou-se apenas aumento da túnica mucosa do jejuno. O número de células caliciformes da mucosa jejunal foi reduzido, em ambos os grupos DM2. Estas alterações podem ser consideradas como uma resposta adaptativa a deficiência metabólica do animal diabético. O número de neurônios da população HuC/D, nitrérgica, e de células gliais mioentéricas foi reduzido em ambos os segmentos intestinais, nos dois modelos de DM2. Também ocorreu hipertrofia no corpo celular dos neurônios e da glia de forma compensatória para a manutenção da inervação desse órgão. A perda de tecido nervoso leva a disfunções, como diarreia e constipação, decorrentes da alteração numérica, que é gerada pela vulnerabilidade neuronal a danos oxidativos, elevados no DM2. A restrição alimentar (RA) promoveu melhora da glicemia no grupo DNR, e da lipidemia no grupo DER, além de reduzir a resistência à insulina e os danos hepáticos em ambos os modelos de DM2. A carbonilação de proteínas foi reduzida em ambos os grupos DM2 sob RA, porém apenas o grupo DER demonstrou benefícios da RA sobre a capacidade antioxidante total. No jejuno e íleo a RA promoveu uma redução geral da morfometria da parede, que ocorre em resposta a menor passagem do quilo na região. A RA também reduziu o número de células caliciformes no jejuno dos animais controle, mantendo os números reduzidos nos grupos diabéticos, sendo também relacionado a adaptação

perante a hipofagia compulsória. Com relação ao plexo mioentérico, a RA promoveu proteção das células neuronais e gliais em ambos os grupos DM2, sendo este efeito mais pronunciado no grupo DER. A proteção fornecida pela RA pode ser atribuída ao reduzido estresse oxidativo.

CONCLUSÕES – Os dois modelos de DM2 utilizados no presente estudo foram eficientes na indução do diabetes, gerando danos gerais e específicos ao jejuno e íleo dos ratos, embora cada modelo afete de maneira distinta determinados parâmetros. Há diferença de respostas entre o jejuno e íleo para com o DM2 e RA. A RA mostrou ser benéfica aos parâmetros fisiológicos e morfológicos gerais, incluindo a inervação entérica, avaliados neste estudo. Assim, sua indicação como terapia complementar às terapias convencionais pode ser utilizada no tratamento do DM2 em humanos.

PALAVRAS-CHAVE: neurônios mioentéricos; insulina; restrição dietética; jejuno, íleo.

ABSTRACT

INTRODUCTION - Type 2 diabetes mellitus (T2DM) raises the glycemic level generating a systemic increase of oxidative stress. The gastrointestinal tract is also compromised by diabetes, generating dysfunctions such as dysphagia and constipation. There are several experimental models of T2DM induction in animals, however, there is no complete similarity with human disease, lacking a better comparative characterization between T2DM models. On the other hand, food restriction generates several benefits to the organism, as in the delay of the aging process and in the fight against diseases, by the reduction of the formation of free radicals and, consequently, of oxidative stress. Despite the various benefits of food restriction on diabetic individuals, there is a lack of studies that demonstrate if this intervention is able to recover the gastrointestinal morphology.

AIMS - The objective of this study was to evaluate the differences of T2DM models, associated or not to food restriction, over physiological and biochemical parameters of the blood, and over the jejunum and ileum segments of the gastrointestinal tract.

METHODS - Thirty adult male Wistar rats were initially distributed in 3 groups (n=10): Controls (group C); Type 2 diabetics (group DE) induced by streptozotocin injection (35 mg/kg) and cafeteria-style diet (33% standard feed, 33% condensed milk, 7% crystal sugar and water); and Type 2 diabetics (group DN) induced by streptozotocin (60 mg/kg) and nicotinamide (80 mg/kg) and a week later a new injection of streptozotocin (30 mg/kg) and nicotinamide (40 mg/kg). After 2 months of T2DM, each group was subdivided into two other groups (n=5/group) in which one of the two groups was submitted to food restriction (FR) and the other group received only the standard diet during 2 months (controls undergoing FR), forming groups: CC (controls), CR (controls submitted to FR), DEC (type 2 diabetics induced by streptozotocin and cafeteria-style diet), DER (type 2 diabetics induced by streptozotocin and cafeteria-style diet submitted to FR), DNC (type 2 diabetics induced by streptozotocin and nicotinamide) and DNR (type 2 diabetics induced by streptozotocin and nicotinamide submitted to FR). The FR consisted of 50% of the average daily intake of group C. During the four months of treatment, biweekly blood glucose, weekly body mass and water consumption, and daily feed intake were evaluated. At the end of each period (pre-FR and FR) the animals were submitted to glucose tolerance test and insulin tolerance test, as well as blood samples were collected via cardiac puncture for the evaluation of biochemical parameters related to the lipid profile, assessment of liver damage and glycation (commercial kits), and insulin dosage (radioimmunoassay). At the end of the treatment, the collected blood was also used for the evaluation of oxidative stress. After euthanasia, the pancreas and samples from the small intestine

(jejunum and ileum) were collected and processed histologically. In the pancreas, the amount of insulin-producing cells was assessed by immunohistochemistry. In the intestine, total wall, mucosa tunica, villi height, crypt depth, submucosa tunica and external muscular tunics morphometry were evaluated in Hematoxylin-Eosin stained sections, and Periodic Acid-Schiff stained sections were evaluated to account for goblet cells producing neutral mucins. Samples of the jejunum and ileum were dissected to obtain the whole-mount that was used to perform fluorescence immunohistochemical staining to reveal the neuronal populations expressing the HuC/D (general population) and nNOS (nitrergic population) proteins as well as the glial cells expressing S-100. The parametric data were submitted to analysis of variance (ANOVA) followed by Tukey's post-test, and the non-parametric Kruskal-Wallis test followed by the Dunns post-test, at a significance level of 95%.

RESULTS AND DISCUSSION – T2DM groups showed the classic signs of the disease in advanced stages, such as hypoinsulinemia, hyperglycemia, altered body mass, hyperphagia, polydipsia, and insulin resistance. Both T2DM groups (DE and DN) had high levels of fructosamine, indicating high blood glycation, and liver damage observed by the high levels of the markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT). It is noteworthy that only the DE group presented dyslipidemia and frequent diarrhea, indicative of neuropathy similar to the characteristics of T2DM in humans. Both diabetic groups also presented high levels of carbonylated proteins and reduced antioxidant capacity in the blood, proving oxidative stress. In the intestine it was observed that both the jejunum and the ileum of the DEC group presented an overall reduction of the tunica componentes thickness of the wall. On the other hand, in the DNC group only there was na increase in the jejunal mucosa. The number of goblet cells of the jejunal mucosa was reduced in both T2DM groups. These changes may be considered as an adaptive response to the metabolic deficiency of the diabetic animal. The number of neurons in the HuC/D, nitrergic, and myenteric glial cells was reduced in both intestinal segments of both T2DM models. Hypertrophy of neurons and glia also occurred in a compensating mechanism for maintenance of the innervation of this organ. The loss of nervous tissue leads to dysfunctions, such as diarrhea and constipation, caused by cellular numeric alteration, which is generated by neuronal vulnerability to oxidative damage, elevated in T2DM. Food restriction (FR) promoted an improvement in glycemia in the DNR group, and lipidemia in the DER group, and reduced insulin resistance and liver damage in both T2DM models. Carbonylation of proteins was reduced in both T2DM groups under FR, but only the DER group demonstrated benefits of FR over total antioxidant capacity. In the jejunum and ileum the FR promoted a general reduction of wall morphometry, which occurs in response to the lower passage of the chyle in the region. FR also reduced the number of goblet cells in the jejunum of the control animals, keeping the numbers reduced in the diabetic groups, and also related to an adaptation to the compulsory hypophagia. With respect to the myenteric plexus, FR promoted protection of neuronal and glial cells in both T2DM groups, and this effect was more pronounced in the DER group.

The protection provided by FR can be attributed to reduced oxidative stress.

CONCLUSIONS - The two T2DM models used in the present study were efficient in the induction of diabetes, generating general and specific damage to the jejunum and ileum of the rats, although each model affected parameters differently. There is a difference in responses between the jejunum and ileum for T2DM and FR. FR showed to be beneficial to the general physiological and morphological parameters, including the enteral innervation, evaluated in this study. Hence, its indication as complementary therapy to conventional therapies can be used in the treatment of human T2DM.

KEYWORDS: myenteric neurons; insulin; dietary restriction; jejunum, ileum.

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LISTA DE ABREVIATURAS E SIGLAS

ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
C	Control
CC	Controls
CR	Control+FR
DC	Diabetic+Cafeteria-style
DEC	Diabetic controls
DER	Diabetics+FR
DN	Diabetic with streptozotocin+nicotinamide
DNC	Diabetic controls
DNR	Diabetic+FR
ENS	Enteric Nervous System
FR	Food Restriction
GIT	Gastrointestinal Tract
GTT	Glucose Tolerance Test
HDL	High Density Lipoprotein
ITT	Insulin Tolerance Test
NIC	Nicotinamide
nNOS	neuronal Nitric Oxide Synthase
ROS	Reactive Oxygen Species
SI	Small Intestine
STZ	Streptozotocin
T2DM	Type 2 Diabetes Mellitus
TAC	Total Antioxidant Capacity
VLDL	Very low Density Lipoprotein

Sumário

Artigo Científico 1

RESEARCH ARTICLE

Food restriction promotes damage reduction in rat models of type 2 diabetes mellitus

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Abstract

There are several animal models of type 2 diabetes mellitus induction but the comparison between models is scarce. Food restriction generates benefits, such as reducing oxidative stress, but there are few studies on its effects on diabetes. The objective of this study is to evaluate the differences in physiological and biochemical parameters between diabetes models and their responses to food restriction. For this, 30 male Wistar rats were distributed in 3 groups (n = 10/group): control (C); diabetes with streptozotocin and cafeteria-style diet (DE); and diabetes with streptozotocin and nicotinamide (DN), all treated for two months (pre-food restriction period). Then, the 3 groups were subdivided into 6, generating the groups CC (control), CCR (control+food restriction), DEC (diabetic+standard diet), DER (diabetic+food restriction), DNC (diabetic+standard diet) and DNR (diabetic+food restriction), treated for an additional two months (food restriction period). The food restriction (FR) used was 50% of the average daily dietary intake of group C. Throughout the treatment, physiological and biochemical parameters were evaluated. At the end of the treatment, serum biochemical parameters, oxidative stress and insulin were evaluated. Both diabetic models produced hyperglycemia, polyphagia, polydipsia, insulin resistance, high fructosamine, hepatic damage and reduced insulin, although only DE presented human diabetes-like alterations, such as dyslipidemia and neuropathy symptoms. Both DEC and DNC diabetic groups presented higher levels of protein carbonyl groups associated to lower antioxidant capacity in the plasma. FR promoted improvement of glycemia in DNR, lipid profile in DER, and insulin resistance and hepatic damage in both diabetes models. FR also reduced the protein carbonyl groups of both DER and DNR diabetic groups, but the antioxidant capacity was improved only in the plasma of DER group. It is concluded that FR is beneficial for diabetes but should be used in conjunction with other therapies.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Diabetes mellitus is a chronic disease that has become an epidemic. It is estimated that more than 420 million adults are affected worldwide with this disease, and this number increases alarmingly [1]. This situation stems from problems related to the modern lifestyle, which include high intake of processed foods, bigger elderly population, reduced physical activity and obesity [2].

The most frequent type of diabetes is type 2 (T2DM) or insulin independent, characterized mainly by chronic hyperglycemia and insulin resistance in peripheral tissues [3]. In T2DM pancreatic beta cells produce insufficient amounts of insulin to maintain normoglycemia [4] or produce excessive amounts due to failure in the peripheral tissues insulin response, which generates insulin resistance [3]. Among the complications of T2DM, oxidative stress has great relevance. Hyperglycemia and metabolic dysregulation increase the production of reactive oxygen species (ROS), damaging tissues [5].

Despite the various forms of study of T2DM in animal models [6–10], few studies aim to compare parameters and treatments between different animal models [11]. In addition to T2DM models based on genetic alterations [11], models involving chemical substances [12], diet alterations [13] or both [14] have been used. Differences between models, mainly related to metabolic changes, can be critical in choosing the best model for the study of a particular treatment.

Changes in diet alone hardly lead to T2DM in rats [15,16]. Therefore, the development of a model of T2DM with chronic characteristics demands the alliance of streptozotocin and altered diet [7,17]. On the other hand, some models that use variations of the chemical substances for diabetes induction seem to generate less characteristics similar to the human condition of T2DM [18–20] when compared to the models involving dietary alterations [10].

Food restriction (FR) consists of reducing food intake while preserving minimum levels of nutrients. FR has already shown benefits for pancreatic beta cell function, maintenance of blood glucose and other factors in patients with T2DM [21,22] and in animal models [9,23]. Therefore, FR could be a less invasive alternative in the control of T2DM compared to other interventions such as bariatric surgeries, which have become more frequent due to the epidemic of obesity and T2DM [24,25].

The oxidative stress has been associated with the development and progression of diabetes mellitus and its complications. This was demonstrated by increases in the production of reactive oxygen species (ROS) associated to a diminished capacity of the antioxidant system in many tissues of both patients and experimental diabetic animals [26–28]. The blood interacts with all tissues of the body and then the oxidative status of the plasma reflects at least in part the oxidative status of the whole body. Therefore, the oxidative stress can be evaluated in the plasma of diabetic rats with the purpose of presenting an overview of the whole body oxidative status in models of T2DM. Hence, this study aimed to evaluate the effects of FR on two distinct T2DM models, induced by streptozotocin and cafeteria style diet or with streptozotocin and nicotinamide, through general physiological characterization, blood biochemical analysis and insulin production evaluations.

Materials and methods

Drugs and chemicals

Streptozotocin, nicotinamide and anti-insulin antibodies (AB 260137) used in this study were obtained from Sigma-Aldrich, USA. The Optium Xceed glucometer and dosing strips were purchased from Abbott, Brazil. Thionembutal was supplied by the Abbott laboratory, USA. The blood laboratory test kits were supplied by Gold Analisa Diagnostics Ltda., Brazil. Recombinant human insulin was obtained from PerkinElmer, Shelton, CT, USA. All reagents used had the best possible quality.

Animals and treatment

Thirty male Wistar rats (*Rattus novergicus*, 90 days, 328.2±21.8 g of initial body mass), from the Central animal house of the State University of Maringá, were kept individually in polypropylene boxes, with light and dark cycles of 12 hours and temperature of 22±2˚C in the Sectorial animal house of the Department of Morphological Sciences. All procedures related to the animals followed the standards established by the Ethics Commission on the Use of Animals (protocol number 7590050415/2015), in order to minimize the suffering of animals.

After one week of acclimatization, the animals were treated for a total duration of 4 months, divided into 2 periods: months 1 and 2 (**pre-food restriction**) and months 3 and 4 (**food restriction**).

Initially, during the pre-food restriction period the animals were divided into 3 groups (n = 10/group): C (control), DE (type 2 diabetes + diet) and DN (type 2 diabetes + nicotinamide). Group C rats received only intravenous saline, and were fed with standard diet and water *ad libitum*.

The diabetization of the DE group rats consisted of intravenous injection of streptozotocin (STZ—35mg/kg) dissolved in citrate buffer (10mM, pH 4.5) after overnight fasting. After confirming hyperglycemia, the animals received a cafeteria-style diet (33% standard ration Nuvilab®, 33% Nestlé® condensed milk and 7% sugar and water), sugar water (32%) and normal water, *ad libitum* (adapted from Sahin et al.[15] and Trammel et al.[29]).

The diabetization of the DN group rats consisted of the initial intravenous injection of STZ (60 mg/kg), and after fifteen minutes intraperitoneal injection of nicotinamide (NIC-80 mg/kg). After seven days they received a new dose of STZ (30 mg/kg), and after fifteen minutes, 40 mg/kg of NIC (adapted from Sharma et al. [19]). After confirming hyperglycemia, these animals received standard diet and water *ad libitum*.

Both diabetic models used produce moderate insulin insufficiency [30]. The confirmation of the diabetic state occurred one week after these protocols, checking the fasting glycemia. Animals with stable glycemia greater than 200 mg/dL of blood were considered diabetic (T2DM) [7].

In the food restriction period, group C was subdivided into groups CC (control) and CCR (control + food restriction with standard diet); the DE group, in DEC (diabetic + standard diet) and DER (diabetic + food restriction with standard diet); and DN formed DNC (diabetic + standard diet) and DNR (diabetic + dietary restriction with standard diet) (n=5/group) (Table 1).

Table 1. Diets of experimental groups during 4 months of treatment.

Months 1 and 2 (pre food restriction period)		Months 3 and 4 (food restriction period)	
Group C	Standard diet (*ad libitum*)	**Group CC**	Standard diet (*ad libitum*)
		Group CCR	Standard diet (16 g)
Group DE	Cafeteria-style diet + sugar water (32%) (*ad libitum*)	**Group DEC**	Standard diet (*ad libitum*)
		Group DER	Standard diet (16 g)
Group DN	Standard diet (*ad libitum*)	**Group DNC**	Standard diet (*ad libitum*)

		Group DNR	Standard diet (16 g)

After the subdivision, groups CC, DEC and DNC received standard diet and water *ad libitum* in the period of months 3 and 4. The CCR, DER and DNR groups were submitted to a food restriction protocol (FR), which consisted of receiving only 50% of the average food intake of the control group (C), which served as the basis for all groups. Therefore, animals under FR received 16 g of standard diet daily, and water *ad libitum* (Table 1). Throughout the treatment it was monitored: daily consumption of food; weekly body mass; and biweekly water consumption and fasting/postprandial blood glucose were measured. During the entire period, the animals were weekly monitored for adverse clinical signs, like hypoglycemia and excessive weight loss, based on these parameters.

Assessments of pre- and food restriction periods and tissue collection

At the end of the pre-food restriction (pre-FR) period (2 first months of treatment), glucose tolerance (GTT) and insulin tolerance (ITT) tests were performed. Three days after these tests, the animals were anesthetized with intravenous ketamine/xylazine (100/10 mg.Kg^{-1}) injection, and 1 mL of blood was collected by cardiac puncture from each rat. The collected blood was used for the measurement of insulin by radioimmunoassay and analysis by means of specific kits of the following biochemical parameters: fructosamine, total proteins, total cholesterol, triglycerides, AST (aspartate aminotransferase), ALT (alanine aminotransferase) and alkaline phosphatase.

At the end of the FR period (4 months of treatment), GTT and ITT were again obtained. Then, the animals were intraperitoneally anesthetized (40 mg/kg of body mass) with intraperitoneal thionembutal and had 5 mL of blood collected by cardiac puncture. All animals died from hypovolemic shock. The blood collected during these experiments was centrifuged for 10 minutes at 3000 rpm to obtain the supernatant. The serum collected was stored in a freezer at -80 ˚C until use.

For the final serological analysis, insulin was evaluated by radioimmunoassay and the following biochemical parameters with a fraction of serum collected: fructosamine, total proteins, albumin, total cholesterol, HDL and VLDL cholesterols, triglycerides, AST, ALT and alkaline phosphatase. Another portion of the serum was analyzed for oxidative stress.

After the euthanasia of the animals, we also collected abdominal fats for weighing: retroperitoneal, mesenteric, periepididymal and subcutaneous. The pancreas was also collected and fixed in 4% paraformaldehyde for 6 hours. After the fixation, the material was embedded in paraffin for the preparation of histological slides with cuts destined to the immunohistochemical technique for the evaluation of the insulin-producing pancreatic cells.

Glucose tolerance test (GTT) and Insulin tolerance test (ITT). For evaluation of the GTT glycemic curve, a solution of glucose (1.5 g.Kg^{-1}) was administered to rats, at night fast- ing, via gavage. Then, the glycemia was measured with a glucometer at 0, 5, 10, 15, 30, 45 and 60 minutes.

The ITT curve was obtained after application of an intraperitoneal injection of regular insulin (Novolin®; 1 U kg^{-1}, Novo Nordisk, Montes Claros, Brazil) to rats at 2-hour fasting. The glycemia was then measured with a glucometer at 0, 5, 10, 15, 20, 25, 30 and 60 minutes.

For both techniques, was obtained a constant of increase of the glycemic rate (for GTT), and of decay of the glycemic rate (for ITT), kGTT and kITT respectively [31].

Plasma oxidative status. The total antioxidant capacity (TAC) of the plasma was measured by spectrophotometry using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) or ABTS [32]. TAC was calculated from the standard curve prepared with Trolox, a water-soluble analog of vitamin E, and the results were expressed as nmol.(mL plasma)$^{-1}$.

Plasmatic thiol contents were measured by spectrophotometry (412 nm) using DTNB (5,5'-dithiobis 2-nitrobenzoic acid) as previously described [32]. Thiol contents were calculated using the molar extinction coefficient (ε) of $1.36 \times 10^4 \bullet M^{-1}.cm^{-1}$ and expressed as nmol.(mg protein)$^{-1}$. Protein carbonyl groups were measured by spectrophotometry using 2,4-dinitro-phenylhydrazine [33]. The levels of protein carbonyl groups were calculated using the molar extinction coefficient (ε) of $2.20 \times 10^4 M^{-1}.cm^{-1}$ and expressed as nmol.(mg protein)$^{-1}$. The groups of carbonylated proteins were measured by spectrophotometry using 2,4-dinitrophe-nylhydrazine. The calculation was done using the molar extinction coefficient (ε) of $2.20 \times 10^4 M^{-1}.cm^{-1}$ and expressed as nmol.(mg of protein)$^{-1}$.

Analysis of insulin-producing pancreatic cells. For this immunohistochemical analysis, pancreas samples were submitted to standard histological treatment, with dehydration in increasing concentrations of alcohol, diaphanization in xylol and inclusion in paraffin. The included tissue was cut into a microtome (Leica[1] RM2245) to obtain semiserial 5 µm thick sections. The immunostaining process, aiming at the labeling of insulin, included stages of hydration, endogenous peroxidase blockade, primary antibody reaction, Meyer hematoxylin counter-staining and, finally, Permount slide mounting. The images were captured under light microscope under 40x objective (Olympus BX41, Olympus America Inc., New York, USA) coupled to high resolution camera (Olympus Q Color 3 Olympus America Inc., New York, USA). Image Pro Plus, version 4.5 (Media Cybernetics, Silver Spring, MD) was used for the analysis of the images and the percentage of immunoreactive cells for insulin was analyzed in 30 areas of 50x50 µm of pancreatic islets per animal, in which the positive and negative insulin cells were counted, resulting in an insulin-positive cell marking index.

Dosage of blood insulin. Plasma insulin concentrations were determined by radioimmu-noassay (RIA) [34] using the Wizard2, TM-2470 automatic gamma counter (PerkinElmer, Shelton, CT, USA). RIA was made using a human insulin standard, a rat anti-insulin antibody, and a radiolabeled recombinant human insulin (125). The coefficients of intra and interassay variation varied by 12.2 and 9.8%, respectively. The limit of detection was 1033 pmol/L.

Statistical analysis

The data were initially submitted to the Kolmogorov-Smirnov test to verify normality. Once the data were normal, the data were submitted to One-way Variance Analysis (ANOVA) fol-lowed by Tukey's *post-hoc* test. The number of immunolabelled cells for pancreatic islet insulin generated non-parametric data that were analyzed under the Kruskal-Wallis test and Dunns *post-hoc* test. The results were presented as mean±standard error (SE) of the mean and level of significance of 5% (p<0.05). Data were statistically evaluated using GraphPad Prism (Graph-Pad Software, version 5.1, San Diego, CA, USA).

Results

Physiological parameters

The physiological parameters analyzed during treatment are presented in Tables 2 and 3, and Figs 1–5, for each period (pre- and food restriction/FR).

Pre-food restriction period. After the initial 2 months of treatment, both diabetic groups (DE and DN) showed typical signs of T2DM [10,12,14], such as hyperglycemia (Fig 1), hyper-phagia and polydipsia (Table 2), which were significantly higher (p<0.05) in comparison to the values of the control group (C).

The glycemia in the fasted state did not differ between the diabetic groups (Fig 1A). The pre-FR postprandial glycemia (Fig 1B) of the DN group was significantly lower (p <0.05) than the pre-food restriction glycemia of the DE group, but remained higher in relation to group C.

Table 2. Average body mass, feed intake and water intake at the end of the first 2 months of treatment (pre-food restriction) of rats. Groups: control (C), diabetic+streptozotocin+cafeteria-style diet (DE) and diabetic+-streptozotocin+nicotinamide (DN) rats.

	C	DE	DN
Body mass (g)	476.8±12 .97	318.3±12.43 [a]	347.8±10.18 [a]
Feed intake (g/day)	31.9±1.46	48.5±1.58 [a]	40.5±1.97 [ab]
Water intake (mL/day)	69.0±4.64	348.0±13.73 [a]	156.0±11.49 [ab]

Results expressed as mean±SE (n = 10/group).

[a] p<0.05 *vs* C;

[b] p<0.05 *vs* DE.

One-way ANOVA and Tukey's *post-hoc* test analysis.

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Table 3. Body mass, feed intake and water consumption of rats after 4 months of treatment (food restriction). Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR).

	CC	CCR	DEC	DER	DNC	DNR
Body mass (g)	489.8±9.31	372.0±9.70	334.8±10.68 [a]	200.4±8.11 [ab]	351.6±14.18 [a]	296.8±12.68 [ac]
Feed intake (g/day)	27.6±1.63	16.0±0 [a]	46.8±2.08 [a]	16.0±0 [ab]	43.4±2.44 [a]	16.0±0 [abc]
Water intake (mL/day)	41.0±5.56	34.0±7.48	152.0±3.74 [a]	52.0±4.89 [b]	172.0±16.55 [a]	48.0±2.00 [bc]

Results expressed as mean±SE (n = 5/group).
[a] p<0.05 *vs* CC;
[b] p<0.05 *vs* DEC;
[c] p<0.05 *vs* DNC.
One-way ANOVA and Tukey's *post-hoc* test analysis.

https://doi.org/10.1371/journal.pone.0199479.t003

Fig 1. Pre-food restriction glycemia. The glycemia of the rats was evaluated in the fasting (A) and postprandial (B) states after the initial 2 months of treatment for the control groups (C), diabetic+streptozotocin+cafeteria-style diet (DE) and diabetic+streptozotocin+nicotinamide (DN). Results expressed as mean±SE (n = 7-10/group). *a p<0.05 vs C; b p<0.05 vs DE. One-way ANOVA and Tukey's *post-hoc* test.

https://doi.org/10.1371/journal.pone.0199479.g001
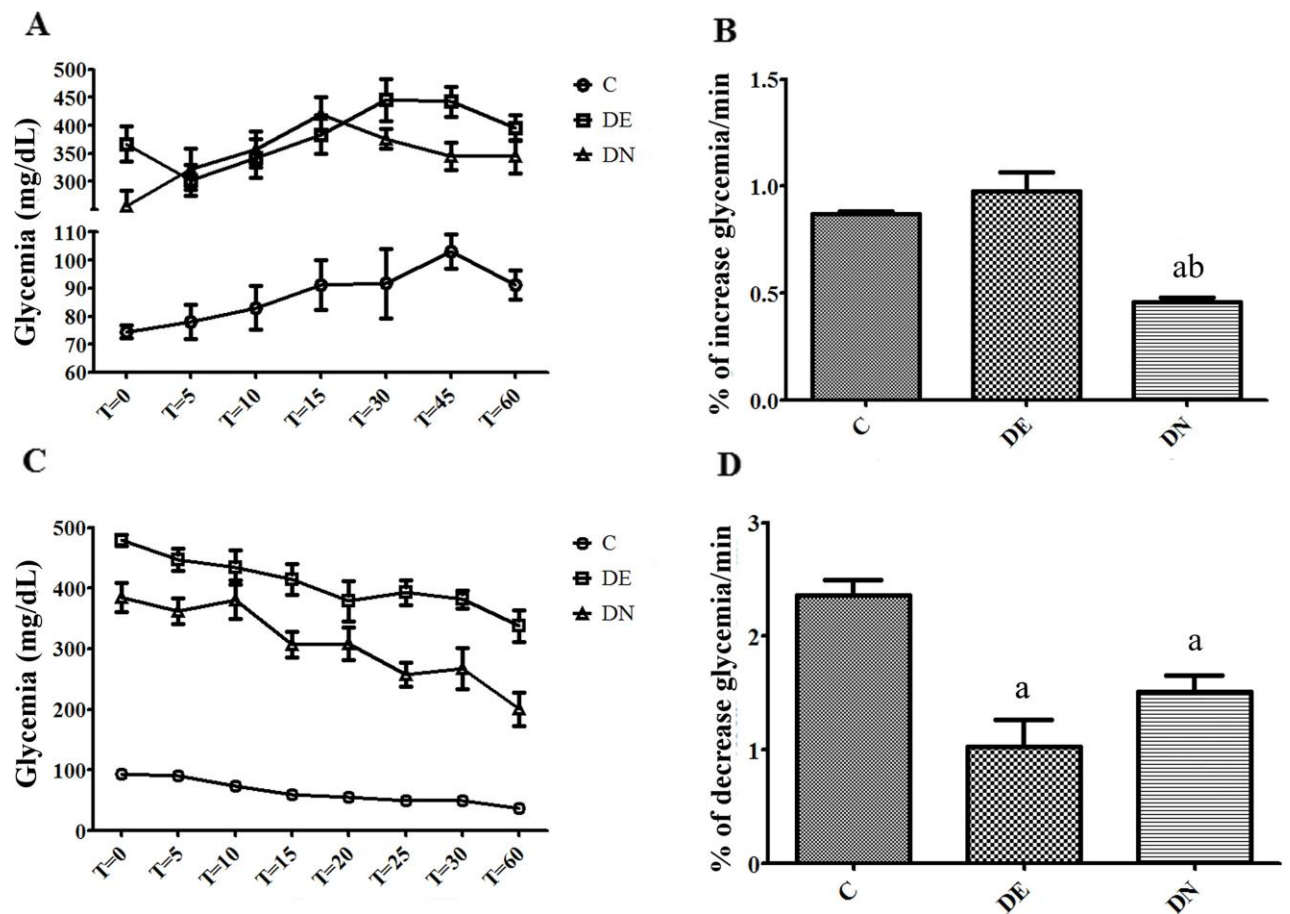
Fig 2. Glucose tolerance and insulin resistance pre-food restriction of rats (after 2 months of initial treatment). (A) Glucose tolerance test (GTT); (B) Rate of increase of glycemia per minute during GTT; (C) insulin-tolerance test (ITT); (D) Rate of blood glucose reduction per minute during ITT. Groups: control (C), diabetic+streptozotocin+cafeteria style diet (DE) and diabetic+streptozotocin+nicotinamide (DN). Results expressed as mean±SE (n = 5/group). *a $p < 0.05$ *vs* group C; b $p < 0.05$ *vs* DE; one-way ANOVA and Tukey's *post-hoc* test.

The groups DE and DN did not differ among themselves ($p > 0.05$) in relation to the body mass. On the other hand, higher food intake and water consumption were observed in the DE group compared to DN. In addition to normal water consumption, the DE group presented a mean intake of sugary water (sucrose 32%) of 38.5±5.32 mL, which aided the development of T2DM. Constant diarrhea was also observed only in the animals of the DE group, demonstrating a more severe diabetic state compared to the DN group.

Fig 2A and 2C presents the data obtained from GTT and ITT techniques after the pre-food restriction period. The kGTT (Fig 2B), obtained from GTT, shows that the DE group did not present a higher glycemic elevation rate than the control group ($p > 0.05$). However, the DN group presented a lower rate of glycemic elevation ($p < 0.05$), compared to the other groups. kITT (Fig 2D) on the other hand, demonstrates that both diabetic groups obtained a lower rate of blood glucose reduction ($p < 0.05$) after insulin application, indicating insulin resistance in diabetic animals.

Food restriction period. At the end of the 4 months of treatment, the animals of the diabetic groups fed *ad libitum* (DEC and DNC) did not present notable differences in the physiological parameters analyzed, compared to the pre-FR period. Body mass (Table 3) and hyperglycemia (Fig 3) were maintained.
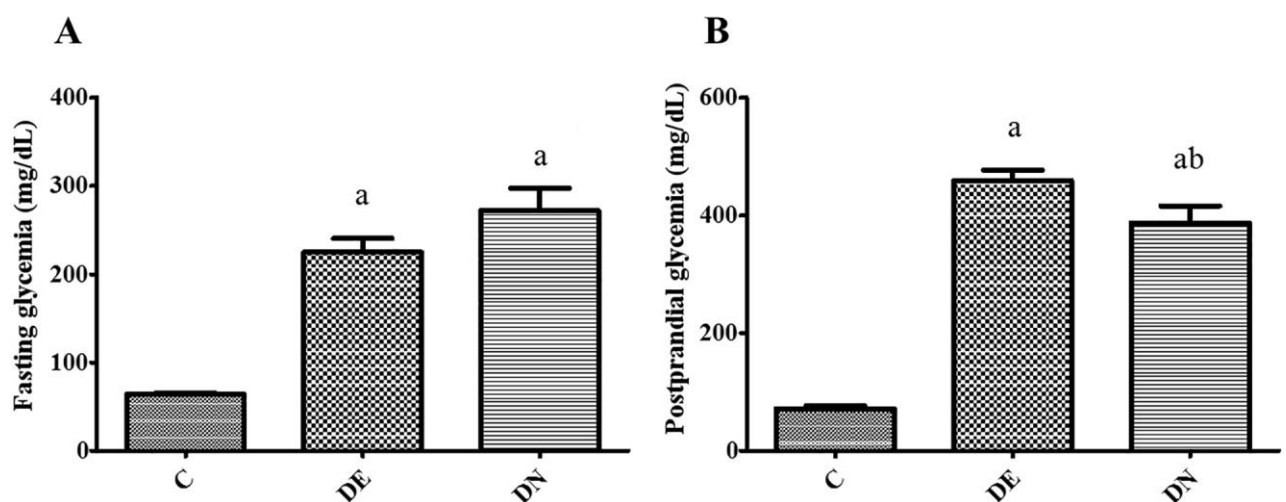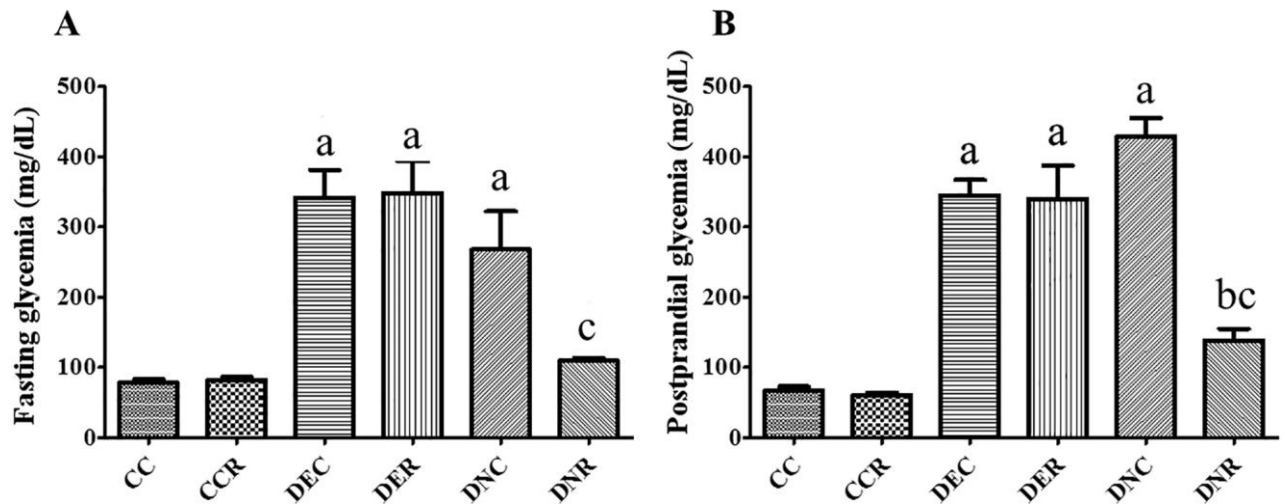
Fig 3. Food restriction glycemia. The glycemia of rats was evaluated in the fasting (A) and postprandial (B) states after 4 months of treatment. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SE (n = 5/group). *a $p < 0.05$ *vs* CC; b $p < 0.05$ *vs* DEC; c $p < 0.05$ *vs* DNC. One-way ANOVA and Tukey's *post-hoc* test analysis.

On the other hand, the DEC group had a reduction in the amount of water ingested in this period, thanks to a change from the cafeteria style diet to the standard diet for rodents. Although the total amount of food ingested was similar during both periods, the type of diet offered was determinant in water intake (Table 3). There was maintenance of the physiological parameters of the DNC group, in relation to the pre-food restriction period.

FR, which occurred in months 3 and 4 for the CCR, DER and DNR groups, promoted strong changes ($p < 0.05$) in the physiological parameters. In the CCR group there was a reduction ($p < 0.05$) in body mass only. The FR in diabetic animals previously submitted to streptozotocin and cafeteria-style diet (DER group) promoted a marked reduction of body mass and water intake when compared to DE (before FR) and DEC (diabetic without FR) groups (Table 3). FR also reduced body mass and water intake in DER group. The glycemia of the DER group presented great fluctuations throughout the treatment (S1 Dataset), with glycemic levels sometimes lower when compared to the DE and DEC groups. However, there was no effective reduction of glycemia as a function of FR for the DER group. In relation to the DNR group, FR promoted a more stable reduction ($p < 0.05$) of blood glucose, besides reduced water intake and body mass. Therefore, FR generated a positive effect on glycemia, despite glycemic control being still deficient.

Fig 4A and 4C present the data obtained from GTT and ITT techniques after the food restriction period. FR kGTT (Fig 4B) showed that glucose tolerance group was compromised ($p < 0.05$) only in the DEC relative to the control. The DNC group did not show a change in the rate of increase of glycemia due to a low change in glycemic index, which was already high at the beginning of the test (Fig 4A). kITT (Fig 4D) demonstrated that the DEC and DNC groups showed insulin resistance, although the values were not significantly different ($p > 0.05$) from the CC group. This is due to the low glycemic variation during the test for these groups, indicating reduced insulin action (Fig 4C). In diabetic groups submitted to FR (DER and DNR), glucose tolerance did not show significant improvement in relation to their respective controls (DEC and DNC). On the other hand, insulin sensitivity was improved ($p < 0.05$) in these groups after 2 months of restriction (Fig 4D).
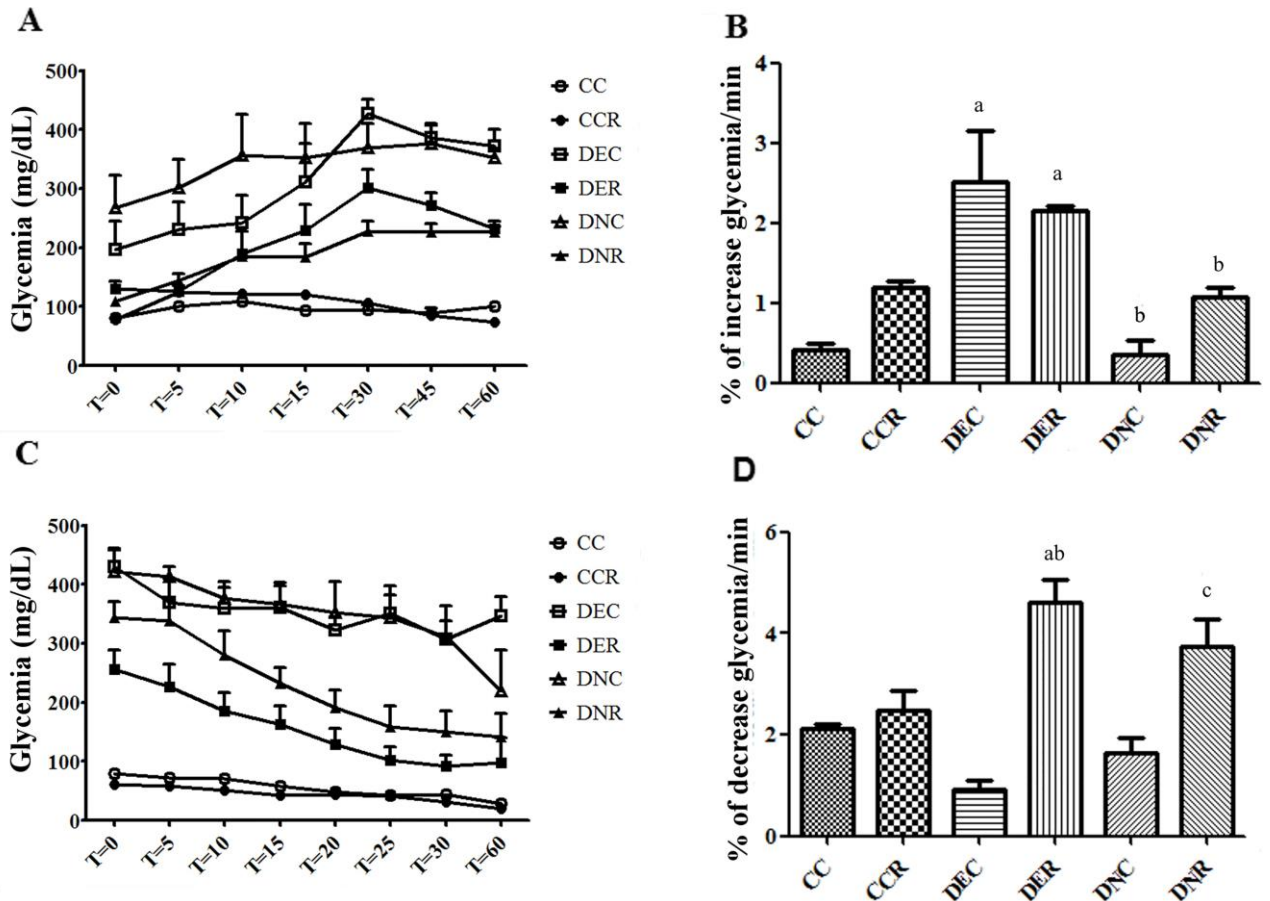
Fig 4. Tolerance to glucose and insulin resistance of rats after food restriction (after 4 months of treatment). (A) Glucose tolerance test (GTT); (B) Rate of increase of glycemia per minute during GTT; (C) Insulin tolerance test (ITT); (D) Rate of blood glucose reduction per minute during ITT. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SE (n = 5/group). *a $p < 0.05$ *vs* CC; b $p < 0.05$ *vs* DEC; c $p < 0.05$ *vs* DNC. One-way ANOVA and Tukey's *post-hoc* test analysis.

Abdominal fat weighing (Fig 5) showed that both diabetic models (DEC and DNC) and FR promoted a marked reduction ($p < 0.05$) in the abdominal fat mass.

Evaluation of pancreatic insulin-producing cells

The detection of insulin-producing pancreatic islet cells (Fig 6) shows that at the end of the 4 months of treatment, both diabetic groups had reduced ($p < 0.05$) number and proportion of insulin-producing cells in the pancreatic islet. FR was not able to improve this parameter.

Evaluation of blood insulin

Radioimmunoassay performed in the serum after the initial 2 months of treatment (pre-FR-Fig 7A) shows that the insulin present in the blood was reduced ($p < 0.05$) in all diabetic groups, in relation to the control groups. This characteristic was maintained after 4 months of treatment (Fig 7B), without interference of FR in the animals.
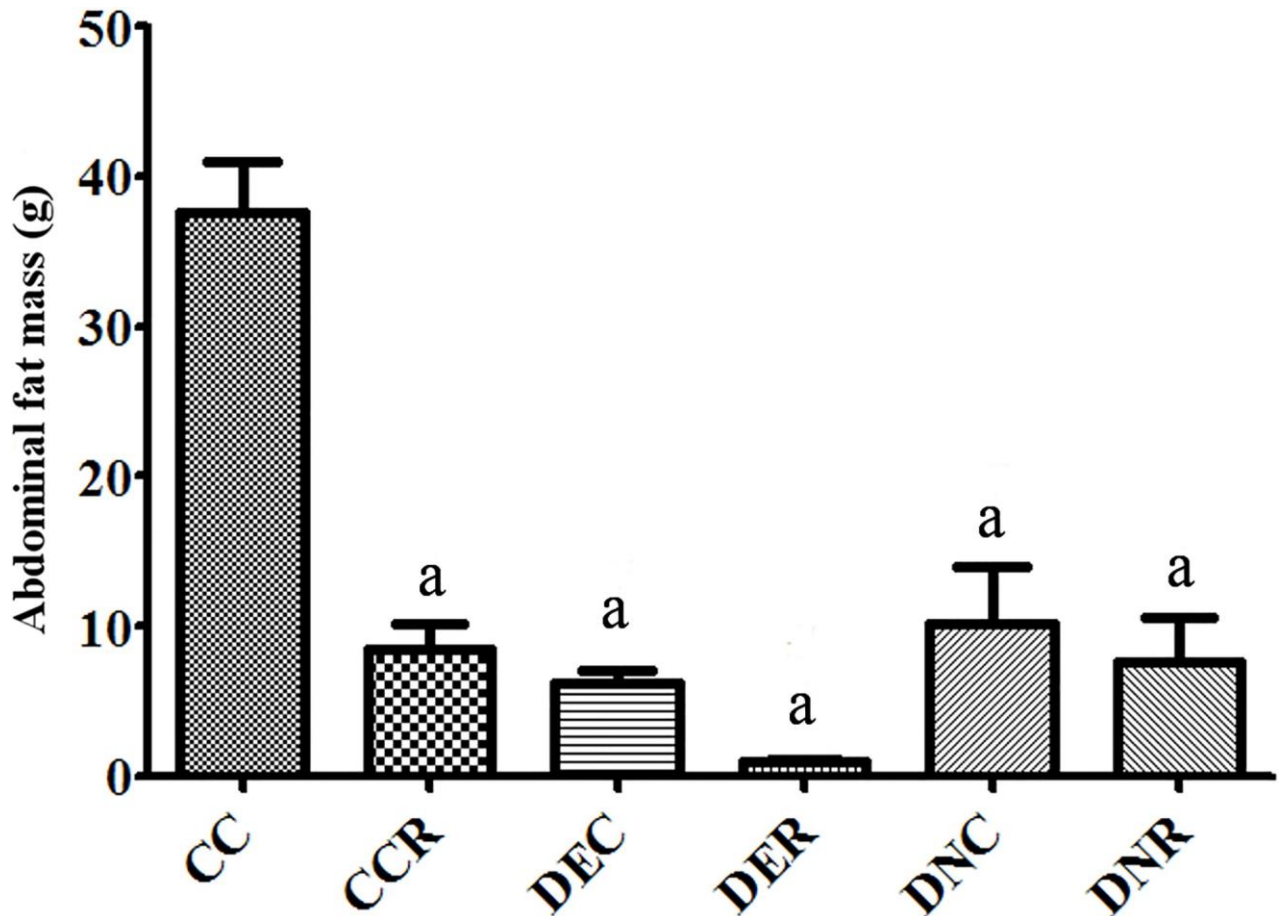
Fig 5. Abdominal fat sum. Added **w**eight of retroperitoneal, mesenteric, periepididimal and subcutaneous fats of rats at the end of the 4 months of treatment for abdominal fat assessment. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SE (n = 5/group). *a $p < 0.05$ *vs* CC. One-way ANOVA and Tukey's *post-hoc* test analysis.

Biochemical parameters

Pre-food restriction period. Table 4 presents the biochemical profile of the blood of the animals after the pre-FR period. Corroborating the diabetic state, there was an increase ($p < 0.05$) in the glycation levels, evaluated through the fructosamine, in both diabetic groups (DE and DN), comparing to group C. Also, there was a rise ($p < 0.05$) of the enzymes AST and ALT, suggesting hepatic damage in both diabetic groups. Elevated levels of alkaline phosphatase also occurred in both T2DM models, although levels of this enzyme were lower ($p < 0.05$) in the DN group than in the DE group. Changes in blood total proteins were only observed in the DN group, which were reduced ($p < 0.05$) when compared to the control group. After 2 months of T2DM, the DE group developed dyslipidemia, with elevated levels of triglycerides and total cholesterol. On the other hand, the DN group did not show changes related to the lipid profile.

Food restriction period. Table 5 shows the blood biochemical profile after 4 months of treatment. The evaluation of fructosamine indicated a maintenance of high glycation levels in the DEC and DNC diabetic groups compared to DE and DN groups, and when compared to
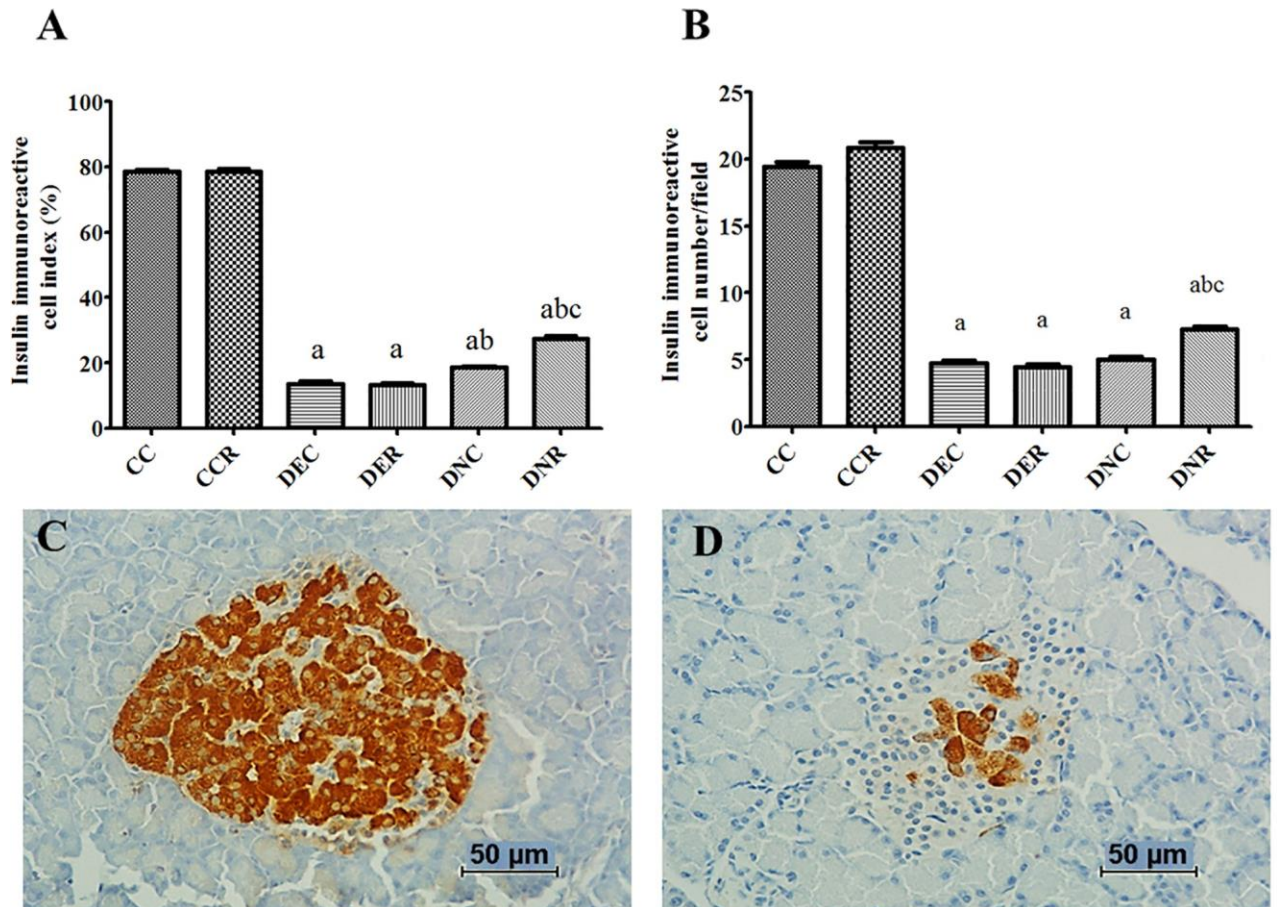
Fig 6. Immunostaining for pancreatic insulin-producing cells of rats after 4 months of treatment. (A) Insulin-producing cells index; (B) Total number of insulin-producing cells; (C) Detail of a pancreatic islet, typical of a non-diabetic animal, immunostained for insulin (200x magnification); (D) Detail of a pancreatic islet, typical of a diabetic animal, immunolabelled for insulin (200x magnification). Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SE (n = 5/ group). *a p<0.05 *vs* CC; b p<0.05 *vs* DEC; c p<0.05 *vs* DNC. One-way ANOVA and Tukey's *post-hoc* test analysis.

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CC group (p<0.05). FR was able to reduce fructosamine levels in both diabetic models (DER and DNR).

AST and ALT were only altered in the DEC group, indicating greater tissue damage (p<0.05) in relation to the CC group. There was also a reduction in the levels of these enzymes in the DEC group in relation to the pre-FR period. The return of these enzymes to normal levels, compared to controls, in the DER and DNR groups indicates a positive effect of FR in reducing this type of damage. A similar result was observed in the evaluation of alkaline phosphatase, which was elevated (p<0.05) in the diabetic groups, and decreased (p<0.05) after application of FR in the DER and DNR groups compared to their respective controls.

Total proteins were not altered in the groups after 4 months of treatment. On the other hand, elevation (p<0.05) of albumin levels in the CCR, DEC and DER groups was observed. This may indicate that the model of T2DM allied to the cafeteria-style diet affects albuminuria, while the model of T2DM without dietary change (DNC group) promotes a reduction (p<0.05) of this parameter. These results also indicate that FR may be linked to elevated blood albumin.
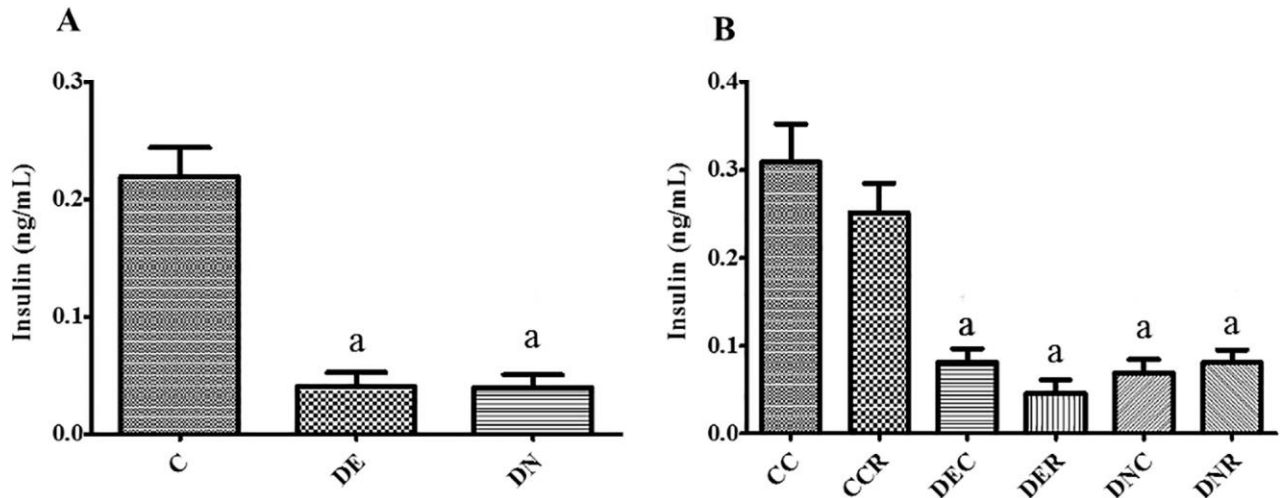
Fig 7. Radioimmunoassay for blood insulin. (A) Amount of immunolabelled insulin in blood of rats after 2 months of treatment for groups control (C), diabetic+streptozotocin+cafeteria-style diet (DE) and diabetic+streptozotocin+nicotinamide (DN). (B) Amount of immunolabelled insulin in blood of rats after 4 months of treatment for groups control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SE (n = 3-5/group). *a $p < 0.05$ *vs* CC; b $p < 0.05$ *vs* DEC; c $p < 0.05$ *vs* DNC. One-way ANOVA and Tukey's *post-hoc* test analysis.

The evaluation of the lipid profile indicates that the triglycerides presented reduction ($p < 0,05$) only in the DNC group and in the groups submitted to FR (CCR, DER and DNR). On the other hand, total cholesterol was high ($p < 0.05$) in the DEC, DNC and DNR groups. The DER group presented a significant reduction ($p < 0.05$) when compared to the CC and DEC groups. Regarding cholesterol fractions, it was observed that there were no significant changes in HDL cholesterol levels, but VLDL cholesterol levels were reduced ($p < 0.05$) for all groups, except the DEC group, in relation to the group CC. These data indicate alteration of lipid metabolism associated with T2DM and FR.

Table 4. Biochemical parameters from pre-food restriction period (2 months of treatment). Total proteins, fructosamine, triglycerides, cholesterol, AST, ALT and alkaline phosphatase from rats' blood were evaluated. Groups: control (C), diabetic+streptozotocin+cafeteria style diet (DE), diabetic+streptozotocin+nicotinamide (DN).

	C	DE	DN
Total proteins (g.dL⁻¹)	6.95±0.16	6.88±0.15	5.78±0.07 [ab]
Fructosamine (mg.dL⁻¹)	0.57±0.04	1.27±0.04 [a]	1.16±0.04 [a]
Triglycerides (mg.dL⁻¹)	106.7±7.08	285.4±77.30	69.8±9.73 [b]
Total cholesterol (mg.dL⁻¹)	77.6±5.04	117.8±9.16 [a]	85.2±6.96 [b]
AST (U.L⁻¹)	22.8±0.75	198.1±14.18 [a]	83.5±9.39 [ab]
ALT (U.L⁻¹)	41.40±2.04	309.4±17.30 [a]	138.8±39.72 [ab]
Alkaline phosphatase (U.L⁻¹)	113.6±9.46	639.9±12.47 [a]	372.6±65.27 [ab]

Results expressed as mean±SE (n = 5/group).
[a] $p < 0.05$ *vs* C;
[b] $p < 0.05$ *vs* DE;
One-way ANOVA and Tukey's *post-hoc* test analysis.

Table 5. Biochemical parameters from food restriction period (after 4 months of treatment). Total proteins, fructosamine, triglycerides, cholesterol, AST, ALT and alkaline phosphatase were evaluated from the blood of rats. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC);

diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR).

	CC	CCR	DEC	DER	DNC	DNR
Total proteins (g.dL^{-1})	5,32±0,19	4,96±0,21	5,5±0,3	5,58±0,59	5,84±0,18	5,85±0,13
Albumin (g.dL^{-1})	2,68±0,05	3,30±0,06 [a]	3,07±0,07 [a]	3,16±0,04 [a]	1,88±0,08 [ab]	2,43±0,02 [c]
Fructosamine (mg.dL^{-1})	0,96±0,05	0,90±0,05	1,80±0,07 [a]	1,20±0,14 [b]	1,68±0,13 [a]	1,38±0,09 [c]
Triglycerides (mg.dL^{-1})	137,3±17,55	41,00±2,93 [a]	126,4±20,10	26,0±3,29 [ab]	68,1±4,91 [ab]	45,38±4,90 [a]
Total cholesterol (mg.dL^{-1})	58,8±1,73	57,1±4,14	77,0±3,76	44,1±4,10 [b]	97,5±5,51 [a]	84,2±7,69 [a]
HDL (mg.dL^{-1})	46,5±5,69	40,5±2,81	58,2±4,44	39,0±3,99 [b]	38,5±1,42 [b]	31,6±4,67
VLDL (mg.dL^{-1})	27,45±3,50	8,20±0,58 [a]	25,28±4,02	5,20±0,65 [ab]	13,63±0,98 [ab]	9,07±0,98 [a]
AST (U.L^{-1})	49,9±1,74	38,2±3,98	110,3±4,64 [a]	43,0±2,62 [b]	57,9±2,38 [b]	37,8±3,93 [c]
ALT (U.L^{-1})	32,4±3,03	31,4±3,19	178,5±14,55 [a]	45,8±5,14 [b]	75,9±5,18 [ab]	25,8±5,44 [c]
Alkaline phosphatase (U.L^{-1})	68,63±3,93	143,6±10,15	1064,0±80,58 [a]	557,9±63,74 [ab]	680,3±52,81 [ab]	256,0±49,84 [c]

Results expressed as mean±SE (n = 4-5/group).

[a] $p < 0.05$ *vs* CC;

[b] $p < 0.05$ *vs* DEC;

[c] $p < 0.05$ *vs* DNC.

One-way ANOVA and Tukey's *post-hoc* test analysis.

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Oxidative status in the plasma

The plasmatic oxidative status was evaluated in the end of the food restriction period. The carbonylation of amino acids has been reported to be the most common oxidative modification in the plasma and thiol protein groups to be the main antioxidant component of the plasma [32]. Thus, the levels of protein carbonyl groups and thiols groups were measured in the plasma of control and diabetic rats. Total antioxidant capacity was additionally and assessed in the plasma of rats. The results are shown in Fig 8. The levels of protein carbonyl groups were 30 and 72% higher in the DEC and DNC diabetic groups, respectively (compared to the control values). Food restriction completely reestablished the levels of protein carbonyl groups in the DER diabetic group, but only partially in the DNR group (compared to the control values). However, it is important to highlight that the levels of protein carbonyl groups in the plasma of the DNC diabetic group were much higher when compared to levels of the DEC diabetic group. Food restriction did not modify the levels of protein carbonyl groups in the plasma of non-diabetic control rats.

The plasmatic levels of thiol groups were reduced only in the DNC diabetic group (-42%; $p < 0.05$), in the same way as the reduction of plasmatic levels of albumin (-31%). Food restriction did not increased the levels of thiol groups in the DNR diabetic group, but it increased the levels of albumin. The total antioxidant capacity (TAC) was strongly reduced in the DEC (-40%) and DNC (-51%) diabetic groups (compared to the controls). Food restriction completely reestablished the antioxidant capacity of the plasma in the DER diabetic group, but it did not improve this parameter in the DNR group.

Discussion

Weir and Bonner-Weir [35], postulated that human T2DM can be divided into 5 stages, according to the degree of development of the disease and its symptoms, mainly related to changes in pancreatic beta cells. Phase 1 is a normoglycemic period of compensation, with onset of insulin resistance (IR). Phase 5 would characterize the time at which the individual
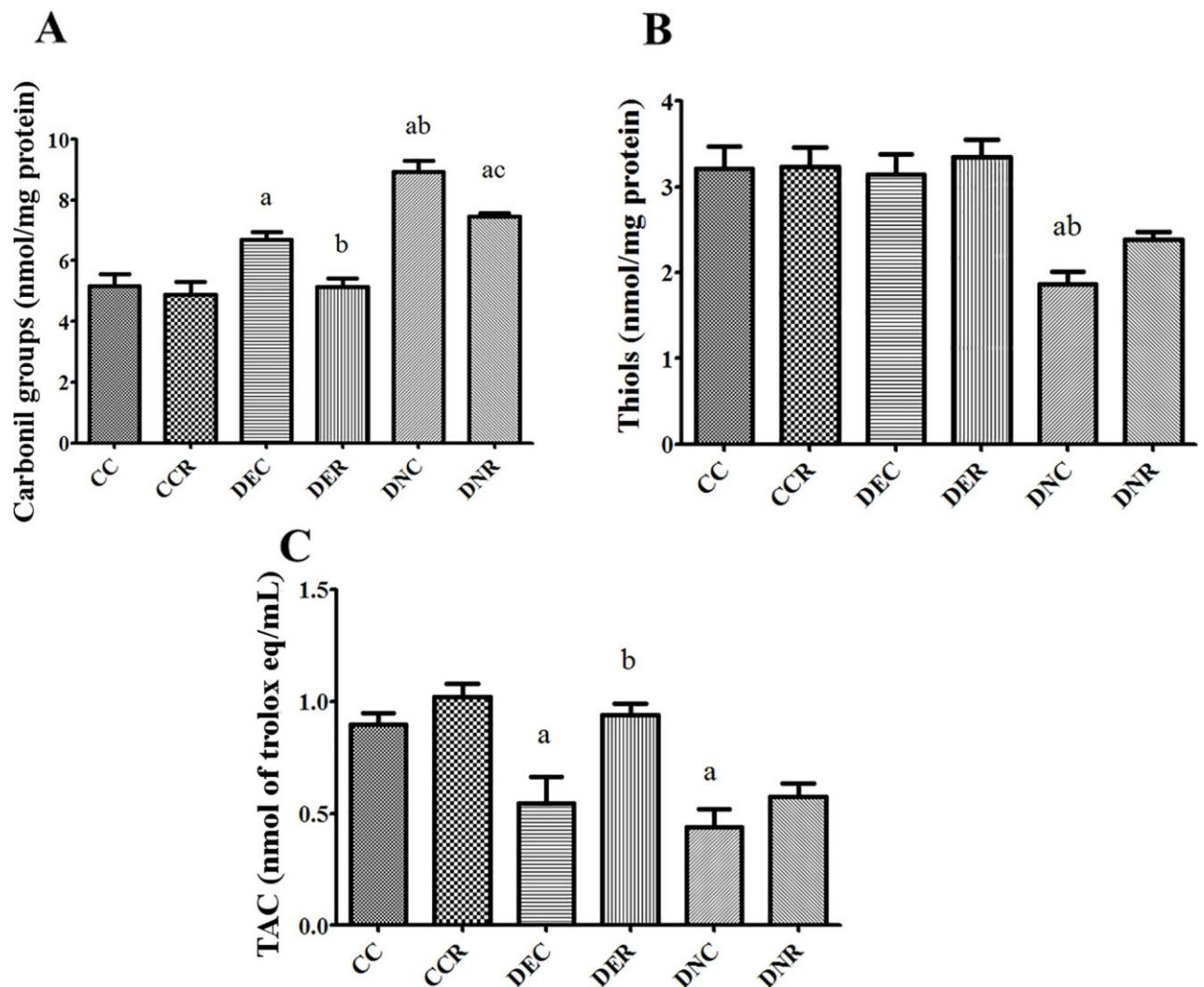
Fig 8. Oxidative stress. Effects of food restriction on the plasmatic oxidative status of type 2 diabetic rats. (A) Protein carbonyl groups, (B) Thiol groups and (C) Total antioxidant capacity (TAC). Oxidative status was assessed at the end of the food restriction period as described in Methods. CC, controls; CCR, controls under food restriction; DEC, diabetic (cafeteria-style diet) rats; DER, diabetic (cafeteria-style diet) rats under food restriction; DNC, diabetic (nicotinamide) rats; DNR, diabetic (nicotinamide) rats under food restriction. Results expressed as mean±SE (n = 4-5/group). *a $p < 0.05$ *vs* CC; b $p < 0.05$ *vs* DEC; c $p < 0.05$ *vs* DNC. One-way ANOVA and Tukey's *post-hoc* test analysis.

has complete failure of pancreatic beta cells, leading to type 1 diabetes, with total absence of insulin and blood ketosis in the individual. The characteristics of the diabetic models evaluated in our study, such as hyperglycemia, reduced number of pancreatic insulin-producing cells, and presence of insulin resistance (IR) lead us to consider both models as representatives of stage 4, with high glycemic decompensation, IR and loss of function pancreatic beta cells.

The characteristics related to advanced T2DM may generate interest in these models for the study of complications associated with T2DM, such as angiopathies and neuropathies, and allow the use of alternative treatments aimed at improving the diabetic condition.

The diabetic model that uses the streptozotocin (STZ) injection associated with the cafeteria-style diet (adapted from Sahin et al. [15] and Trammel et al. [29]) efficiently simulated the advanced T2DM condition. The use of STZ to induce T2DM is important to replicate some T2DM late metabolic disorders, like hyperglycemia, avoiding the prediabetes situations [7,15,16]. The STZ use assisted in obtaining the advanced T2DM condition and fulfill the pur- pose of this study, since in literature there are a lot of studies with T2DM in initial stages. The cafeteria-style diet simulated the modern pattern of food intake, with excessive carbohydrates and lower amounts of proteins and fibers. The large amount of

carbohydrate associated to this diet was a determinant of the greater proximity of this model to the disease in humans, due to a greater alteration of the lipid profile (triglycerides and cholesterols) and liver damage (AST, ALT and alkaline phosphatase) detected in comparison to the other T2DM model. Characteristics associated with angiopathies (retinopathies) and neuropathies (altered gastrointestinal activity observed as frequent diarrhea, probably derived from oxidative stress) were also present in this model. Models similar to this are found in the literature. Okoduwa et al. [17] found similar characteristics to our model in rats fed with diet added of margarine and sucrose solu- tion. Similar results were found in another study involving induction with hyperlipidic diet, STZ and nicotinamide [10]. Trammel et al. [29] demonstrated that the association of the hyperlipidic diet and STZ was more effective in promoting effects associated with T2DM in mice compared to the isolated hyperlipidic diet, presenting similar characteristics to our model. IR is one of the pathophysiological bases of T2DM [36], and the cafeteria-style diet has already shown potential to generate this resistance [15,16]. The presence of insulin resistance, along with the other characteristics presented, shows that the model that associates STZ and cafeteria-style diet is an alternative for easy simulation of human T2DM in advanced stages, with potential for further development and other studies. This model could also be an alterna- tive to using the conventional cafeteria diet.

Interestingly, Zhou et al. [37] obtained expressive results in certain parameters related to T2DM only with a hypercaloric diet supplied to Sprague-Dawley rats, whereas previous studies demonstrated that only the hyperlipidic diet [14] or cafeteria-style diets alone [15,16,38] were not able to generate T2DM models, despite the presence of obesity or even of prediabetic states. This leads us to conclude that, in general, studies involving diets only in rats are relevant only for the study of prediabetic periods and their prevention. The study for the reversal or cure of T2DM, however, requires more robust and advanced models of T2DM.

An additional feature of our work was the exchange of diets offered to animals, cafeteria style for standard diet only, during the food restriction period (DEC group). The purpose of this amendment was to analyze the effects of altering a hypercaloric diet to a balanced diet over T2DM. The amount of feed ingested was similar for these animals during treatment, but the nutritional quality of the feed was improved. Changes in diet led to a significant reduction in water intake, improvement in overall lipid profile, and in tissue damage markers (AST and ALT). This indicates that only dietary change is sufficient to alleviate such changes, although these levels are still altered in relation to the control. Nevertheless, this isolated alteration was not able to improve the general characteristics of the induced T2DM, such as glycemia, IR and glucose tolerance. Positive results related to dietary changes and/or habits are associated with the improvement of symptoms associated with T2DM, despite the great difficulty of being applied in humans [39]. Female rats showed improvement in several parameters associated with obesity and IR, only with the change from a cafeteria diet to a standard diet for rats [40], reinforcing the benefits of improving eating habits against the development of T2DM.

The strictly chemical induction diabetic model (DN - adapted from Sharma et al.[19]), without dietary intervention, was also efficient in promoting an advanced T2DM. The two consecutive injections of STZ and NIC were applied to ensure the stable T2DM hyperglyce- mia, since NIC promotes some protection from STZ damage over pancreatic beta cells [12,19]. However, several of the previously mentioned human-associated characteristics were more discreet or distinct when compared to the diet-associated model (DE). The low glycemic varia- tion observed during GTT for this group reflects the lower glycemic fluctuation observed

during the whole treatment, allied to high basal glycemia, even after fasting. The data of this model referring to the physiological parameters, insulin, glycation state and oxidative stress are in agreement with the results of the work of Badole et al. [12], with the same model of T2DM, but in Sprague-Dawley rats. On the other hand, these same authors observed a general elevation of the lipid profile of diabetic animals, while our data demonstrated a general reduction for this model. This difference can be due to the difference in lineage or age of the animals.

Therefore, although both models of T2DM have already been used in the literature, the model with dietary intervention is more reliable for the study and extrapolation for the human condition.

A negative point of both models was the absence of obesity, typically associated with T2DM. Although lipid profile alterations were detected, insulin deficiency confirmed by RIA and immunohistochemical techniques seems to have been a limiting factor in the body mass gain in the organism of our diabetic animals. This could also be observed by the reductions of abdominal lipid deposits. The amount of lipid deposits is directly associated with reduction of body mass, which is impacted by the metabolic dysfunction generated by T2DM and/or reduced energy intake.

Lim et al. [21] surprisingly showed that only dietary energy restriction was able to reverse the abnormalities of T2DM in humans, mainly related to pancreatic beta cell function and hepatic insulin sensitivity. However, these benefits were observed only in short-term T2DM, justifying the necessity for studies that simulate long-term T2DM models. Jazet et al. [41] demonstrated that the benefits of the restriction are persistent in the long run. Caloric restriction would also be the main responsible for the benefits of bariatric surgeries on obese and diabetic patients [42,43], as well as by preventing the development of T2DM [44]. Beneficial effects of FR have also been demonstrated [45] and its variations [46–48] in diabetic rats. Our study aimed to complement this scenario, with data on the effect of FR on a more advanced T2DM condition.

In the literature, there are variations of FR with the objective of treating T2DM. In addition to restricted types of nutrients, such as carbohydrates [49] and proteins [48], variations of the restriction periods can be found. Barnosky et al. [50] suggest intermittent fasting and alternate-day fasting as alternatives to common FR. However, since FR would have the same efficacy as intermittent fasting [51], and a smaller number of large daily meals would generate more benefits over T2DM [52], we opted for the choice of continuous FR.

In this study, FR showed potential to improve the diabetic condition. Although the impact on body mass was great, the animals maintained a healthy condition throughout the period. This is supported by total protein levels that were stable throughout the treatment, indicating absence of protein malnutrition. The positive effects of FR on insulin resistance, glycosylation, lipid profile, tissue damage and oxidative stress in diabetic groups are directly related to glucose levels. In the model of T2DM without diet, greater benefit of FR over final glycemia and insulin resistance was observed. The hypoglycemic benefits of FR are mainly related to the negative energy balance that reduces the hepatic glucose production [21]. On the other hand, both models showed elevated oscillation of glycemia during the treatments. This effect may be due to the lower severity of chemically induced T2DM. On the other hand, in relation to biochemical parameters and oxidative stress the FR promoted more significant benefits in T2DM plus cafeteria-style diet.

There are many ways to assess oxidative stress. Analyzing the blood can be an alternative since some oxidation-modified serum components are relatively stable and can be related to the severity of the disease [32]. In the present study, both DEC and DNC diabetic groups presented higher levels of protein carbonyls associated to lower antioxidant capacity of the plasma

(Fig 8). These results were already reported in the plasma of poor glycemic control diabetic patients and it has been associated with the development of diabetic complications [53,54]. In fact, the hyperglycemia induces the generation of reactive oxygen species (ROS) in the cells and plasma and proteins are major targets of ROS in the plasma [55]. Thus, the increased levels of protein carbonyl groups in the plasma of both DEC and DNC diabetic animals indicate an increased ROS-mediated injury to serum proteins. The oxidation of plasma thiol groups is itself a manifestation of protein oxidation because the serum albumin contributes with 80% to the plasma thiol groups [56]. Therefore, the lower levels of plasma thiol groups in the DNC diabetic animals possibly reflect an excess of ROS allowing the albumin-SH groups (thiol) to be oxidized [53]. Indeed, the plasmatic thiols serve an antioxidant function and an inverse relation of the plasma thiols and antioxidant capacity of the plasma in diabetic patients is a direct evidence of increased protein oxidation [53]. The plasmatic thiols were not reduced in the DEC diabetic groups, but the protein carbonyl groups increased only slightly (compared to DNC diabetic group). The latter shows the oxidative stress was lesser pronounced in the plasma of DEC diabetic group, in which the reduced antioxidant capacity seems to be not associated to reduced thiols in the plasma. In this regard, plasma contains many antioxidant compounds and the combined action of all these molecules in the plasma represents the antioxidant capacity of the plasma[53].

One of the causes of the oxidative stress observed in this study may be the high glycemic variation observed in our data. It is known that high blood glucose fluctuations, common in patients with uncontrolled T2DM, lead to higher production of ROS and proinflammatory molecules than a regularly elevated glycemia, increasing their circulating levels [57].

Food restriction has been reported to improve the oxidative stress in the plasma of aging rats and in type 2 diabetic rats [47,58–60]. In the present study, food restriction reduced the levels of protein carbonyl groups in the plasma of both DER and DNR diabetic animals and it shows that the ROS-oxidative injury to proteins was reduced in the plasma. The fasting and postprandial glycemia were not improved in DER diabetic group, however, both parameters represent only one punctual dosage in the end of the food restriction period. On the other hand, the levels of fructosamine in the plasma were reduced by food restriction in both DER and DNR diabetic groups, an indicative that the chronic hyperglycemia-induced stress may have reduced in both models of type II diabetes. Regarding the antioxidant status, the total antioxidant capacity (TAC) was improved only in DER diabetic group, in which the levels of albumin and thiols were not diminished in the plasma. The reduced levels of albumin and thiol groups in DNC diabetic rats may occur due to the onset of renal injury, which is associated with an increase in permeability of plasma albumin through a damaged glomerular filtration barrier [61]. The food restriction improved the levels of albumin in the plasma of these animals (DNR), however, it did not improve the thiols and TAC. Therefore, increasing the albumin levels was not enough to increase the antioxidant capacity and it is possible that the sulfhydryl groups of the plasma albumin remained largely in the oxidized state. The latter was also verified in a previous study evaluating the effects of natural oils on the plasmatic oxidative status of rats with adjuvant-induced arthritis and TNBS-induced colitis [62,63].

There is a direct relationship between hyperglycemia and decreased pancreatic islet function, which is reversible [4]. Isolated FR may not be as effective on symptoms related to glycemia and insulin sensitivity, as these benefits are also related to the secretion of incretins produced in the intestine [64]. Troy et al. [65] have demonstrated that bariatric surgery of enterogastric anastomosis type is more efficient in the control of T2DM than simple gastric contrition, a type of forced FR, due to the associated intestinal and hepatic effects. Our results indicate that FR would interfere with the secretion of incretins by the small amount of food

that reaches the intestine. Therefore, the benefits on the islet, and consequently on glycemia and insulin resistance, would be reduced.

The distinct forms of T2DM induction can explain the difference in the damages generated by each model of T2DM. The DE groups presented higher glycemic variation and lipid dysfunction, as well as signs of neuropathy, probably due to high carbohydrate intake. On the other hand, the animals of the DN group presented higher oxidative damages and alterations that may reflect nephropathy, common in T2DM [48,66]. Therefore, both these models demonstrate specific metabolic changes that must be considered for possible treatments to be tested.

Conclusions

Both T2DM models produced the general characteristics of the disease, such as hyperglycemia, insulin resistance, glycation, hepatic damage, hyperphagia and polydipsia. However, some metabolic differences should be observed for its use. The model associated with the cafeteria-style diet (DE) generates greater alterations in the lipid profile, and changes related to neuropathies and retinopathies, being also more susceptible to the benefits of FR. On the other hand, the model without dietary intervention (DN) was more vulnerable to oxidative damage and showed signs of nephropathy, with less benefit of FR. The oxidative stress was increased in both models of type 2 diabetes, but it was more pronounced in the DNC diabetic rats. Food restriction reduced the protein carbonyls in the plasma of both DER and DNR diabetic groups, but the antioxidant capacity was improved only in the plasma of DER group. FR has great potential to improve diabetes-related parameters, although it is not able to reverse the conditions of our models in isolation. Therefore, FR should be associated with other treatments.

Supporting information

S1 Dataset. Excel spreadsheets containing, in separate files, the numerical data for Tables 2–5, and Figs 1–8.

(RAR)

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Artigo Científico 2

Food Restriction Protects The Myenteric Nervous Population Of Rats With Type 2 Diabetes Mellitus

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Abstract

Type 2 diabetes mellitus affects the morphophysiology of the gastrointestinal tract, including the enteric nervous system. Food restriction (FR) promotes benefits such as reducing oxidative stress, but there are few studies on its effects over intestine morphology of type 2 diabetes rats. The objective of this study is to evaluate the influence of two type 2 diabetes models over the intestine morphology and their responses to food restriction. Thirty male Wistar rats were distributed in 3 groups (n=10/group): control (C); diabetes with streptozotocin and cafeteria-style diet (DE); and diabetes with streptozotocin and nicotinamide (DN), all treated for two months (pre-food restriction period). Then, the 3 groups were subdivided into 6, generating the groups CC (control), CCR (control+food restriction), DEC (diabetic+standard diet), DER (diabetic+food restriction), DNC (diabetic+standard diet) and DNR (diabetic+food restriction), treated for additional two months (food restriction period). The FR used was 50% of the average daily dietary intake of group C. At the end of the treatment, samples of ileum and jejunum were collected for evaluation of wall and tunics morphometry, goblet cell number, number and profile of myenteric neurons and glial cells. Both diabetic models produced typical characteristics of type 2 diabetes. However, DE model promoted wall reduction in both intestinal segments, while DN model promoted increase of the wall only in jejunum. Goblet cell number was reduced only in jejunum for both diabetic models and FR. Both intestinal segments presented reduction of neuronal HuC/D, nNOS and glial S-100 positive populations of myenteric plexus in both models. FR promoted a general reduction of jejunum and ileum morphometries but it was able to protect myenteric neurons and glial cells against diabetic damage. The jejunum and ileum respond in distinct ways to diabetes and FR. It is concluded that FR has positive effects over the small intestine, mainly over the enteric nervous system.

Keywords: dietary restriction; streptozotocin; enteric neurons; enteric glia; small intestine

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by metabolic disorders. The incidence and prevalence of this disease has increased considerably in recent years. Among the main factors responsible for this increase are population growth, older elderly population, urbanization, obesity and lack of physical activity [1].

T2DM can be induced in various ways: genetic or spontaneously [2], quimically, or by association of factors [3]. The induction also can occur by a carbohydrate and/or lipid-rich diet [4], or by the combination of hypercaloric diet and streptozotocin injection (STZ) [5].

The oxidative stress can affect individuals with T2DM. The increase in production of reactive oxygen species (ROS) and the reduction in the antioxidant capacity of the tissues are mainly results from hyperglycemia [6,7].

Although T2DM is the more frequent type of diabetes, it is the less studied with relation to the gastrointestinal tract (GIT) [8]. In the GIT, T2DM favors motor problems like constipation, diarrhea and fecal incontinence. This symptoms are mainly originated from diabetic neuropathy, derived from oxidative stress [9]. The neuropathy consists in neuronal death or alteration of its expression, which generates dysfunction and the common symptoms of T2DM [7].

The enteric nervous system (ENS) is involved in GIT neuropathy. It is arranged in two main ganglionated plexus: the submucous and myenteric plexus. They modulate processes like absorption, secretion and motility in the GIT [10]. Both plexus are affected by T2DM [11].

Stenkamp-Strahm *et al*. [12] observed diabetic neuropathy in mice with a model of T2DM achieved by hyperlipidic diet. Besides the structural damage (neurofilament loss and axon swelling), there was reduction of myenteric neuron number and alteration in the expression of vipergic and nitrergic subpopulations of duodenum [12]. Spangéus and El-Salhy [13] also found abnormalities in neuronal expression, like reduction in the myenteric neurons that produce vasoactive intestinal peptide and nitric oxide synthase (NOS), in the GIT of a spontaneous diabetic mice model.

In the small intestine (SI) of diabetic rats, it can also be observed numerical increase of serotoninergic enteroendocrine cells [14], besides morphologic (like hyperplasia) and enzymatic increase [8,15]. The mechanism of these alterations is not clear yet.

Food restriction (FR) consists of reducing food intake, maintaining minimum levels of nutrients. It benefits pancreatic β-cell function, the maintenance of blood glucose and other factors in patients with T2DM [16,17], and in animal models [2,18]. Therefore, FR is an easy alternative in the control of T2DM compared to other interventions such as bariatric surgeries, which have become more frequent due to the epidemic of obesity and T2DM [19].

In our previously study [20] we observed that both models of T2DM (association between STZ and cafeteria-style diet, and association of STZ and nicotinamide) produced consistent alterations such as weight deregulation, hyperglycemia, hyperphagia, polydipsia, biochemical parameters (glycation and hepatic damage), insulin production and response (insulin resistance), and oxidative stress. FR was able to partially reverse the observed damage of this study. From this, in the present study we aimed to evaluate the morphological alterations caused by these two models of T2DM in the jejunum and ileum, as well the effects of FR in these segments.

Materials and methods

Drugs and chemicals

Streptozotocin and nicotinamide used in this study were obtained from Sigma-Aldrich, USA. Thionembutal was supplied by the Abbott laboratory, USA. All reagents used had the best possible quality. The antibodies used were: primary anti-HuC/D (Molecular Probes; Cat# A-21271, AB_221448), anti-nNOS (Santa Cruz Biotec.; Cat# ABIN460169, AB_10789440) and anti-S100 (Sigma-Aldrich; Cat# S2644, AB_477501); and secondary Alexa Fluor 546 and 488 (Invitrogen Cat# A10036 and 488 Cat# A21206 respectively).

Animals and treatment

Thirty male Wistar rats (*Rattus novergicus*, 90 days, 328.2±21.8 g of initial body mass), from the Central animal house of the State University of Maringá, were kept individually in polypropylene boxes, with light and dark cycles of 12 hours and temperature of 22±2°C in the animal house of the Department of Morphological Sciences. All procedures related to the animals followed the standards established by the Ethics Commission on the Use of Animals (protocol number 7590050415), in order to minimize the suffering of animals.

After one week of acclimatization, the animals were treated for a total duration of 4 months, divided into 2 periods: months 1 and 2 (pre-food restriction) and months 3 and 4 (food restriction).

Initially, during the pre-food restriction period the animals were divided into 3 groups (n=10/group): C (control), DE (T2DM with diet) and DN (T2DM). Group C rats received only intravenous saline, and were fed with standard diet and water *ad libitum*.

The diabetization of the DE group rats consisted of intravenous injection of streptozotocin (STZ - 35mg/kg) dissolved in citrate buffer (10mM, pH 4.5) after overnight fasting. After confirming hyperglycemia, the animals received a cafeteria-style diet (33% standard ration Nuvilab®, 33% Nestlé® condensed milk and 7% sugar and water), sugar water (32%) and normal water, *ad libitum*, adapted from [21] and [22].

The diabetization of the DN group rats consisted of the initial intravenous injection of STZ (60 mg/kg), and after fifteen minutes intraperitoneal injection of nicotinamide (NIC-80 mg/kg). After seven days they received a new dose of STZ (30 mg/kg), and after fifteen minutes, 40 mg/kg of NIC, adapted from [23]. After confirming hyperglycemia, these animals received standard diet and water *ad libitum*.

Both diabetic models used produce moderate insulin insufficiency [24]. The confirmation of the diabetic state occurred one week after these protocols, checking the fasting glycemia. Animals with stable glycemia greater than 200 mg/dL of blood were considered diabetic [5].

In the food restriction period, group C was subdivided into groups CC (control) and CCR (control + food restriction with standard diet); the DE group, in DEC (diabetic + standard diet) and DER (diabetic + food restriction with standard diet); and DN formed DNC (diabetic + standard diet) and DNR (diabetic + food restriction with standard diet) (n=5/group) (Table 1).

After the subdivision, groups CC, DEC and DNC received standard diet and water *ad libitum* in the period of months 3 and 4. The CCR, DER and DNR groups were submitted to a food restriction protocol (FR), which consisted of receiving only 50% of the average food intake of the control group (C), in the pre food restriction period, which served as the basis for all groups. Therefore, animals under FR received 16 g of standard diet daily, and water *ad libitum*.

Throughout the treatment it was monitored: daily consumption of food; weekly body mass; and biweekly water consumption and fasting/postprandial blood glucose were measured [20].

Table 1. Diets of experimental groups, controls and diabetics, during the treatment with food restriction.

	Months 1 and 2 (pre food restriction period)	Months 3 and 4 (food restriction period)
Group CC	Standard diet (*ad libitum*)	Standard diet (*ad libitum*)
Group CCR	Standard diet (*ad libitum*)	Standard diet (16 g)
Group DEC	Cafeteria-style diet + sugar water (32%)	standard diet (*ad libitum*)
Group DER	Standard diet (*ad libitum*)	Standard diet (16 g)
Group DNC	Standard diet (*ad libitum*)	Standard diet (*ad libitum*)
Group DNR	Standard diet (*ad libitum*)	Standard diet (16 g)

Small intestine samples collection

At the end of the FR period, the animals were anesthetized (40 mg/kg of body mass) with intraperitoneal thionembutal and had blood collected by cardiac puncture to obtain the serum to access biochemical analysis [20]. All animals died from hypovolemic shock.

After vertical laparotomy was made, small intestine (SI) was collected and measured in its length. Subsequently, samples of jejunum (right after the duodenojejunal flexure) and ileum (in the final quarter of SI) were separated and washed in phosphate buffered saline (PBS, 0.1 M, pH 7.3), and fixed in 4% paraformaldehyde for histological and immunohistochemical processing.

Processing and histological analysis

After 6 h of fixation in 4% paraformaldehyde, jejunum and ileum samples were dehydrated in increasing series of alcohols, cleared in xylene and embedded in paraffin. Then, 5 μm thick semiserial transversal sections were made with Leica RM 2145 microtome.

Morphometric analysis of the intestinal wall components.

The histological sections were stained with hematoxylin and eosin (HE) for morphologic and morphometric analysis of jejunal and ileal wall components. The images were captured under a light microscope (Olympus BX41, Olympus America Inc., New York, USA) coupled to a high resolution camera (Olympus Q Color 3 Olympus America Inc., New York, USA) under a 10× objective to analyze the total wall thickness, mucosa, height of the villi, crypt depth, submucosa and muscular externa. One hundred measurements of each parameter were performed (10 points per section) per animal per intestinal segment, using the program Image Pro Plus 4.5 (Media Cybernetics, Maryland, USA).

Goblet cells counting

To assess goblet cell number, another set of slides was submitted to Periodic Acid-Schiff (PAS) histochemical staining method to evidence neutral mucines of jejunum and ileum. The slides were analyzed under light microscope, under 20× objective. 2500 epithelial cells per animal were recorded to obtain the percentage of labeled to unlabeled cells. The goblet cell index was calculated by the number of labeled cells × 100 / total number of counted cells.

Labeling and quantification of immunoreactive myenteric neuronal and glial cells

After fixation in 4% paraformaldehyde, jejunum and ileum samples were dissected under a stereomicroscope with trans illumination to obtain whole-mounts from the muscular externa tunic. Subsequently the whole-mounts were washed (2×10 min) with PBS plus detergent Triton X-100 0.5% (T) and incubated in blocking solution containing 2% bovine serum albumin (BSA) and 10% donkey serum (1 h). Then, the tissues were incubated for 48 h with primary anti-HuC/D and anti-nNOS antibodies diluted (1:500) in a mother solution (PBS, 1% BSA and 10% donkey serum), washed in PBS+T (2×10 min), incubated for 2 h with secondary antibody (Alexa Fluor 546 and 488 respectively) diluted in mother solution (1:500) and washed with PBS (2×10 min), to mark general population and the nitrergic subpopulation, respectively. Another set of whole-mounts from each intestinal segment was incubated with an anti-S-100 antibody, followed by a secondary antibody (Alexa Fluor 488) to mark glial cells. Subsequently the whole-mounts were mounted in slides with Prolong Gold Antifade Reagents. All of the procedures were performed at room temperature. The slides were examined under a fluorescence microscope (Olympus FSX100). The neurons and glia found in 40 captured microscopic fields under 20× objective were counted, with a total analyzed area of 5.87 mm^2 /animal. Additionally, 100 cell bodies of HuC/D, nNOS and S-100 positive cells per animal were measured in their area. These evaluations also used the program Image Pro Plus 4.5 (Media Cybernetics, Maryland, USA).

Statistical analysis

The obtained data were subjected to the Kolmogorov-Smirnov normality test. Parametric data were subjected to analysis of variance (ANOVA) followed by Tukey's post-test. For nonparametric data the Kruskal-Wallis test followed by Dunns *post-hoc* test was adopted. The results were presented as mean ± standard error (SEM). The statistical data were

analyzed using GraphPad Prism program (GraphPad Software, version 5.0, USA) and considered significant at p<0.05.

Results

T2DM was obtained in both types of induction, STZ+cafeteria-style diet and STZ+NIC. In general, both models presented hyperglycemia, insulin resistance, reduced pancreatic insulin, altered serum biochemical parameters and oxidative stress [20]. GIT function was also affected, as seen by the frequent episodes of diarrhea, mainly in DEC group. Both models affected the small intestine.

Small intestine length

The small intestine (SI) length was significantly increased (p<0.05) by T2DM only in DEC group (15,74%), regarding CC group. Food restriction (FR) significantly reduced (p<0.05) SI length by 20,66% in DER group, when compared to DEC. The SI length of DNC group had no difference (p>0.05) when compared to CC group, but was 19,84% lower compared to DEC. FR did not affects this parameter on DNC group (Fig. 1).

Jejunum and ileum wall morphometry

The histological organization of the intestine was preserved in all groups, for both ileum and jejunum. However, there were differences in the intestinal morphometric parameters between the groups.

The jejunum wall morphometry data is presented in Table 2. It was observed that the jejunum of DEC rats was reduced (p<0.05) in every parameter analyzed (total wall, mucosa, submucosa, muscularis externa, villus height and crypt depth) when compared to CC group. On the other hand, in the second T2DM model, the DNC group showed an increase in these jejunum wall parameters, except in submucosa and crypt depth, which were reduced, regarding CC group. When we compared the two diabetic groups, DEC and DNC, we noted significantly higher (p<0.05) total wall, mucosa, villi height and muscularis externa, while the submucosa and crypt depth parameters were reduced.

Table 2. Morphometry of jejunum wall of control and diabetic rats submitted to food restriction. Measurements of total wall thickness (TW), mucosa (M), submucosa (SM) and muscularis externa tunics (ME), villus height (VH) and crypt depth (CD) after 4 months of treatment. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR).

	CC	CCR	DEC	DER	DNC	DNR
TW (μm)	653.8±3.09	637.9±2.99	548.1±3.28[a]	582.2±3.89[ab]	757.7±3.68[ab]	583.9±3.81[ac]
M (μm)	535.0±2.46	526.0±2.79	457.9±2.52[a]	485.3±3.02[ab]	644.2±3.15[ab]	500.4±3.07[ac]
VH (μm)	382.2±50.19	375.6±61.05	349.4±46.97[a]	375,5±49.92[b]	509.3±56.83[a]	401.9±58.95[ac]
CD (μm)	109.6±0.79	95.09±0.76[a]	99.39±0.59[a]	92.22±0.39[ab]	96.30±0.76[ab]	83.18±0.56[ac]
SM (μm)	40.14±0.34	41.18±0.34	35.61±0.40[a]	41.29±0.40[b]	26.38±0.21[ab]	32.53±0.37[ac]

ME (µm)	74.37±0.79	75.47±0.67	61.51±0.81[a]	64.59±0.73[a]	78.30±0.75[ab]	51.04±0.72[ac]

Results expressed as mean±SEM (n=5/group).
a $p<0.05$ *vs* CC;
b $p<0.05$ *vs* DEC;
c $p<0.05$ *vs* DNC.
Kruskal-Wallis and Dunn's *post-hoc* test analysis.

FR did not produces significative morphometric changes in the jejunum of CCR group (Table 2), except a reduction ($p<0.05$) in crypt depth. With regard to diabetic groups, FR presented an increase ($p<0.05$) of most wall parameters, except in muscularis externa, of DER group when compared to DEC. However, most of these values still presented significative difference in relation to CC group, except in submucosa and villus height. The DNR group showed that FR promotes great reduction ($p<0.05$) of jejunum layers in this specific type of T2DM, regarding the DNC group. This was not true only for the submucosa tunic that showed an increase ($p<0.05$). Except in the villus height, all jejunum parameters of DNR group remained lower than the values of CC group.

Table 3 presents the data of ileum wall morphometry. DEC group showed that T2DM induced by streptozotocin and cafeteria-style diet promotes general reduction ($p<0.05$) of ileum tunics. In the other model, DNC rats showed no alteration ($p>0.05$) of total wall and mucosa measurements, while we observed a reduction ($p<0.05$) of submucosa, muscularis externa and crypt depth, compared to CC group. The villus height was increased ($p<0.05$) in the DNC group, regarding the control group.

FR promoted changes in the ileum wall. CCR group showed a general reduction ($p<0.05$) in the ileum, when compared with CC group, except for the submucosa tunic. FR also promoted a general reduction ($p<0.05$) in the DER group, except for the submucosa tunic, which increased regarding CC group. When compared to DEC group, DER rats showed that FR exacerbates the ileum mucosa reduction ($p<0.05$). In DNR group, FR promoted strong reductions ($p<0.05$) in all wall parameters analyzed, when compared to CC and DNC groups, except for the submucosa tunic, which was reduced only when compared to the CC group.

Table 3. Morphometry of ileum wall of control and diabetic rats submitted to food restriction. Measurements of total wall thickness (TW), mucosa (M), submucosa (SM) and muscularis externa tunics (ME), villus height (VH) and crypt depth (CD) after 4 months of treatment. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR).

	CC	CCR	DEC	DER	DNC	DNR
TW (µm)	666.6±2.75	588.8±2.61[a]	604.2±2.62[a]	544.5±3.19[ab]	659.3±3.35[b]	486.6±2.92[ac]
M (µm)	551.6±2.11	472.2±2.11[a]	499.8±2.07[a]	429.9±2.70[ab]	542.7±2.32[b]	410.9±2.53[ac]
VH (µm)	330.5±1.3	272.1±1.72[a]	291.1±1.35[a]	243.2±1.80[ab]	351.9±2.26[ab]	264.5±1.59[ac]
CD (µm)	189.6±1.04	156.5±0.88[a]	173.4±1.00[a]	156.9±1.26[ab]	162.6±0.99[ab]	125.0±0.83[ac]
SM (µm)	30.73±0.33	34.90±0.24[a]	28.24±0.25[a]	32.47±0.30[ab]	20.40±0.21[ab]	20.37±0.21[a]
ME (µm)	76.26±0.51	76.99±0.64	69.53±0.55[a]	72.56±0.67[a]	73.73±0.83[a]	49.79±0.55[ac]

 Results expressed as mean±SEM (n=5/group).
a p<0.05 *vs* CC;
b p<0.05 *vs* DEC;
c p<0.05 *vs* DNC.
Kruskal-Wallis and Dunn's *post-hoc* test analysis.

Goblet cell number analysis

The goblet cell data of jejunum and ileum is presented in Fig. 2. The number and index of PAS$^+$ epithelial cells of jejunum (Fig. 2 A and B) was reduced (p<0.05) due to both T2DM models and FR, regarding CC group. We also observed lower number of goblet cells of DNC and DNR groups when compared to DE group. The goblet cell number and index of ileum (Fig. 2 C and D) did not change between groups.

Immunohistochemistry for myenteric neurons and glial cells

Samples of the immunohistochemistry staining are presented in Fig 3. The immunohistochemistry data are presented in the Figs. 4 and 5. In the jejunum (Fig. 4) we observed a reduction (p<0.05) in the number of HuC/D$^+$ neurons and in the nNOS$^+$ subpopulation, in both groups with T2DM (DEC and DNC), when compared to the control (CC group). The neuron reduction associated with the T2DM as accompanied by a reduction (p<0.05) in the number of glial cells (S-100$^+$). With relation to the neuronal profile (morphometry of cellular area), we observed an increase (p<0.05) of neuronal cell size in HuC/D$^+$ neurons and in glial cells only in the DEC group, and not in the DNC. The neuronal profile of nNOS$^+$ neurons was increased (p<0.05) in both diabetic groups, DEC and DNC, regarding CC.

FR did not influence (p>0.05) the jejunum of the control group (CCR). In T2DM, FR prevented the decrease in number and the increase of neuronal profile (p<0.05) of HuC/D$^+$ neurons only in DER group. Regarding the nNOS$^+$ subpopulation, FR avoided the decrease in number and the increase of neuronal profile (p<0.05) in both T2DM models (groups DER and DNR), when compared to untreated diabetics. In the S-100$^+$ glia population we observed a preservation (p<0.05) of cell number in both diabetic groups (DER and DNR) compared to the diabetic controls. The glial profile was unaltered (p>0.05) in DER and reduced (p<0.05) in DNR when compared to DNC and CC groups.

The ileum (Fig. 5) presented a reduction (p<0.05) in the number of HuC/D$^+$ and nNOS$^+$ neurons, in both diabetic groups (DEC and DNC), regarding CC group. The reduction in neuronal number was accompanied by an increase (p<0.05) of neuronal profile for HuC/D$^+$ and nNOS$^+$ neurons in DEC and DNC groups, when compared to the control. With relation to the S-100$^+$ glia we observed a reduction (p<0.05) of cell number and an increase (p<0.05) of cell profile only in DEC group, regarding the CC group.

The only alteration observed in the ileum of CCR group, by the action of the FR, was an increase (p<0.05) of glial profile. FR promoted a preservation (p<0.05) of HuC/D$^+$ neurons in the ileum of DER and DNR groups, when compared to their respective diabetic control groups (DEC and DNC). This effect was not observed in the nNOS$^+$ neurons. The glial S-100$^+$ cell number was not affected by FR in the DER group, in comparison with DEC group. On the

other hand, the DNR group presented an increase (p<0.05) of glial cell number, and a reduction (p<0.05) of glial profile when compared to the normal (CC) and diabetic (DNC) controls.

Discussion

Both diabetic models (STZ+cafeteria-style diet and STZ+NIC) presented hyperglycemia, insulin resistance and oxidative stress, characterizing the T2DM state on our previous results, as well [20]. Some works already showed that T2DM affects the GIT. These changes occurs in the intestine wall morphology [25,26], in the enteroendocrine cell number [27], carbohydrate-related enzymes [8] and in the myenteric neuron population [13,28]. However, the literature is still scarce about T2DM impacts over the small intestine. The present study focused on the alterations, generated by two types of T2DM models, in the jejunum and ileum, concerning morphological characteristics of the wall and the ENS.

The SI length were increased only in the DEC group (STZ + cafeteria-style diet). It is known that the increase of the intestine lenght is linear accordingly to the glycemia [29]. The higher levels [20] of advanced glycation ending products (AGE), when compared to the other T2DM model, probably are the responsible for this greater morphological remodeling in diabetes [30]. FR was able to prevent this AGE-related increase, probably by the positive impact of FR in glycation previously evaluated [20].

The biomechanical proprieties of the wall, also related to the integrity of the tunics, are important to the intestine functions [31]. However, results involving T2DM and intestine morphometry are scarce in the literature. In our study, both jejunum and ileum morphometry presented a reduction of the intestinal wall in the DEC group. This goes against the increase in the SI length observed in the same group. These results could mean a total deregulation in the proliferation control of SI. T2DM affects the intrinsic innervation (ENS), the responsible for this regulation, and it probably affects the intestine regulation [32]. Another possible factor is the failure in the mechanical control of the wall, mainly the muscularis externa, which could affect these morphological characteristics. Hadzijahic et al. [33] confirmed that both mucosa and muscularis externa thickness are influenced by the reduction of myenteric neuron number. On the other hand, STZ+NIC-induced diabetes (DNC) showed an increase of jejunum total wall, even with crypt depth reduction.

With relation to the ileum, this model of T2DM caused a disruption of mucosa balance, with an increase of villus height and reduction of crypt depth. This could occur by changes in proliferation and apoptosis, which compromises the normal function of the organ. Verdam et al. [25] showed that human patients with obesity and T2DM presented increased enterocyte mass and simultaneous enterocyte loss, inferred by blood markers, corroborating our hypothesis about the deregulation of the wall. Others studies already showed that obesogenic diet promotes hypertrophy in the jejunum of rats [34], as well as T2DM causes villus edema [26], impaired mucosal barrier integrity [35] in the small intestine of human subjects, and increased small intestine weight, length [8] and proliferation [36] in rodents.

All results means that different models of T2DM, with different durations and intensities, promote disparate characteristics, and even affects the distinct intestinal segments in several ways. The difference in T2DM severity, besides the disturbance of the diet could be responsible for our results, since each model affected the blood parameters, and the whole body differently [20].

Some studies relate FR with pancreatic beta-cell parameters [2,17,37] and oxidative stress [38,39], but few considerate its effects over the intestine wall. Goodlad et al. [40] observed that just three days of starvation promoted a great reduction of crypt cell production rate and proliferation of small intestine epithelial cells. Another study showed that a type of restriction promoted reduction of parameters of mucosa and an increase of apoptosis of small intestine epithelial cells of mice [41]. Schoffen et al. [42] showed that FR during three months, beginning from weaning, promotes increase of the mucosa, while decreases the muscularis externa layer of the proximal colon. These data corroborates with our study, with a general reduction observed in the small intestine, due to FR. Also, in our study, the ileum seems more vulnerable to FR effects than jejunum, as seen in CCR group. This could be explained by the smaller role of ileum concerning absorptive functions, compared to the jejunum, saving nutrients and energy for the other segments more required [43]. Also, the wall reduction alone cannot be considered a type of damage neither causes apparent physiological changes.

The relation between intestine, diabetes and FR is scarce in the literature. Mao et al. [36] found that caloric restriction can reverse the morphometric alterations of intestine which are related to diabetes. In the present study, FR promoted contrary effects in the jejunum of the two models of T2DM. FR partially avoided the diabetes-induced reduction of wall in DER, while promoted a strong reduction in DNR. Regarding the ileum, all groups submitted to FR showed a general reduction of the wall. This could mean that the jejunum can adapt to different circumstances, while the ileum would have a limited adaptive capacity.

The goblet cells are important in maintaining a good environment to the intestinal activities. In the jejunum and ileum, they are responsible for protecting the epithelium from damage [44]. However, data about goblet cells related to T2DM and FR are scarce. Our results show that both T2DM and FR caused a reduction in the goblet cell number in jejunum. However, the ileum did not change with any of the variables. The reduction of goblet cells in the jejunum indicates that probably there are less neutral mucines available in the luminal surface, which affects the normal functions of SI. The goblet cell and mucines reduction were observed in mice subjected to high-fat diet, being related to the alteration of microbiota and inflammation [45,46]. The FR could reduce the goblet cell and mucus of the epithelial surface by the reduced food intake and consequent lower demand. Schoffen et al. [42] also observed the reduction of neutral mucines, but in the proximal colon. Based on this, FR has an overwhelming effect over T2DM changes. However, this cannot be considered a positive effect, but a simple adaptive response. The difference between the intestinal segments should be further studied in the future.

High-fat diets [47,48] and the main diabetes types, types 1 [49] and 2 [12,22,28], promote losses in the enteric neuron population, generating neuropathy [50]. The neuropathy disrupts the normal regulation of the GIT, generating alterations in function, like diarrhea [7], and can affect its morphology as well [33]. Our results corroborate the literature, showing reduction of general and nitrergic neuron number, as well as the glial density, both in jejunum and ileum from both T2DM models, except in the glia from ileum of DNC group. Except in the HuC/D$^+$ neurons and glial cells from jejunum of DNC, all other populations analyzed, in both intestinal segments, presented an increase in their cellular profile. Even after two months of standard diet, the diabetic animals from DEC group maintained the adaptations caused by the T2DM. This indicates that only normalizing dietary conditions has no efficacy over this type of damage.

The neuron and glia loss are attributed to the oxidative stress, since these cells are more vulnerable this type of damage [6]. Glial cells are important in the maintenance of neuronal

environment, including in diabetes [51] and therefore their alteration is related to neuronal disturbance [52,53]. However, contrary to our results, another study showed maintenance of glial population number in the ileum of type 1 diabetic rats [53], even with both diabetes types showing similar factors influencing the ENS, like oxidative stress [6,20] and advanced ending products [20,49,54]. This glial resistance is attributed to the endogenous production of an antioxidant system, not present in the neurons [55]. Stenkamp-Strahm et al. [56] showed that high-fat diet affected only mucosal glial cell populations, and while myenteric glia was unaffected. This difference could be related to the type of damage, as well the duration of the study. The increase of neuronal profile, caused by the neuronal loss, can be related to a compensatory adaptation to the lack of cells in order to maintain the minimal control over the tissue.

FR had little or no effect over the small intestine of control animals regarding the neuronal and glial population. The literature shows that FR is able to protect neurons during a post-weaning period [42]. In the other hand, FR promoted great benefits over the two diabetic models, preventing alterations in the number and cellular area of neurons and glia in most of the situations. The jejunum was more susceptible to FR protection, mainly in DER group. The nNOS[+] subpopulation of ileum had no benefits from FR, while S-100[+] glia reduced its size and increased its number. The protective effect of FR over neurons is considered by other studies [57], but to our knowledge this is the first study correlating the effects of T2DM and FR over intestine and enteric neurons.

The alterations observed in the nervous tissue of the present study explains, in part, the other results. The reduced innervation decreases the control over the absorption and musculature, leading to the observed characteristics like diarrhea and altered morphology of the intestine wall.

Conclusion

Both T2DM models cause alterations in the morphology of intestine wall and in the enteric nervous system, leading to reduction of neuron and glia number of myenteric plexus, as well their morphometry. FR protects the intestine from diabetic damage, although FR itself generates some adaptations. Different segments of the gut present distinct responses to T2DM and FR. More studies are necessary to verify the full potential and safety of FR over the intestine, since it is a complex and vital organ.

Acknowledgments

Competing Interests

The authors have declared that no competing interest exists

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Figure 1. Small intestine length (cm) of control and diabetic rats submitted to food restriction after 4 months of treatment. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). *a $p<0.05$ *vs* CC; b $p<0.05$ *vs* DEC. One-way ANOVA and Tukey's *post-hoc* test analysis.
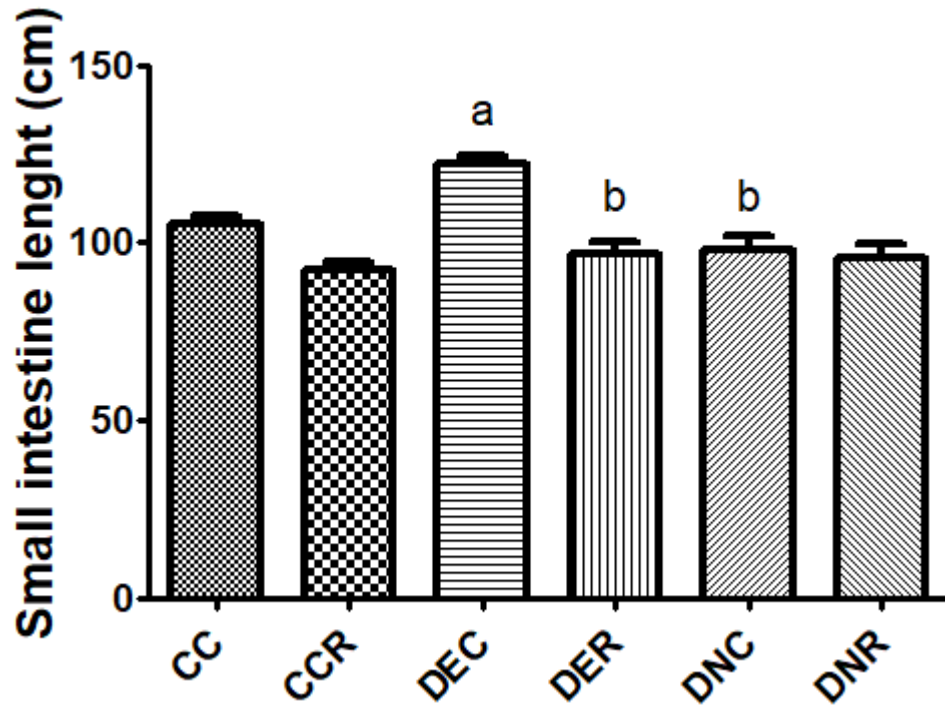
Figure 2. Goblet cell number of small intestine of control and diabetic rats submitted to food restriction after 4 months of treatment. (A) Total goblet cell number counted in 2500 epithelial cells of jejunum. (B) Goblet cell index of jejunum. (C) Total goblet cell number counted in 2500 epithelial cells of ileum. (D) Goblet cell index of ileum. (E) Representative photomicrograph of PAS-stained goblet cells (arrows). Periodic Acid-Schiff histochemical staining; 100X magnification. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). *a $p<0.05$ *vs* CC; b $p<0.05$ *vs* DEC. One-way ANOVA and Tukey's *post-hoc* test analysis.
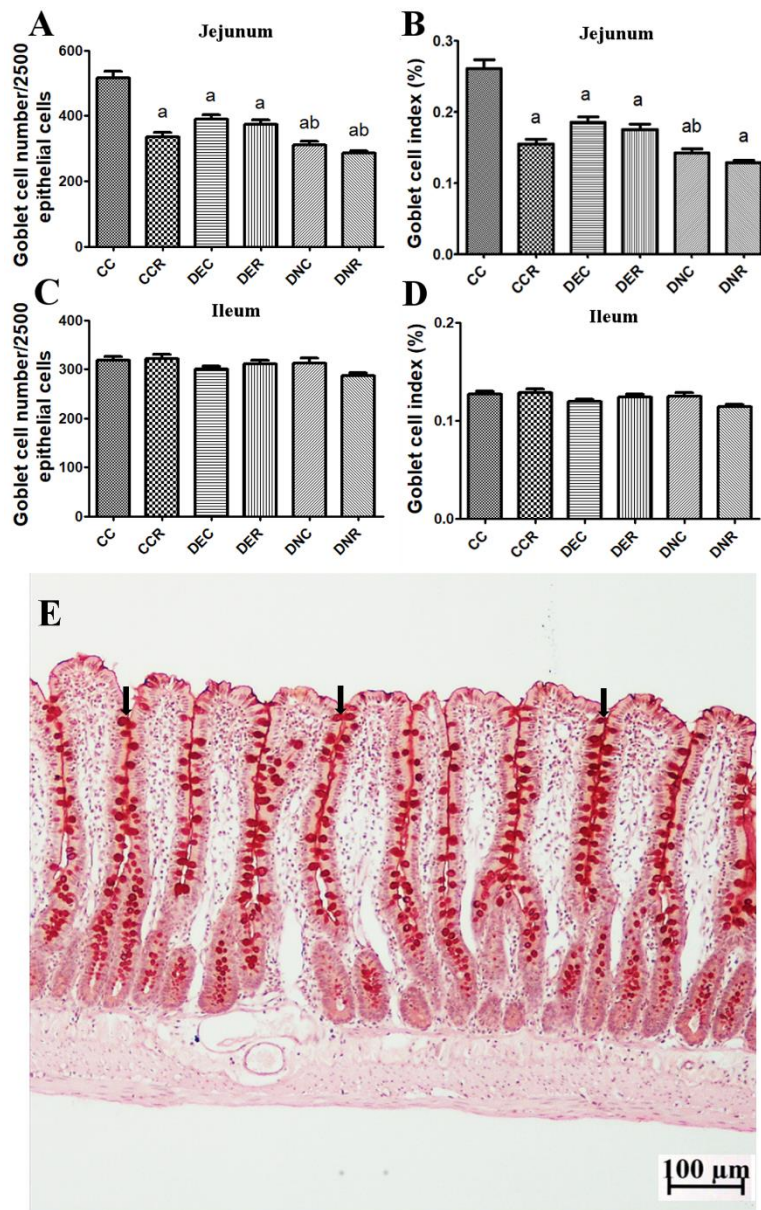
Figure 3. Representative photomicrographs of myenteric ganglia. Myenteric ganglia (200x magnification) from jejunum of diabetic rats submitted to food restriction (after 4 months of treatment). Myenteric ganglia (A) double immunostained myenteric ganglia for HuC/D$^+$ (red) and nNOS$^+$ neurons; (B) HuC/D$^+$ single immunostain; (C) nNOS$^+$ single immunostain; and (D) S-100$^+$ glial cells.
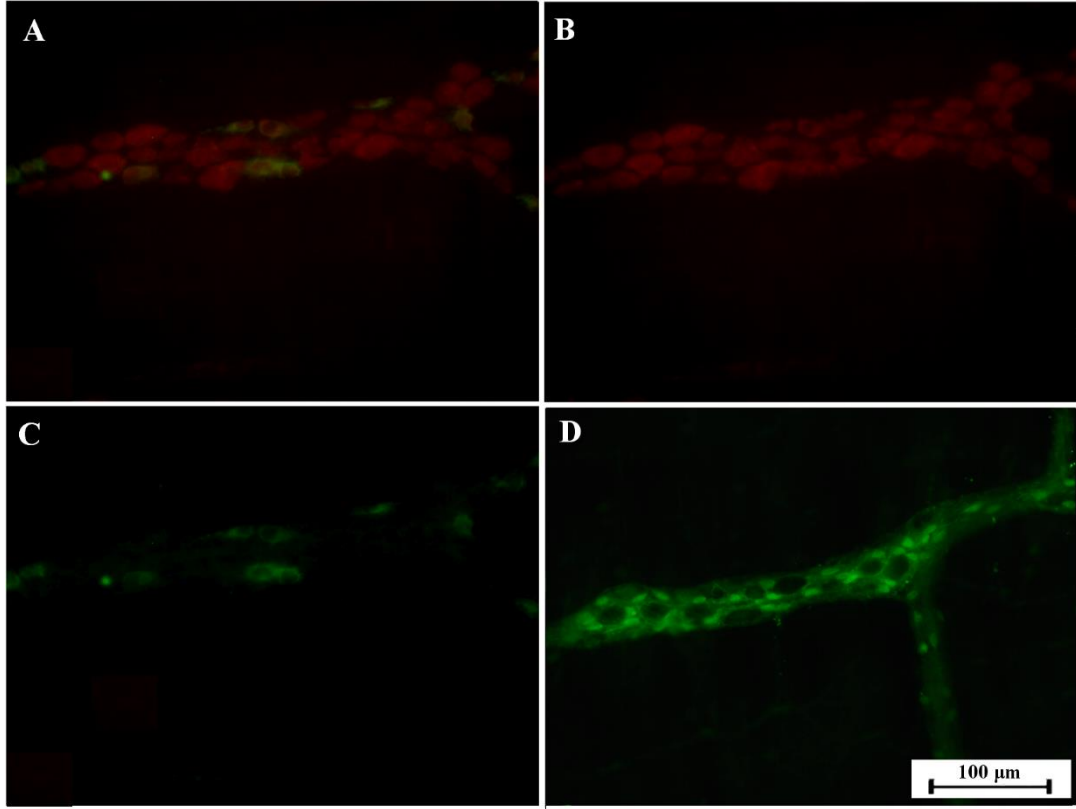
Figure 4. Myenteric neurons and glia from jejunum of control and diabetic rats submitted to food restriction after 4 months of treatment. (A) Number of HuC/D$^+$ neurons. (B) Cell body area of HuC/D$^+$ neurons. (C) Number of nNOS$^+$ neurons. (D) Cell body area of nNOS$^+$ neurons. (E) Number of S-100$^+$ glia. (F) Cell body area of S-100$^+$ glia. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). *a p<0.05 *vs* CC; b p<0.05 *vs* DEC; c p<0.05 *vs* DNC. One-way ANOVA and Tukey's *post-hoc* test analysis.

Figure 5. Myenteric neurons and glia from ileum of control and diabetic rats submitted to food restriction after 4 months of treatment. (A) Number of HuC/D$^+$ neurons. (B) Cell body area of HuC/D$^+$ neurons. (C) Number of nNOS$^+$ neurons. (D) Cell body area of nNOS$^+$ neurons. (E) Number of S-100$^+$ glia. (F) Cell body area of S-100$^+$ glia. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). *a p<0.05 *vs* CC; b p<0.05 *vs* DEC; c p<0.05 *vs* DNC. One-way ANOVA and Tukey's *post-hoc* test analysis.
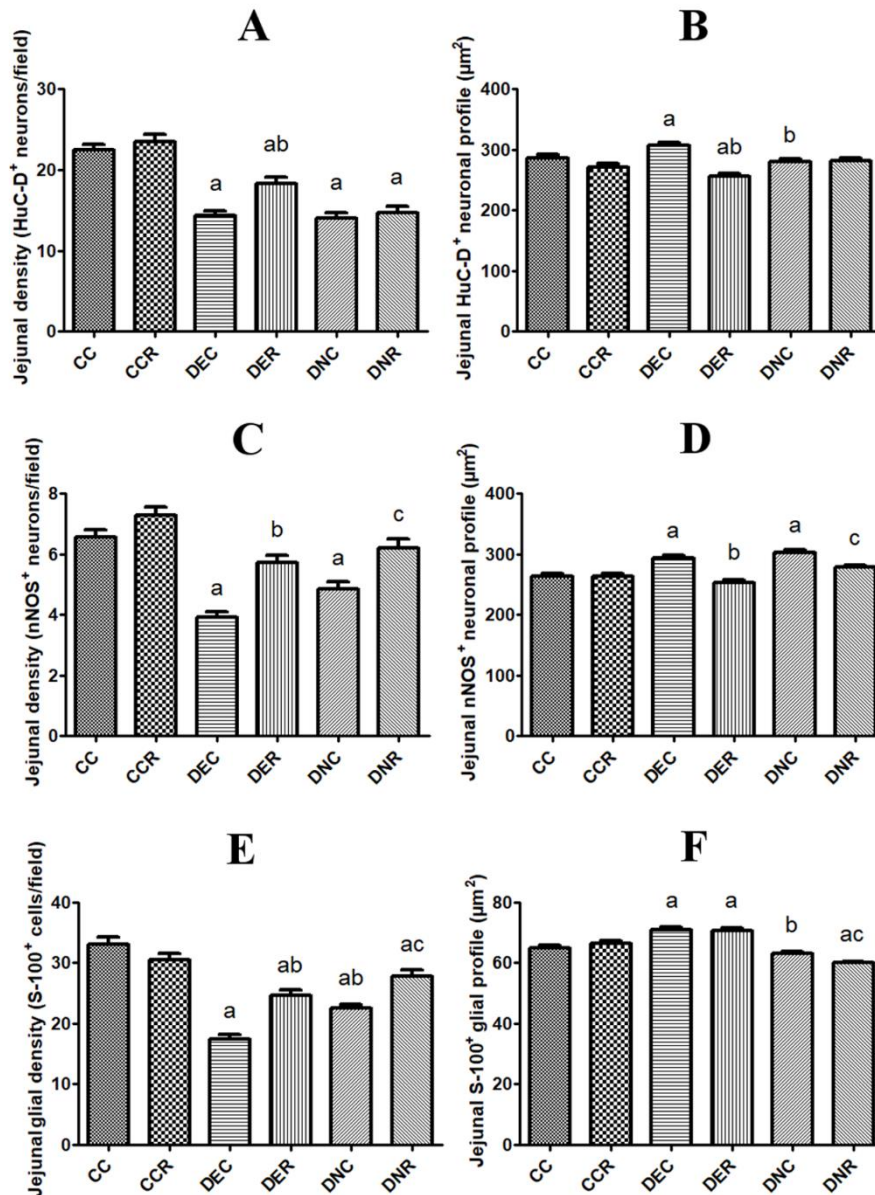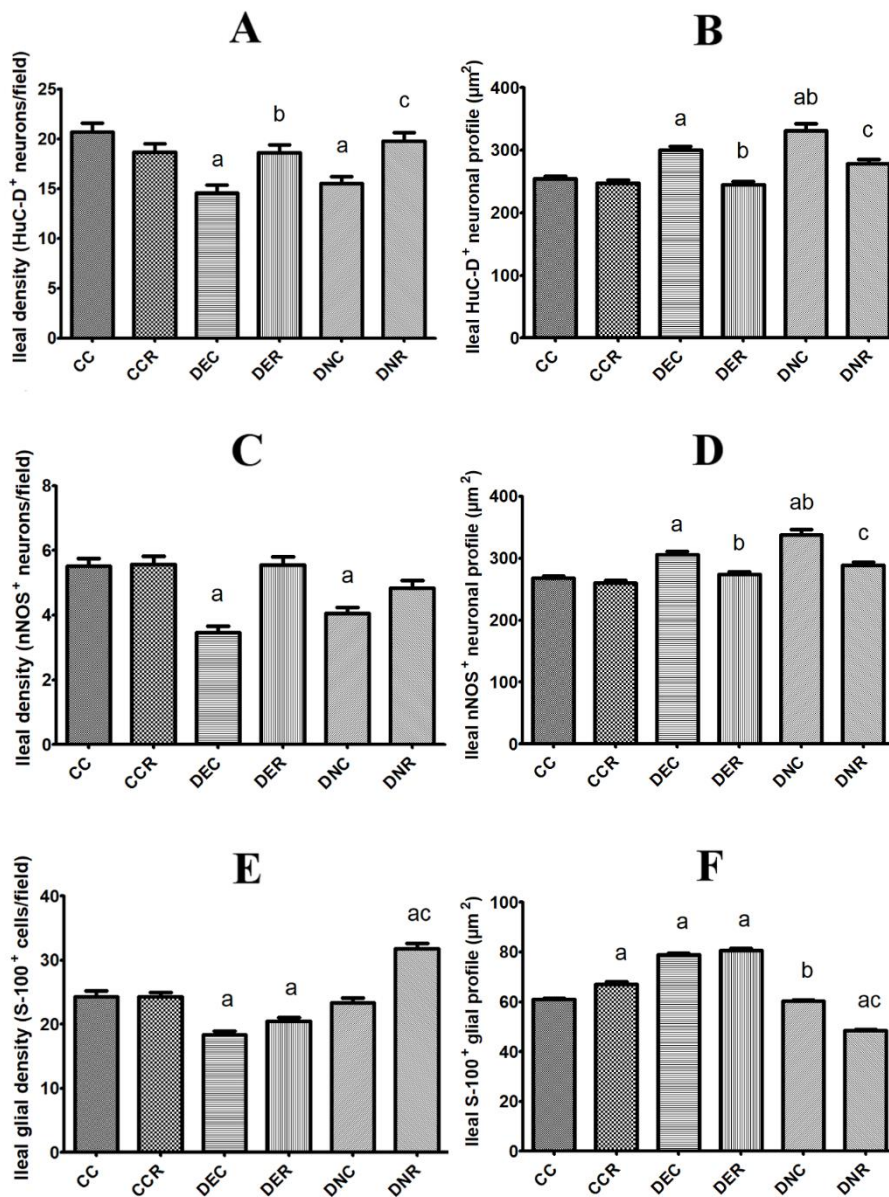
Considerações Finais

O diabetes é uma doença que afeta a humanidade há séculos. Entretanto, recentemente observou-se aumento preocupante da incidência desta doença, em decorrência do estilo de vida atual. Este aumento gerou a necessidade do desenvolvimento de tratamentos e terapias que amenizassem os danos do diabetes, considerados até pouco tempo atrás como irreversíveis.

Para tratar os danos do diabetes é necessário o entendimento da real extensão de seus efeitos no organismo, porém apenas alguns órgãos e tecidos recebem a devida atenção no desenvolvimento da doença. O trato gastrointestinal muitas vezes é ignorado nesse quesito, mesmo sofrendo alterações notáveis que geram grande impacto na qualidade de vida do indivíduo acometido pelo diabetes.

Outro fator importante é a limitação das formas de estudo do diabetes. Apesar da semelhança metabólica e estrutural do humano com outros animais, como os ratos, o desenvolvimento e a resposta ao diabetes apresenta diferenças. Por este motivo a literatura nos mostra diversos modelos diferentes para auxiliar o entendimento desta doença.

A partir disso, neste trabalho optamos pelo estudo comparativo de dois modelos distintos de diabetes mellitus do tipo 2: um induzido pela associação de uma droga diabetogênica (estreptozotocina) e uma dieta estilo cafeteria, simulando a principal origem deste doença em humanos, a má alimentação; enquanto o outro modelo foi obtido através do uso exclusivo da droga diabetogênica associado a uma droga mitigante (nicotinamida). A utilização destes dois modelos nos dá uma idéia mais clara do comportamento do organismo em face de duas causas diferentes, embora o resultado final do processo seja, na prática, semelhante, o diabetes. A utilização de drogas para a indução de diabetes, embora já documentada na literatura, ainda enfrenta alguma resistência no meio acadêmico, em função dos efeitos sistêmicos que as drogas geram no animal. No caso da indução via estreptozotocina e nicotinamida, notamos ser necessárias duas instâncias de aplicação das drogas para que o quadro diabético e a hiperglicemia fossem mantidos estáveis. Ambos os modelos foram previamente testados com o objetivo de encontrar uma dose de drogas mais efetiva, evitando o desenvolvimento de diabetes do tipo 1 pela destruição total das células beta pancreáticas, ou um efeito demasiado fraco para obtermos alterações importantes. Ainda, desde o princípio objetivamos estabelecer um quadro diabético com hiperglicemia constante, já que queríamos estudar os efeitos do diabetes estabelecido de maneira mais rápida que os modelos convencionais, principalmente nos neurônios entéricos.

Com relação a dieta estilo-cafeteria, alguns estudos já foram feitos com essa dieta com vários objetivos, mas principalmente com o intuito de gerar obesidade e até resistência à insulina nos animais. Por este motivo escolhemos aliar a dieta a estreptozotocina para produzir um quadro de sintomas mais estável e condizente com a condição humana. Outro motivo para testarmos essa dieta pouco famosa foi a substituição da tradicional dieta de cafeteria, que envolve o fornecimento de diversos alimentos processados e industrializados, por esta dieta mais simples e de características mais controladas. Embora a associação da dieta estilo-cafeteria e estreptozotocina tenha gerado um quadro de diabetes tipo 2 estável, não houve desenvolvimento de obesidade em função da aplicação da estreptozotocina. A aliança desta dieta com a estreptozotocina é algo inédito na literatura, que nos forneceu dados importantes para comparação com outras formas de indução ao diabetes, permitindo a comunidade científica dar um passo a mais em direção ao "modelo animal perfeito de diabetes mellitus tipo 2".

Os resultados obtidos desta tese, além de mostrar novas formas de estudo do diabetes, nos possibilitaram avaliar o comportamento geral do intestino delgado, com enfoque nos segmentos jejuno e íleo, em face desta doença. Nossos resultados mostraram que cada modelo interfere de maneira distinta os parâmetros avaliados neste estudo, assim como a resposta a restrição alimentar também foi diferente. Ainda mais, os diferentes segmentos do intestino delgado observados tem comportamentos distintos, corroborando outros estudos. Os dados nos mostraram que o diabetes do tipo 2 geram comportamento intestinal diferente em comparação ao diabetes do tipo 1, cujo dados estão presentes na literatura. Esses dados permitem a extensão dos estudos na área com o objetivo de implementar novas metodologias que visem o tratamento eficaz do paciente diabético.

Também demonstramos que os modelos utilizados neste trabalho, com apenas dois meses de diabetes estabelecido, já geram alterações significativas nos neurônios entéricos. O comprometimento destas populações neuronais corrobora com diversos outros estudos que mostram a fragilidade dessas populações, bem como o impacto da qualidade de vida que isto gera.

Corroborando outros dados, principalmente relacionados ao envelhecimento, a restrição alimentar protegeu o metabolismo geral e a morfologia dos neurônios, reforçando seus efeitos positivos em diversas situações. Como observado em nossos dados, os efeitos benéficos da restrição alimentar são principalmente relacionados a proteção contra o estresse oxidativo no organismo, o qual é elevado em diversas situações, incluindo o diabetes.

Embora a restrição alimentar de 50%, utilizada no presente trabalho, seja considerada severa, sua utilização controlada já mostrou benefícios em diversas situações. Ainda, mais

estudos são necessários para encontrarmos a melhor forma de aplicação desta intervenção, já que a literatura mostra uma grande diversidade e falta de padronização neste assunto, já há algumas décadas. Por fim, acreditamos ter contribuído para a expansão destas áreas de estudo, esperando que o potencial dos modelos de diabetes e da restrição alimentar possam ser continuamente explorados no futuro.

ANEXO I – Parecer da Comissão de Ética no Uso de Animais/UEM

Comissão de Ética no Uso de Animais
da
Universidade Estadual de Maringá

CERTIFICADO

Certificamos que o Projeto intitulado "INVESTIGAÇÃO DOS EFEITOS DA RESTRIÇÃO ALIMENTAR SOBRE A NEUROPATIA DIABÉTICA EM MODELO ANIMAL DE DIABETES MELLITUS TIPO 2", protocolado sob o CEUA nº 7590050415, sob a responsabilidade de **Maria Raquel Marçal Natali** *e equipe; Carlos Vinicius Dalto Da Rosa; Jéssica Men De Campos* - 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 11.794, de 8 de outubro de 2008, com o Decreto 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá (CEUA/UEM) em reunião de 14/08/2015.

We certify that the proposal "INVESTIGATION OF THE FOOD RESTRICTION EFFECTS ON DIABETIC NEUROPATHY IN ANIMAL MODEL OF TYPE 2 DIABETES MELLITUS", utilizing 60 Isogenic rats (60 males), protocol number CEUA 7590050415, under the responsibility of **Maria Raquel Marçal Natali** *and team; Carlos Vinicius Dalto Da Rosa; Jéssica Men De Campos* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes (or teaching) - it's in accordance with Law 11.794, of October 8 2008, Decree 6899, of July 15, 2009, with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the State University of Maringa (CEUA/UEM) in the meeting of 08/14/2015.

Vigência da Proposta: de 05/2015 a 12/2017   Laboratório: Ciências Morfológicas (dcm)

Procedência: BIOTÉRIO CENTRAL DA UEM

Espécie: Rato isogênico   Gênero: Machos   idade: 90 dias

N:60

Linhagem: Wistar   Peso: 250g

Resumo: O diabetes mellitus tipo 2 eleva o nível glicêmico gerando aumento do estresse oxidativo em vários tecidos. O trato gastrointestinal também é comprometido pelo diabetes, gerando disfunções, como disfagia e constipação. Há vários modelos animais de indução do diabetes, porém, não há completa similaridade com a doença humana, carecendo de melhor caracterização e comparação entre modelos. A restrição alimentar gera vários benefícios ao organismo, como no processo de envelhecimento e no combate a doenças pela redução da formação de radicais livres e, consequentemente, do estresse oxidativo. A restrição alimentar já mostrou benefícios sobre os indivíduos diabéticos, mas a literatura não considera se este modelo é capaz de recuperar as funções gastrointestinais. O objetivo deste estudo é avaliar as diferenças de modelos de diabetes sobre o jejuno e o íleo, e suas respostas à restrição alimentar. Para isto serão utilizados 60 ratos Wistar, dos quais 20 serão animais controle, distribuidos em animais não tratados e animais sob restrição alimentar. Os animais restantes serão submetidos a dois protocolos de diabetização, com indução química por meio de injeção de nicotinamida e estreptozotocina, ou dieta de cafeteria por dois meses seguida de injeção de estreptozotocina. Para cada protocolo 10 animais receberão ração padrão ad libitum e 10 sofrerão restrição alimentar baseada no grupo controle restringido. A restrição alimentar utilizada será de 50% do consumo diário de ração. Após 60 dias de restrição os animais serão eutanasiados, e terão seu sangue

coletado para realização de dosagens bioquímicas de proteínas totais, uréia, frutosamina, níveis de aspartato aminotransferase e alanina aminotransferase, malondialdeído e atividade de GLP-1. O intestino delgado será coletado e terá o jejuno e íleo separados e fixados para técnicas imunohistoquímica de avaliação da população geral e subpopulações colinérgica, nitrérgica e vipérgica neuronal, e glia do plexo mioentérico. Outra porção destes segmentos serão destinadas a técnicas de imunoidentificação para avaliar células enteroendócrinas serotoninérgicas e GLP-1 imunoreativas, além da apoptose por meio do TUNEL em cortes histológicos. Também será realizada determinação de alguns parâmetros do estresse oxidativo tecidual por meio das técnicas da superóxido dismutase e do ácido tiobarbitúrico. Os dados serão submetidos à análise estatística utilizando o teste K-S, seguidos pelos testes de análise de variância (one-way ANOVA) e pós-teste de Tukey, ou teste de Kruskal-Wallis e pós-teste de Dunn's, conforme a normalidade dos dados.

Maringá, 18 de agosto de 2015

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CEUA Nº 7590050415

Prof. Dr. Alexandre Ribas de Paulo
Coordenador da Comissão de Ética no Uso de Animais
Universidade Estadual de Maringá

ANEXO II – Normas da revista International Journal of Biological Sciences

Instructions for Authors

International Journal of Biological Sciences publishes papers of high quality in any area of biology and biological sciences. Submission to the Journal is on the understanding that the article has not been previously published in any other form and is not under consideration for publication elsewhere.

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- **Short Research Communication** presents a concise study, or sometimes preliminary but innovative research findings that might be less substantial than a full research paper. Short Research Communication is limited to 2500 words. It should have a set of keywords and an abstract summarizing background of the work, the results and their implications. Results and Discussion Section should be combined and followed by Conclusion. Materials and Methods will remain as a separate section. The number of references is limited to 30 and the number of figures and/or tables combined is limited to 4.
- **Letter**: Description of novel findings that might not be suitable for a regular research paper or short research communication may be published as letter. Letter is limited to be under 500 words and 5 references. There should be not more than two figures or tables combined, and no supplementary material.
- **Commentary**: This is the place for discussing controversial and/or interdisciplinary topics, viewpoints and any other issues of interest. It is limited to a maximum of 10 references, 1 display (figure/table), and 1000 words including all contents.
- **Review or mini-review** should be authoritative and of high interest. A minimum of two figures/illustrations should be included in the review or mini-review that should be some 3000 or 5000 words long (excluding references and figure legends). High quality reviews from leading researchers in their fields are particularly welcome.

New! Graphical Abstract: authors should provide a graphical abstract (a beautifully designed feature figure) to represent the paper aiming to catch the attention and interest of readers. Graphical abstract will be published online in the table of content. The graphical abstract should be *colored*, and kept within an area of 12 cm (width) x 6 cm (height). Image should have a minimum resolution of 300 dpi and line art 1200dpi. Note: *Height of the image should be no more than half of the width.* **Please avoid putting too much information into the graphical abstract as it occupies only a small space**. Graphical abstract can be provided in the format of jpg (preferred), PDF, Word, PowerPoint, or png, after a manuscript is accepted for publication. See more sample graphical abstracts in http://www.thno.org.

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Please include figures at the end of manuscript Word file, or provide a high quality PDF containing all figures. Make sure images are of highest resolution, and any letters inside are clear and legible. If the figures or tables are created using PowerPoint, Photoshop, Microsoft Excel etc, please also provide the original files from those programs.

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The first letter of each word in title should use upper case. Include in the first page the article title, author's names, affiliations, corresponding author's phone/fax number and/or email. Abstract should not contain citations to references. Also provide 3-6 keywords.

Author's names: please provide full names. Important! *Please write first name and middle name before surname or lastname,* e.g., "Mike Johnson" (*not* "Johnson, Mike"; *not* "Johnson M."; *not* "M. Johnson").

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Text

Please do not add number before subtitles (This does not apply for Review or mini-review).

Citation of Tables and Figures

Tables and Figures should be numbered consecutively. Citation of tables and figures should use the format: Table 1, Table 2, ... Figure 1, Figure 2, ... (or Fig. 1, Fig. 2, ...). Parts in a figure can be identified by A, B, C, D, ... and cited as Figure 2A, Figure 2B, Figure 2C. Please do not abbreviate the word "Table" to "Tab.".

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    1. Kiloh LG, Smith JS, Johnson GF, et al. Physical treatment in psychiatry. Boston, USA: Blackwell Scientific Publisher; 1988.
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    0. Beckenbough RD, Linscheid RL. Arthroplasty in the hand and wrist. In: Green DP, ed. Operative Hand Surgery, 2nd ed. New York: Churchill Livingstone; 1988: 167-214.
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E.g. HIV: human immunodeficiency virus; SIV: simian immunodeficiency virus; ....

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Experimental research involving human or animals should have been approved by author's institutional review board or ethics committee. This information can be mentioned in the manuscript including the name of the board/committee that gave the approval. Investigations involving humans will have been performed in accordance with the principles of Declaration of Helsinki. And the use of animals in experiments will have observed the *Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education* by the New York Academy of Sciences, Ad Hoc Animal Research Committee.

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