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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio
Mestrado Integrado em Medicina Veterinária

Structural and functional intestinal changes in STZ-induced diabetic rats

Marisa Manuela Esteves Monteiro

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Abstract

Enteric dysmotility is a long-term complication of diabetes mellitus (DM) that causes significant discomfort in 75% of diabetic outpatients.

Considering this alarming reality, we decide to study structural and functional intestinal changes in diabetic rats, two weeks after type 1 DM induction. DM was induced in adult male Wistar rats by a single intraperitoneal injection of streptozotocin (STZ, 55 mg/kg). In vivo physiological parameters were registered daily (body weight, food and water intake and fecal pellets production), and the ileum and colon of STZ-induced rats collected for histological analysis and evaluation of the contractile response to acetylcholine (ACh) and angiotensin II (AngII).

Rats that had an initial glycaemia of 99.30±3.29 mg/dL became hyperglycemic (395.09±13.80 mg/dL, n=23) within 48 hours. Diabetic animals experienced a consistent weight loss during the protocol, despite drinking and eating more than controls. Moreover, we were the first to report that STZ-induced rats gradually increase their fecal excretion, reaching, on the 14th day of the protocol, values 4 times higher than the initial ones.

Regarding the macroscopic evaluation, abdominal cavity observation showed an enlarged cecum, loss of visceral fat and distended bladder. The intestines of diabetic rats were heavier than the same intestinal parts of control animals and presented bigger longitudinal length and circumferential perimeter. The microscopic evaluation revealed thickening of intestinal wall in all regions (mainly mucosa and muscular layers). Both diabetic and control portions of intestine had the same response to exogenous ACh, but DM tissue of proximal and middle colon presented smaller Emax in response to Ang II.

Taken together these data indicate that STZ-induced rats exhibit typical type 1 DM signs only two weeks after induction: polydipsia, polyuria, polyphagia and loss of body weight. Diabetic rats have a higher fecal excretion and display structural intestinal differences such as distension, increased length and circumferential perimeter, corroborated by increased thickness of the intestinal wall. Finally, our study also showed functional alterations, since a lower contractile force was observed in the proximal and middle colon (but not in the distal colon) of STZ-induced rats, in response to Ang II. The structural differences and altered contractile response observed could underline the enteric dysmotility seen in diabetic patients and should be taken into consideration in future studies.

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A todos, o meu sincero obrigado!

List of Abbreviations

ACE	Angiotensin Converting Enzyme
ACh	Acetylcholine
Ang II	Angiotensin II
AGEs	Advanced Glycation Endproducts
AT1R	Angiotensin Receptor type 1
AT2R	Angiotensin Receptor type 2
DALYs	Disability-Adjusted Life Years
DAN	Diabetic Autonomic Neuropathy
DC	Distal Colon
DM	Diabetes mellitus
ENS	Enteric Nervous System
GI	Gastrointestinal
ICC	Interstitial Cells of Cajal
MC	Middle Colon
NO	Nitric Oxide
PC	Proximal Colon
PDGFRα+	Platelet Derived Growth Factor Receptor Alpha+
RAGE	Advanced Glycation Endproducts Receptor
RAS	Renin-Angiotensin System
SMC	Smooth Muscle Cells
STZ	Streptozotocin
T1DM	Diabetes mellitus Type 1
T2DM	Diabetes mellitus Type 2

Index

List of Figures

List of Tables

Introduction

Diabetes mellitus (DM) is a complex chronic progressive metabolic disorder, medically incurable, that can affect almost every organ system[1]. The prevalence of DM in humans of all age groups is at alarming proportions worldwide and has nearly doubled since 1980. The number of people with diabetes went from 108 million in 1980 (a global prevalence of 4.7%) to 422 million in 2014 (8.5% of the adult population)[2–4]. In Portugal, the total prevalence of DM in the adult population (20 to 79 years old) was of 13,3% in 2015[5,6]. DM is also one of the most common metabolic diseases diagnosed in canines and felines, with an estimated prevalence ranging from 0.21% to 1.24% in cats[7] and 0.34% to 1.2% in dogs[8,9].

Besides the substantial economic impact of the disease, the importance of DM as a public health problem is also related to the significant morbidity and mortality rates. The costs of diabetes are measured through not only medical expenses, but also indirect costs associated with productivity loss and premature mortality, with an estimation of US$825 billion worldwide anually[3]. There were 1.5 million deaths due to diabetes in 2012, plus an additional 2.2 million deaths related to the increasing risks of cardiovascular events and other associated comorbidities[2]. Additionally, in 2015, diabetes was considered the sixth leading cause of disability[2,10] and was the third most common global risk factor for disability-adjusted life years (DALYs), accounting for 143 million DALYs, representing a 22% increase from 2005 to 2015[11].

Classification

DM is characterized by a state of hyperglycemia caused by insulin deficiency, defect in insulin action, or both, resulting in abnormal metabolism of carbohydrates and subsequent elevated levels of glucose both in blood and urine. There are two main forms of DM: type 1 DM (T1DM) and type 2 DM (T2DM)[12,13].

T1DM accounts for 5-10% of all DM cases[12,14]. It results from a cellular-mediated autoimmune inflammatory response against pancreatic β-cells, which causes absolute insulin deficiency. Since the pancreas is unable to produce insulin, patients with T1DM require daily insulin administration. The cause of pancreatic β-cells destruction is still a matter of debate[15], but the incidence rate seems to depend of genetic susceptibility and environmental factors[16].

T2DM is a combination of insulin resistance in target organs and relative deficiency caused by dysfunctional pancreatic β-cells, and accounts for 90 to 95% of all cases. The specific etiologies are not known, but the occurrence of this form of DM is determined by a combination of genetic and metabolic factors, of which obesity is one of the strongest risk factors[12,14,17]. Therefore, physical inactivity and the consumption of high-energy diets are considered the main causes of the rising prevalence of T2DM[18]. Other risk factors include smoking, alcohol consumption, older age, family history of T2DM, gestational diabetes, some medications,

stress and depression[19]. So, the prevention of this form of diabetes is mostly based on maintaining a normal body weight and a healthy lifestyle[19].

In small animals the etiopathogenic mechanisms of DM are not identical to humans, but the classification used is roughly the same. Diabetes in dogs is mostly comparable to T1DM, while the majority of diabetes in cats resembles T2DM[8].

Symptoms and complications

The most common symptoms of DM are polyuria, polydipsia, polyphagia (the three P's of diabetes) and weight loss. The reduced insulin secretion results on impairment of cell glucose uptake from the blood, causing hyperglycemia. Additionally, the absence of glucose in the cells induces lipolysis, proteolysis, glycogenolysis and neoglucogenesis, leading to higher blood glucose levels and reduced body mass[20].

Since glucose is an osmotically active compound, diabetic patients will experience dehydration, because there is: a) increased osmotic pressure in the extracellular fluids, causing osmotic transfer of water from the cells and b) decreased tubular reabsorption and increased urinary debit, causing glycosuria and osmotic diuresis - thus explaining polyuria and polydipsia[21]. The lack of glucose in the cells also creates a state of starvation that causes polyphagia as a compensatory response, without increasing body weight. Actually, it will only boost hyperglycemia and associated polyuria and dehydration[22].

In the long-term, chronic hyperglycemia will damage different organs, especially eyes, kidneys, nerves, heart and blood vessels, causing their dysfunction or failure[12,23]. The mechanism associated to vascular and nerve damage is based on their permeability to the entrance of glucose without the presence of insulin, meaning that the level of glucose in the vascular endothelium and nerve tissue will be similar to the level found in the plasma[22]. Long-term complications of DM can be divided into microvascular, macrovascular and neuropathic[16,24]. Microvascular complications may occur all over the body; they include retinopathy (one of the most important causes of adult blindness) and nephropathy that can result in renal function impairment[22,12,23]. Regarding macrovascular complications, the most common manifestation is atherosclerosis of the coronary arteries, the most frequent cause of death in diabetic patients[23]. As for neuropathic complications, the mechanisms associated to nerve damage include neurovascular deficiency, metabolic insult due to hyperglycemia, autoimmune damage and increased oxidative stress with increased free radical production. These may cause diabetic autonomic neuropathy (DAN), a condition that affects many organ systems, including cardiovascular, genitourinary and gastrointestinal[25].

The Streptozotocin-Induced Experimental Model of DM

Considering the prevalence and severity of DM, it is easy to understand the importance of using animal models of this disease to study what causes it and to test new antidiabetic drugs. There are several animal models of diabetes in different laboratory animal species, which include surgical (pancreatectomy), chemical or genetic models[26]. Chemical models are one of the most common methods for inducing DM, and generally use Streptozotocin or Alloxan as the trigger (69% and 31% of the published studies, respectively)[27]. Streptozotocin (STZ) has been the agent of choice to induce diabetes since 1963, because of its ability to induce structural, functional and biochemical alterations that resemble those seen in DM[28]. The induction of diabetes with STZ is most frequently used in rats and mice, but has also proven to be efficient in rabbits, hamsters, guinea pigs, pigs and gerbils[26,29].

STZ is a naturally occurring compound synthetized by *Streptomyces achromogenes*, with antimicrobial and chemotherapeutic properties[30]. The diabetogenic effect of STZ is due to the selective destruction of pancreatic β-cells. These cells are in constant need for glucose, uptake via GLUT-2 transporters - that STZ also uses to enter pancreatic β-cells, causing their destruction[29]. STZ toxicity occurs through three main mechanisms: a) STZ intracellular accumulation causes DNA alkylation, resulting in cell necrosis, b) STZ acts as a nitric oxide donor, which is known to cause damage in pancreatic β-cells and c) STZ produces reactive oxygen species that accelerate β-cell destruction[26,28]. STZ is considered a relatively selective drug, since it does not affect pancreatic parenchyma or α-cells but may cause injury in other cells that express GLUT-2 transporters, like hepatocytes and renal tubular cells. For this reason, this model is not recommend to study renal or hepatic effects of DM[26].

Diabetes mellitus and the gastrointestinal tract

Gastrointestinal (GI) complications are relatively common and very important as they can be associated with significant morbidity, affecting up to 75% of patients, although it is uncertain if the prevalence differs between T1 and T2DM[31]. Though this high prevalence, the knowledge and treatment options for this complications are considerably scarse[1,32,33]. The most common GI complications include esophageal and gastroesophageal dysmotility, gastroparesis, enteropathy and colonic disorders, most probably due to morphological and biomechanical remodeling of the GI tract[1].

Upper GI tract

The esophageal alteration of the morphological and biomechanical properties outcomes as increased stiffness and reduced compliance and sensitivity to distension that leads to dysmotility. Esophageal dysmotility (characterized by abnormal peristalsis and reduced sphincters tone) affects up to 63% of diabetic patients[34,35], but the percentage of those with

symptoms (dysphagia or heartburn) is much lower[25]. As for gastroesophageal dysmotility, it often leads to reflux, affecting up to 41% of patients[1]. But of all the upper digestive complications, the most frequent symptoms are related to gastroparesis, which causes nausea, vomiting, early satiety and epigastric pain, and can affect up to 65% of diabetic patients. Food retention in the stomach is combined with accelerated gastric emptying, contributing to poor post–prandial glycemic control that leads to alternating hyper and hypoglycemic episodes[1,36]. Surprisingly, there seems to be no correlation between esophageal and gastric transit time[37].

Small intestine and colon

Enteropathy and colorectal dysfunctions are mostly found in patients with long-standing diabetes and are often associated with gastroparesis[1]. The pathogenesis of intestinal dysfunction is multifactorial, and it may be related to the accumulation of advanced glycation end-products (AGEs), enteric nervous system (ENS) or Interstitial Cells of Cajal (ICCs) injury, and muscular layers fibrosis[38].

The increased formation of AGEs is considered one of the main mechanisms responsible for DM complications[39]. AGEs are naturally formed in our organism, but their production is accelerated when intracellular glucose levels are chronically elevated[32,40]. Thus, in diabetic patients AGEs accumulation disturbs the cell's metabolic activities and modifies the extracellular matrix. Furthermore, the activation of the AGEs receptor (RAGE) also modulates cellular functions, causing oxidative stress and the production of inflammatory factors[41]. It was previously demonstrated that AGEs and RAGE are up-regulated in duodenum, jejunum, ileum[42] and colon[40] of STZ-induced rats. This high density of AGE/RAGE found in crypts, villus and brush border probably contributes to the digestion and absorption disorders observed in these animals. In addition, accumulation of AGEs in smooth muscle and RAGE in neurons may contribute to intestine and colon motor disorders[42,43]. There also seems to be an association between AGE/RAGE accumulation and hypertrophy of the intestinal layers, through the overexpression of connective tissue growth factor, vascular endothelial growth factor and platelet-derived growth-factor[42]. The level of colon remodeling seems to be proportional to the abnormal expression of AGEs and RAGE in the different wall layers[40].

The ENS is present through the entire GI tract and works as an autonomous entity that controls the majority of physiological processes such as motility, blood flow, immune response, intestinal barrier and secretory functions[44,45]. The motor and sensory enteric neurons are organized in two major plexi: the submucosal (between the submucosa and the mucosa – controls for example GI secretions) and the myenteric plexus (between the circular and the longitudinal muscular layers - the main responsible for GI motility control)[31,46]. More than 30 neurotransmitters have been identified in the ENS, like acetylcholine (ACh), dopamine and

serotonin. In general, neurons that secrete ACh are excitatory, stimulating muscle contraction[44]. The intestinal response to cholinergic stimulation (induced by cholinergic agonists, like acetylcholine or carbacol) depends on muscarinic activation of M_2 and M_3 receptors, that are preferentially expressed in the smooth muscle[47,48]. The cholinergic response is essential to maintain peristalsis, the aboral movement of luminal content that can be inhibit by muscarinic antagonists, like atropine[49].

GI motility is determined by a complex collaboration between enteric neurons, smooth muscle cells (SMC) and interstitial cells (Interstitial Cells of Cajal (ICCs) and PDGFRα+ cells), which form an integrated unit called the SIP syncytium[50,51] SIP cells are responsible for GI pacemaker activity, propagation of slow waves, transduction of inputs from motor neurons, and mechanosensitivity. Loss or improper function of these cells has been linked to several GI tract disorders[52,53].

Several studies indicated that DAN causes damage to the ENS, not only in diabetic animals models but also in humans with DM[20,54,55]. Several authors reported changes in the number and size of myenteric neurons through the entire GI tract, including stomach[56], duodenum[51], jejunum[55], ileum[46], cecum[57] and colon[20]. The phenotypic changes of neurons were associated with unviable extracellular conditions like hyperosmolarity, low nutrient availability, and oxidative stress[20,54,55]. Regarding the mechanisms proposed for the neuronal loss, they include increased apoptosis, oxidative stress and AGE/RAGE, and decreased levels of nerve growth factors. Furthermore, the ratio between inhibitory and excitatory neurons seems to be altered in the diabetic GI tract. Inhibitory neurons are more severely affected, namely those that produce nitric oxide (NO), a potent gaseous nonadrenergic, noncholinergic neurotransmitter that mediates smooth muscle relaxation in the GI tract[31]. Loss of nitrergic neurons in the diabetic GI tract[23,48,54,58] may occur via some of the mechanisms described previously: a) phenotypic switch; b) AGE/RAGE accumulation[59]; increased production of reactive oxygen species [60] and apoptosis increase [61]. The reduction of these inhibitory neurons was directly implicated in motility alterations in diabetic patients, due to disturbed signaling in the myenteric plexus. It has also been described a deficit in the intestine's cholinergic neurotransmission, since the response to exogenous ACh seems to be impaired in ileum[62] and colon[48] of diabetic rats. This impairment may be due to: a) a decrease in ACh release or production[49] or b) a reduction of acetylcholinesterase action, with a subsequent down-regulation of muscarinic receptors[62].

Additionally, diabetes has also been linked to changes and loss of ICCs in humans and animal models[17,54,63–65]. In the rat colon, ICCs presented swollen mitochondria, partial depletion of cells bodies with many of them losing their connections with enteric nerves, which also displayed marked injury. Damage of the ICCs may cause increased excitability of the diabetic colon[65], contributing to smooth muscles dysrhythmia[48,63].

Finally, mechanical factors may also contribute to intestinal and colonic disorders, since diabetes seems to cause structural remodeling that can affect biomechanical properties[34]. It has been reported that the proliferation of the different layers of the rat colon (mainly the mucosa) can increase stiffness and decrease the resting compliance and relaxation capacity of the intestinal wall, in proportion to the duration of DM[66,67]. The main mechanisms proposed to explain this remodeling are related to the increased production of collagen type 1 and, again, accumulation of AGE[68].

The interaction between all the DM related changes in intestine and colon described previously results in symptoms like chronic constipation, diarrhea, and in clinical conditions like colorectal cancer and inflammatory bowel disease (figure 1). Constipation alternating with diarrhea is one of the most common symptoms[1,14]. Constipation can be explained by slower motility of the large bowel and affects up to 60% of DM patients[1]. However, possible complications like megacolon, pseudo obstruction, stercoral ulcer or perforation may (rarely) occur[1,69]. Diarrhea affects up to 22% of patients with DM and is typically painless and nocturnal, being occasionally associated with fecal incontinence[1]. The dysfunction of internal and external sphincters is secondary to DAN and can be boosted by an acute hyperglycemia episode, which inhibits the sphincters and decreases rectal compliance[1,14]. The modification of GI motility can also affect the amount, composition and function of gut microbiota, which may affect the integrity of the intestinal mucosa, the interaction with the ENS, and the function of the smooth muscle layers[38].

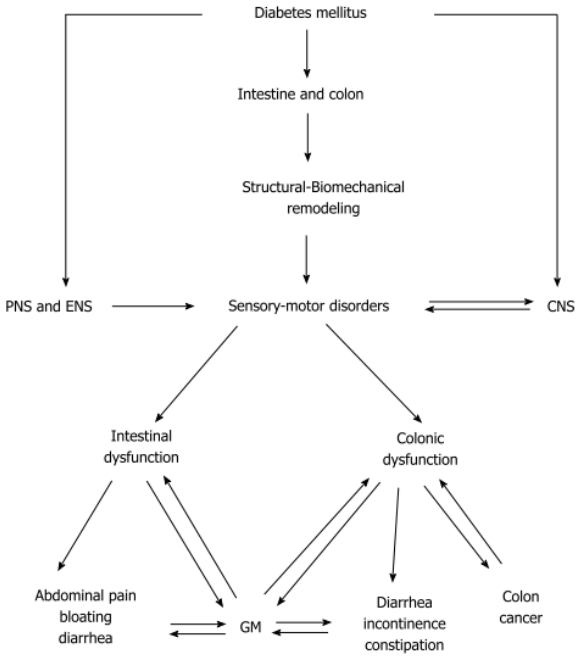

Figure 1 – DM-induced intestinal and colonic changes and clinical consequences. CNS:Central nerve system; PNS: Peripheral nerve system; ENS: Enteric nervous system; GM: Gut microbiota. Source: Zhao et al 2017

Renin–angiotensin system and the gastrointestinal tract

The renin–angiotensin system (RAS) is mostly known for its effects in the cardiovascular and renal systems, regulating blood pressure and fluid homeostasis. Nevertheless, it also has an influence in other systems, like the GI tract[70]. In a classical view, angiotensinogen is cleaved by renin into angiotensin I, which is then converted to angiotensin II (Ang II) by the Angiotensin-Converting Enzyme (ACE). Ang II is the final effector of this system, causing vasoconstriction, aldosterone release, promoting inflammation and fibrosis, amongst other effects. These functions are mostly mediated via the Ang II receptor type 1 (AT_1R) while activation of the Ang

II receptor type 2 (AT_2R) usually counteracts them. The view of the RAS has been expanding with new peptides, enzymes and receptors identified[71,72]. For the purpose of this study the focus has been in the classical RAS.

The GI tract expresses all of the RAS components, indicating that it must influence the function of this system. In the colon, AT_1R were found on crypt bases, mucosal vessel walls, lamina propria macrophages and myofibroblasts; AT_2R were identified in epithelium surface, in crypts and mesenchymal cells (with less expression)[72]. Functionally, besides increasing sodium and water reabsorption through NaCl coupled transport, Ang II seems to have a role in colonic motility. Ang II contracts circular and longitudinal smooth muscle in response to direct activation of post-junctional AT_1R and indirect activation of pre-junctional AT_1R in myenteric and submucosal neurons, inducing tachykinines and acetylcholine release[72,73,74,75]. Curiously, the human colonic smooth muscle is more sensitive to Ang II than to acetylcholine[76,77], but the physiological importance of Ang II in the GI tract is not completely understood. There is some evidence showing that Ang II is more important to sustain muscular tone than to induce phasic contractions, but further studies are needed to demonstrate it[72,78].

Regarding DM, there could be an association with RAS, since ACE inhibitors or AT_1R blockers have shown to reduce the incidence of vascular complications, nephropathy and cardiovascular disease in these patients[79]. Also, increased levels of tecidual ACE were found in DM patients, resulting in additional formation of Ang II[80,81]. In addition, in patients with T2DM RAS inhibition improves insulin sensitivity allowing better control of glycemic values[82]. In fact, infusion of Ang II seems to induce insulin resistance[79,83]. However, to date there is no information involving RAS with diabetic intestinal and colonic alterations.

AIM

Considering the actual knowledge on GI complications in diabetic patients, the aim of this study was to evaluate the structural and functional impact of STZ-induced DM in the gut of Wistar rats.

During the induction period animals' welfare was evaluated daily, and *in vivo* physiological parameters registered (body weight, food and water intake, fecal excretion). Two weeks after DM induction the ileum and colon of these animals were collected for histological analysis and the functional response to ACh and Ang II was characterized. In all cases, data from STZ-induced rats was compared to that of control animals.

Materials and Methods

Animals and housing

Thirty-two male Wistar rats with 10 to 18 weeks of age (weighing 300-400 g) were used in this study. Animals were maintained at ICBAS rodent animal house facility and the project was approved by the local animal welfare body (ORBEA ICBAS-UP). In order to reduce the number of animals used, all the rats evaluated in this study were already assigned to another experimental protocol.

Animals were exposed to a 12 hours light / 12 hours dark cycle, with controlled ventilation, temperature (20-24ºC) and relative humidity (40-60%). All animals used in this protocol were kept in Sealsafe Plus GR900 Tecniplast® cages with proper bedding (Corncob ultra 12, Ultragene), in groups of two, with free access to autoclaved water (two bottles for each cage) and laboratory rodent food (4 RF21, Mucedola S.r.l., Italy). All cages were also provided with nesting paper and paper tunnels as environmental enrichment, in addition to cereal seeds and flakes mixed with the bedding material.

Diabetes induction

On the day of DM induction (d0), animals were fasted for 4 hours with free access to autoclaved tap water. The STZ solution (S0130, Sigma-Aldrich) (55 mg/ml in citrate buffer pH 4.5) was prepared just prior to the injection, since a freshly prepared solution is considered to be more effective[29]. Diabetes was induced by a single intraperitoneal injection of 55 mg/kg of STZ[29], under the analgesic effect of tramadol (20 mg/kg, PO), administered moments before. Rats maintained *ad libitum* access to water and food through the remaining protocol. Animals were considered diabetic if 48h after STZ injection their blood glucose was ≥ 250mg/dL. Glycemia was evaluated using a FreeStyle Precision Neo glucometer (small sample size > 0.6 µL blood) and compatible individually wrapped test strips (both from Abbott, Canada). The blood glucose level of diabetic rats was measured by puncturing one of the tail veins at d0 (control value), d2 (to confirm or discard DM) and d7. On d14, glycemia was obtained after the animal's sacrifice, collecting blood from the abdominal aorta. From 32 animals that were injected with STZ 23 became diabetic (blood glucose ≥ 250mg/dL), representing a rate of induction of 72%. Different animals of similar age and body weight (n=8), that did not undergo any of these procedures, were used as controls.

Animal monitorization and welfare evaluation

The animals included in this project were daily monitored (11:00h to 13:00h) throughout the entire protocol, and all information was registered in an individual evaluation table.

Our evaluation started in the maintenance room, where we evaluated coat's appearance, piloerection, animal's posture before and after a brief stimulus, abdominal discomfort and changes in the breathing pattern. Then, already in the observation room and with the box open, the same parameters were observed, and the animals' hydration status evaluated. Monitoring proceeded by weighting the animal, which also occurred prior to the fasting period on day 0. Then, water and food were weighed to calculate daily intake. The appearance of the feces was also evaluated, and fecal pellets counted, collected and later weighted.

Whenever the cages became excessively wet (due to classical signs of diabetes – polydipsia and polyuria) they were changed (usually every 2 days).

Macroscopic evaluation

On day 14, control and STZ-induced rats were euthanized by decapitation, using a guillotine suitable for that species (Small Guillotine, Harvard Apparatus). The abdomen was opened and the overall appearance of the viscera was evaluated and photographed. The abdominal aorta was identified and punctured to collect blood to measure glycemia. Then, the part of the gut from the anus to the ileum (10 cm proximal to the ileocecal junction), including the cecum, was removed and weighed, and the longitudinal length of the colon was measured (figure 2). Then, the intestine was separated into ileum, cecum and colon. All of those segments were gently cleaned of their content using Krebs-Henseleit solution (in mM: 118 NaCl; 4.8 KCl; 2.5 $CaCl_2.2H_2O$; 1.2 $NaH_2PO_4.H2O$; 1.2 $MgSO_4.7H_2O$; 25 $NaHCO_3$; 0.02 Na_2EDTA; 0.3 Ascorbic acid; 11 monohydrated glucose) and individually weighed without content. The difference in weight was taken as the fecal content weight. A 1 cm portion of the rectum, middle colon and ileum (figure 2 – green portions) was opened through the non-mesenteric border and laid flat to measure the circumferential perimeter (mm).

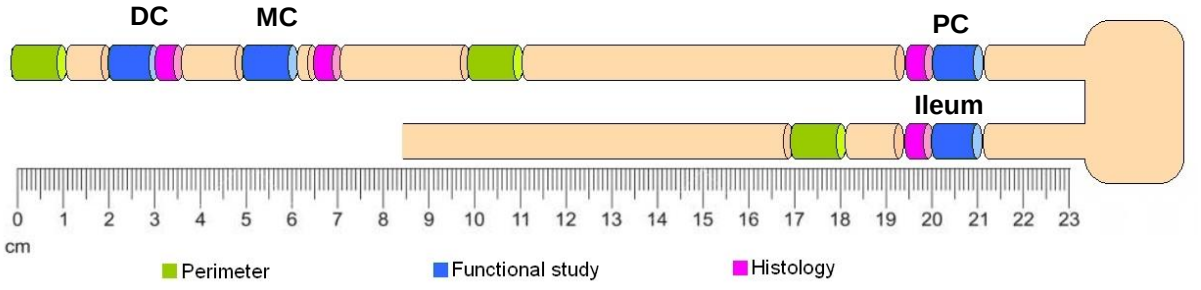

Figure 2 - Different portions of ileum and colon used throughout the protocol. PC - Proximal Colon; MC - Middle Colon; DC - Distal Colon.

Histology

Samples (0.5 cm long) of the colon and ileum of diabetic and control animals were collected for histological examination (figure 2 – pink portions). More precisely, the portion of the ileum was collected 3 cm proximal to the ileocecal junction; the proximal colon (PC) 3 cm distal from the cecum; the distal colon (DC) 3 cm proximal to the anus; and the middle colon (MC) 3 cm proximal to where the DC was collected. Each sample was opened through the anti-mesenteric border and fixed in 10% formalin.

All samples were dehydrated in consecutive 70%, 96% and 99% ethanol solutions and embedded in paraffin. Then, 3 µm-thick cuts were made perpendicularly to the mucosa using a microtome and mounted in sterilized glass slides. Finally, the sections were rehydrated in a series of graded ethanol (99, 96, 70%), washed in water, and stained with hematoxylin and eosin (H&E).

Each section was evaluated under a microscope and photographed in 2 or 3 different representative regions with objective lens of 4x, 10x and 20x (magnification of 40x, 100x and 200x). The images were used to measure the thickness of the mucosa, submucosa, circular muscle and longitudinal muscle, always by the same person, using the free ImageJ® software. For each image, the layer thickness was measured 3 to 5 times and averaged. The measurements were only carried out in images where all the intestinal wall could be observed.

Functional study

Four 1 cm long portions were harvested from the colon and ileum of diabetic and control animals to evaluate smooth muscle contraction (figure 2 - blue portions). The ileum was taken 2 cm proximal to the ileocecal junction; the PC 2 cm distal from cecum; DC 2 cm from anus and MC 2 cm proximal to the DC. Each sample was mounted in a vertical organ bath along its longitudinal axis, fixed to the bottom of the bath and to an isometric transducer (UGO BASILE S.R.I., Italy, Model 7004) using sewing threads. The bath was continuously aerated with carbogen (95% O2 and 5% CO2) and maintained at 37±1ºC. Tissues were stretched to an initial resting tension of 1 g and mechanical responses were recorded using a PowerLab system (ADInstruments). All tissues were washed twice, every 15 minutes, and triggered with 10µM of acetylcholine. They were then washed and allowed to stabilize for 15 min more. Each intestinal portion could then follow one of these protocols:

a) a cumulative concentration-response curve to ACh (1nM to 10mM)
b) a non-cumulative concentration-response curve to AngII: ileum, PC and MC: 300pM to 100nM; DC: 1nM to 300nM. Between each AngII concentration tissues were washed for 1 hour (every 15 min), to avoid receptor desensitization.
c) the response to a single concentration of AngII (Ileum, PC and MC: 30nM, DC: 100nM) in the absence and presence of Candesartan (10nM, AT_1R antagonist) or

PD123319 (100nM, AT_2R antagonist). Antagonists were added to the bath 20 min before AngII to evaluate the role of AT_1R and AT_2R.

The range of concentrations tested was previously determined in other studies of this research group[77].

At the end of every protocol, the contractile response to potassium chloride (KCl, 125 mM) was recorded. Finally, each portion used in the functional study was weight immediately after the functional protocol (fresh weight) and after 48 hours on a filter paper, at room temperature (dry weight). The fresh weight was used to normalize the contractile response and, together with the dry weight, to calculate the wet-to-dry ratio as an edema index. The ratio was computed according to the following equation: $WtDr = \frac{WetWeight - DryWeight}{DryWeight}$.

Statistical analysis

The GraphPad Prism® 8.1.2 (Graph Pad Prism Software, Inc.) was used for statistical analysis of data, using unpaired Student's t-test and two-way ANOVA as appropriate. The unpaired Student's t-test was used to analyze animal monitorization and macroscopic evaluation. The two-way ANOVA was used to look for interaction in the data from histological evaluation and functional data. For comparison between 2 experimental groups (CTRL and STZ) the Student's t test was used for variables with a Gaussian distribution and the Mann-Whitney test for those with a non-Gaussian distribution. Accordingly, data was expressed as mean±SEM for the former and median [95% CI] for the latter. The two-way ANOVA was used to look for interactions between experimental group and intestinal portion. In all cases, a p value of less than 0.05 was considered to denote a statistical significant difference. All results were presented as mean ± SEM and "n" refer to the number of experimental animals.

Drugs and solutions

Drugs used were tramadol (Tramal® oral suspension, 100 mg tramadol/ml, Grünenthal) and Streptozocin (S0130, Sigma-Aldrich).

Krebs-Henseleit solution consists in NaCl (José Manuel Gomes dos Santos, Portugal), KCl, CaCl2, MgSO4, glucose, NaHCO3 (all from Pancreac Quimica, Spain), NaH2PO4, Na2EDTA (both from MERCK, Germany) and ascorbic acid and is equilibrated with 95% O_2 and 5% CO_2.

In the functional studies we used acetylcholine, angiotensin II and PD123319 dissolved in distilled water, in order to obtain the desired concentrations. Candesartan was dissolved in a mixture of physiological saline and was a kind gift of Professor Fredrik Palm (Uppsala University, Sweden).

Results and discussion

Animal welfare and monitorization

Overall, this protocol started with the collection of a drop of blood (from one of the tail veins) to assess basal glycemia, before the fasting period. According to the literature, prior to STZ induction of DM, rats should not eat for 6 - 16 hours (preferably during the morning of the induction day), in order to minimize competition between the intake of glucose and STZ by pancreatic β-cells[84]. However, other authors have shown the same diabetogenic effect of STZ between fasted and fed mice[30,85]. So, considering animal welfare without jeopardizing the experimental objective, we decided for 4 hours fasting. After this period, DM was induced (STZ, 55mg/kg IP) under analgesia with tramadol, 20 mg/kg PO.

Before fasting, basal glycemia of control and STZ-induced rats was similar (105.63 ± 6.31 mg/dL vs 99.30 ± 3.29 mg/dL, respectively, n=23 $p>0.05$). STZ-induced rats had an initial glycemia of 99.30 ± 3.29 mg/dL that increased to 395.09 ± 13.80 mg/dL within 48 hours ($p<0.0001$, n=23), while control rats had an initial glycaemia of 105.63 ± 6.31 mg/dL that was roughly the same within 48 hours (111.14 ± 5.41 mg/dL; $p>0.05$, n=8). At d7 and d14, almost all STZ rats presented with a glycemia above 500mg/dL with ketone bodies, while control animals presented glycemic values of 105.57 ± 4.76 mg/dL (n=8) at the 14th day. The fact that the glucometer used considered glycemic values above 500mg/dL as “HIGH”, was one of the limitations of this study, since it was not possible to define the exact blood glucose values when above 500mg/dL. However, there was no doubt that STZ-induced rats maintained a diabetic condition through all the entire protocol.

These data shows a significant increase in the glycemic values of STZ rats only 48 hours after induction, which increased even more until d7 (499.5mg/dL at d7, $p<0.0001$ vs 48h) and then decreased by d14, although still higher than at 48h (463.6mg/dL at d14, $p<0.05$ vs d7). Control animals maintained normoglycemic values over time. The observed hyperglycemia was most probably due to STZ-induced toxicity towards pancreatic β-cells that resulted in insulin deficiency. The long-standing hyperglycemia in STZ-induced rats suggests a progressive cell damage, reaching blood glucose values four times higher than non-diabetic controls[46,66].

The parameters documented during the daily monitorization (body weight, water intake, food intake and fecal excretion) are shown in Figure 3.

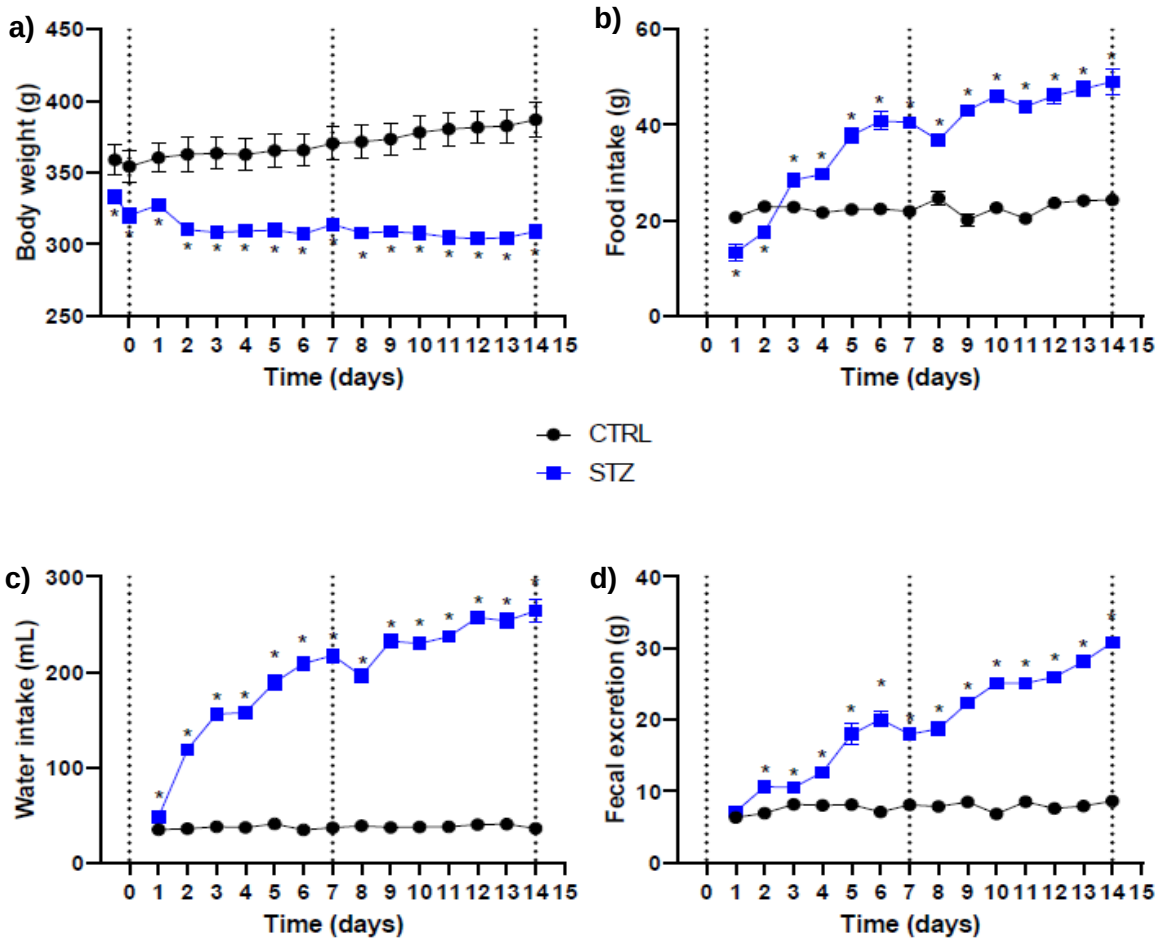

Figure 3 – Variation of body weight (a), food intake (b), water intake (c) and fecal excretion (d) in both STZ and controls rats through the protocol (- statistical difference (p<0,05)).*

In the control group, rats progressively gained weight, their weight being 7.8% ± 0.73% higher by d14 than on d0 (before fasting). Diabetic rats had a consistent weight loss that was more pronounced on d2 (less 5% compared to the day before) and then maintained that relatively stable lower weight. Their weight was 7.66 ± 1.04% lower at d14 when compared to the initial weight (before fasting). Chen and colleges[42] also observed a marked weight loss two days after DM STZ-induction in rats (then the weight remained stable), while Akbarzadeh and coauthors[86] only observed weight loss 3 days after induction. Other study showed that two weeks after diabetes induction with STZ the animals tend to recover some of the weight lost in the first days (recovering from 11% total body weight lost to approximately 5%)[46].

The food intake was significantly higher in STZ rats than controls after the 3rd day. Diabetic rats started the experimental protocol eating 13.25 ± 1.86 g in the first day, and progressively increased food consumption until the last day, when the intake was 49.08 ± 2.64 g/rat/day. The control group maintained a constant food intake during the experimental time, with a mean consumption of 22.44 ± 0.38 g/day. Both groups were always fed *at libitum*. These results are in accordance with others that showed that food intake of the STZ-induced diabetic group was

between 45 ± 5g[86] and 65.8 ± 2.6g[65], while that of non-diabetic rats was between 10 ± 2g and 31.3 ± 1.7g[65,86]. Curiously, it has also been reported that the body weight loss of STZ-induced rats is accompanied by body fat loss, leading to leptin deficiency[87,88]. Leptin is considered the "satiety hormone", as it decreases food intake. Deficiency in this hormone is seen in both diabetic patients and STZ-induced rats, thus contributing to hyperphagia[65]. The fact that diabetic rats ate less in the first day after induction (compared to controls) was also observed in other study, and may be related to animals' manipulation and discomfort associated to STZ administration[89].

Chronic hyperglycemia associated with DM results in an increase of glucose in the glomerular filtrate. Non reabsorbed glucose acts as an urinary osmotic solute, producing osmotic diuresis, polyuria and thirst[90]. The volume of urine produced by rats in our study was not measured, but it was clearly much higher than that of the control group. This became obvious because the bedding of STZ-induced rats cages was visibly wet just a day or two after they were changed, while the control animal cages were still dry one week after the last change.

Polydipsia is another classical signs of DM[21] (alongside polyuria and polyphagia), and is described in all animals models of diabetes[91]. As a consequence of osmotic diuresis, diabetic rats experience more thirst so, as expected, water intake was significantly higher in our STZ group comparing to controls that maintained a relatively constant water intake through all the experimental protocol: 37.54 ± 0.53 mL/day. Diabetic rats drank more water since d1 (48.38 ± 1.16 mL), but their water intake increased progressively throughout the protocol, reaching values 7 times higher than those of control animals at d14: 264.08 ± 12.18 mL/day. The average water intake observed in the control group was similar to that described by others (30 ± 5mL/day[86] or 41 ± 3.1mL/day[92], for example), but diabetic animals in our protocol drank more water than reported in other studies (197.57 ± 16.12mL/day in our study compared to 145 ± 5mL/day[86] or 113.3 ± 15.5mL/day[92], for example). This difference could be related to the high blood glucose levels that were consistently higher than 500mg/dL in our study (compared to 500mg/dL[86] and 333mg/dL[92]).

So, STZ-induced rats drink more water and eat more food, but still lose weight. And what about fecal excretion? To our knowledge, this is the first study to quantify fecal excretion in STZ-induced diabetic animals. Non-diabetic animals maintained a relatively stable fecal excretion during the entire experimental period (7,75±0.18 g/day/rat), whereas diabetic rats gradually increased their fecal excretion, reaching values 4 times higher than those obtained in the first day (d14: 30.79 ± 0.73g/rat; d1: 7.11 ± 0.34g/rat; $p<0.0001$; $n=16$). Besides the increase in quantity, the fecal pellets also presented visual qualitative differences. The fecal pellets from the diabetic group were well formed but were bigger, larger and darker. Cuervas-Mon and collaborators also observed some differences in the feces of STZ-induced diabetic

rats, but they described them as being thick and amorphous[93]. We have no explanation for this interesting finding by now but it could eventually be ascribed to the polyphagia and the reported below distension of the intestinal wall. Further studies will embrace this question.

Macroscopic evaluation

Comparing to control animals (Figure 4a), all parts of the intestines of STZ rats (Figure 4b) seemed enlarged, a description already done by Cuervas-Mon and collegues[93]. Also, upon the opening of the abdomen of STZ-induced rats it was easily perceived that the intestine was distended and that there was almost no abdominal fat (Figure 4b). This observation is in agreement with other authors, that concluded that in this animal model visceral fat is reduced to half only 7 days after STZ induction[87]. Moreover, in those animals it was possible to observe an extremely dilated cecum that produced a "mass effect", pushing the intestine to the side. Curiously, the bladder of these diabetic rats was also distended.

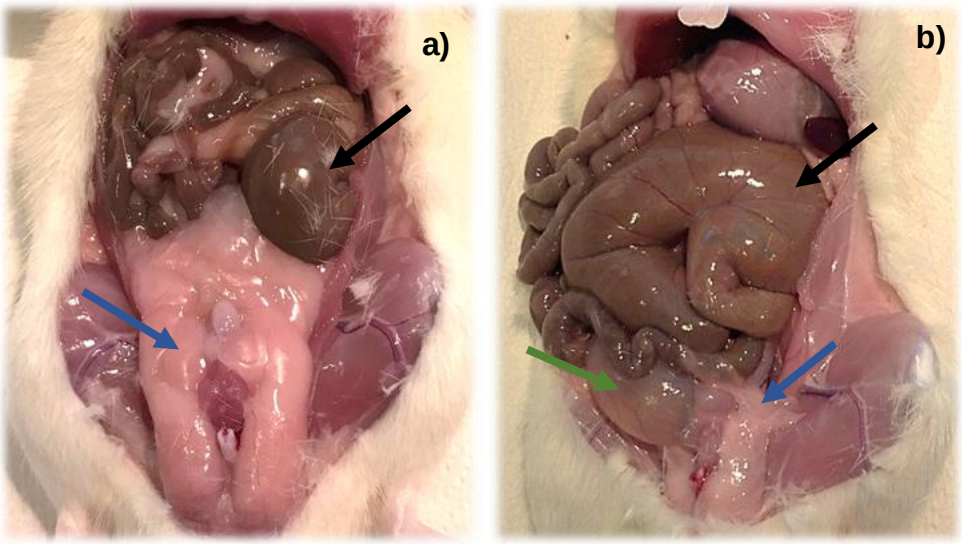

Figure 4 – Macroscopic observation after abdominal cavity opening of control (a) and STZ (b) rats (representative images). The most preponderant findings in diabetic animals are an enlarged cecum (black arrows), loss of visceral fat (blue arrows) and distended bladder (green arrow).

We further observed that the colon length was significantly higher in diabetic animals compared to the control group (25.75 ± 0.77cm, n=14 vs 19.63 ± 0.47cm, n=12, $p<0.05$) (Figure 5). Since some animals were heavier than others, we measured colon length *per* body weight and the difference between the two groups was maintained (Figure 5c). To our knowledge, this is the first study showing that, comparing with control animals, the colon length of STZ animals increased in such a premature phase of diabetes (only 2 weeks after induction). Colon length was also assessed in a previous study that used rats 8 weeks after STZ induction; in this experimental protocol the colon of diabetic rats was on average 4 cm longer than that

of control animals[65]. Some authors found an increase in the length of the small intestine of diabetic rats, 3, 9,[55] and 10 weeks after STZ induction (not seen in diabetic animals treated with insulin, compared to controls)[58], while others only described an increase in ileum lenght[94]. In our study we decided to only measure colon length, since it was difficult to find an exact macroscopic difference that would allow us to accurately distinguish portions of the small intestine (duodenum, jejunum and ileum).

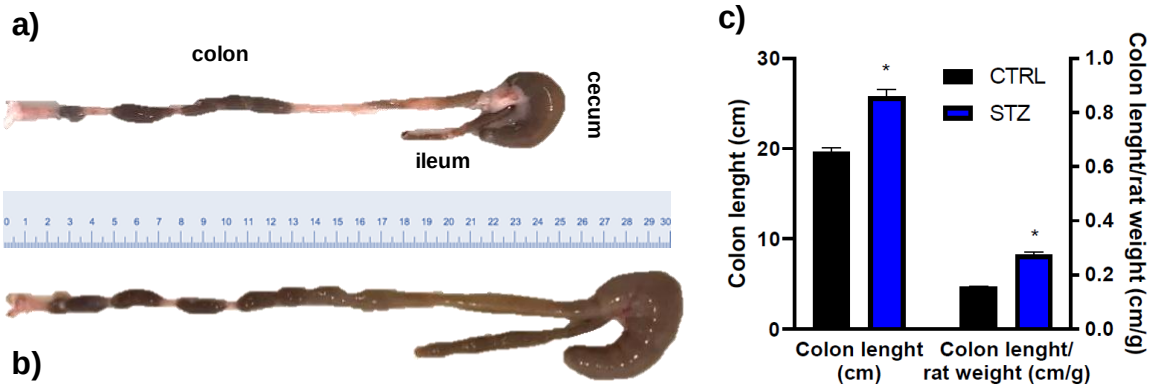

*Figure 5 – Representative images of the colon length of control (a) and STZ (b) rats and quantitative analysis of colon length (c-left y axis) and colon length per rat weight (c-right y axis). Values are mean±SEM; * Statistical difference (p<0,05).*

Additionally, we also measured the circumferential perimeter of the intestinal portions, and found out that it was significantly higher in the rectum (16.91 ± -0.81mm vs 12.13 ± 0.46mm, p<0.001), middle colon (15.45 ± 0.58mm vs 11 ± 0.46mm, p<0.0001) and ileum (12.55 ± 0.31mm vs 9.38 ± 0.32mm, p<0.0001) of STZ-induced rats (*n*=11) compared to non-diabetic rats (*n*=8) (Figure 6). Previous studies have shown higher circumferential lengths in the distal colon (6 weeks after induction)[68] and ileum (35 days[94] and 8 weeks[46] after induction) of STZ-induced rats. One of these studies revealed that the ileum was the intestinal portion that showed the greatest difference regarding circumferential length between diabetic rats and controls[94], an observations that our data do not corroborate (p>0.05 for interaction between experimental group and intestinal portion).

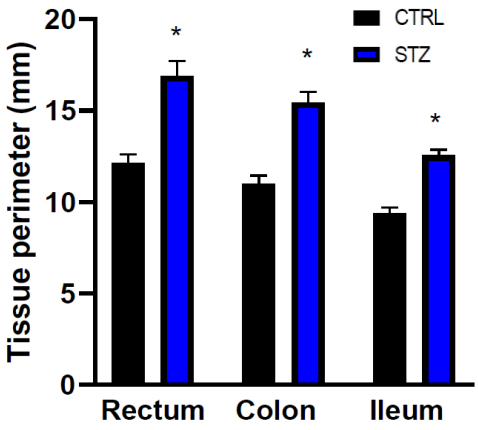

*Figure 6 – Tissue circumferential perimeter of intestinal portions of STZ versus controls rats: rectum, colon and ileum. Values are mean±SEM; * Statistical difference (p<0,05).*

A large part of the intestine, from the anus to 10 cm proximal to the ileocecal junction (ileum), was weighed with its content. The relative weight of the whole intestine segment studied (with fecal content) was higher in STZ-induced animals than in controls (2.69 ± 0.10 g/g of body weight, *n*=21 vs 1.80 ± 0.05 g/g of body weight, *n*=12; p<0.0001). This increase was also observed when we looked at the individual intestinal segments cleaned of fecal content (Figure 7). The 2-way ANOVA results showed an interaction between the experimental group (Control or STZ) and the intestinal segment (p<0.0001); the increase in relative tissue weight was more marked in the cecum than in the colon or ileum. This quantitative data is in accordance with our visual observation of the marked dilatation of the cecum in STZ-induced animals (Figure 4). The relative fecal content weight was also higher in STZ-induced animals than in controls (7.10 ± 0.15 g/g of body weight, *n*=21 vs. 2.66 ± 0.11 g/g of body weight, *n*=12; p<0.0001). To our knowledge, this is the first time that the weight of intestinal content is reported in STZ rats.

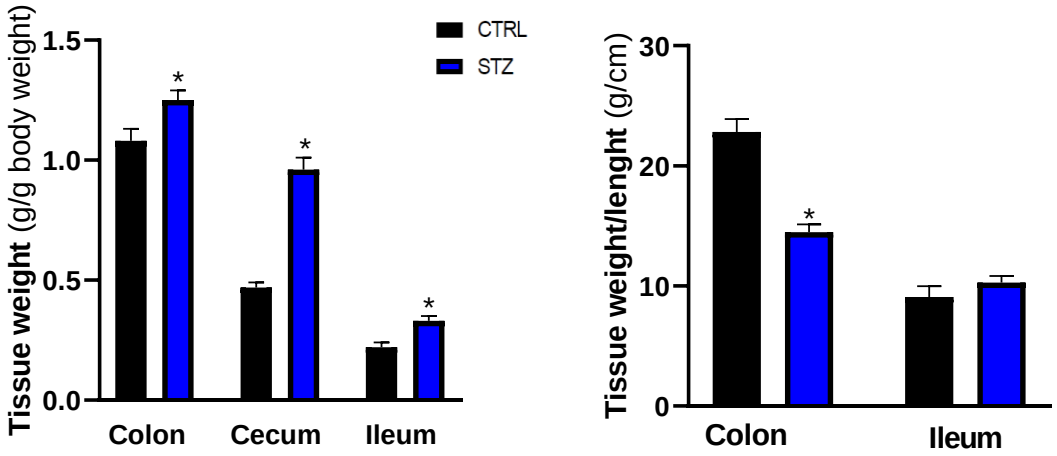

*Figure 7 - Relative weight of intestinal segments of control and STZ-induced animals, expressed as g of colon, cecum or ileum / g of body weight (left) or as g of colon or ileum / cm (right) Values are mean±SEM; * Statistical difference (p<0,05).*

So, our data shows that just 2 weeks after induction, STZ-induced rats already present intestinal macroscopic alterations, namely increased length, perimeter and weight, which are also accompanied by an increased weight of the intestinal content. Forrest *et al*[65] also found that dry colon weight increased significantly in diabetic animals compared to the corresponding control group, and suggested that this could be related to increased colon length, since weight *per* length did not differ between the two experimental groups. This hypothesis is in agreement with a previous study that also described the existence of a greater intestinal mass in diabetic animals, associated with changes in intestinal diameter and length[95]. Zhao and colleagues, in a more detailed study, concluded that until the 4th day the ileum weight of diabetic rats was similar to that of controls, but that a difference was progressively more marked from the 1st to the 4th week after STZ induction[66]. Other authors have also found that colon[58,96] and ileum[58] weight was significantly higher in diabetic animals (treated or not with insulin) when compared to controls. Considering that the intestine length of insulin-treated diabetic rats was not different from that found in controls, but the weight was still higher, this study contradicts Forrest *et al.* suggestion, since it shows that intestinal weight increase might not be related to length increase[58,96]. That was also the conclusion of another study that showed an weight-to-length ratio increase in STZ-induced rats, but not in diabetic animals rats treated with insulin[97]. Intriguingly, our results show a lower weight-to-length ratio in the colon of STZ-induced rats when compared with controls, whereas ileum weight-to-length ratio remained similar for both experimental groups (Figure 7). A possible explanation for the intestinal wall weight increase could be related to the tissue water content, which has been reported to be higher in diabetes[95]. However, we did not observe any difference between control and STZ-induced animals in the wet-to-dry ratio of the intestinal segments studied (Figure 8), in line with another study that was not able to establish a relationship between intestinal weight and edema in diabetic rats[65].

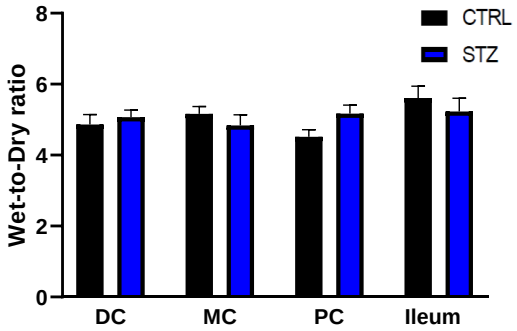

Figure 8 - Wet-to-dry ratio of four intestinal segments: distal colon (DC), medium colon (MC), proximal colon (PC) and ileum (I) of control and STZ-induced rats. Values are mean±SEM.

Taken together, our data indicate that just 2 weeks after STZ administration, the intestines of diabetes rats increase in weight, longitudinal length and circumferential perimeter. For the time being, there is no clear answer as to which mechanisms are triggering this increase. The

results presented in this dissertation suggest that it is not a consequence of tissue edema and that it may be more or less important, according to the intestinal section considered.

Also interesting in this context is the view that intestinal smooth muscle cells are plastic and adapt to functional demand, by remodeling[65]. Jervis and collaborators suggested that the enlargement of the intestine represents an adaptation to polyphagia of diabetic animals. In a study with diabetic rats induced by alloxan, these authors described that abdominal distention was due to a large amounts of feces, concomitant with an enlargement of the diameter and length of small intestine and colon[98]. The same authors also described that the intestine of diabetic animals were heavier than those from control animals, especially the small intestine[98]. Curiously, other causes of polyphagia like lactation[99,100] or hypothalamic lesions[101,102], may also induce GI enlargement - similar to the one seen in diabetes. However, this hypothesis was contradicted by another study that showed that when the food intake of diabetic rats was matched to that of controls (avoiding polyphagia), the intestinal mass of diabetic animals continue to be higher[66]. Therefore, it seems prudent to assume that only part of the intestinal growth will depend on food consumption. One way to explain how polyphagia can promote intestinal growth is through the increased expression of glucagon-like peptide 2 (GLP-2), an intestinal trophic hormone. It has been demonstrated that there is a relationship between GLP-2 and the enlargement of the GI tract in diabetic animals[103]. It seems that the increase of nutrients in the luminal content of diabetic animals nearly doubles the plasma concentration of GLP-2 and enteroglucagon, which stimulates intestinal epithelial proliferation that precedes intestinal growth[103,104]. But besides food intake, what else could explain intestinal mass increase in diabetic animals? It has been proposed that the megacolon and ileum may be secondary to DAN, as it seems that the reduction in the number of neurons can cause hyperplasic and hypertrophic changes in intestinal walls[55], thus opening new research hypothesis.

Histology

The results of the microscopic evaluation of the intestines of STZ-induced animals were compatible with the previously described macroscopic data, showing an increase in the thickness of the intestinal layers of the ileum, PC, MC and DC (Figure 9). The histopathological measurements were also in line with the microscopic observations, indicating that the thickness of the four intestinal segments studied was higher in diabetic animals (Figure 10 and 11). The increase was similar for all the intestinal segments, as 2-way ANOVA showed a non-significant ($p=0.1681$) interaction between experimental group and intestinal segment.

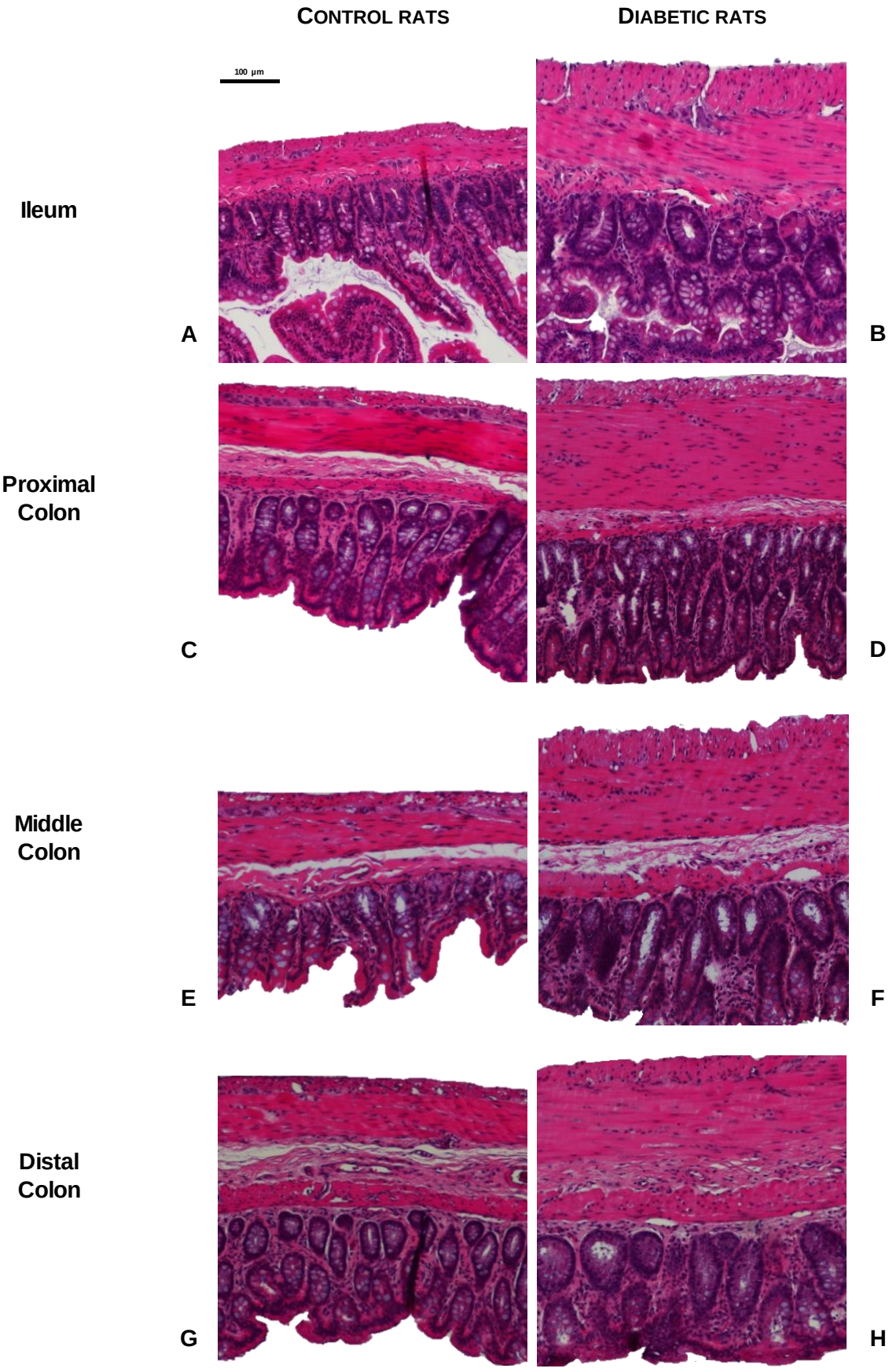

Figure 9- Representative photographs of H&E-stained cross-sections of the ileum (A, B), proximal colon (C, D), middle colon (E, F) and distal colon (G, H) of control (A, C, E, G) and STZ-induced (B, D, F, H) animals. The scale bar (100 µm) is valid for all images.

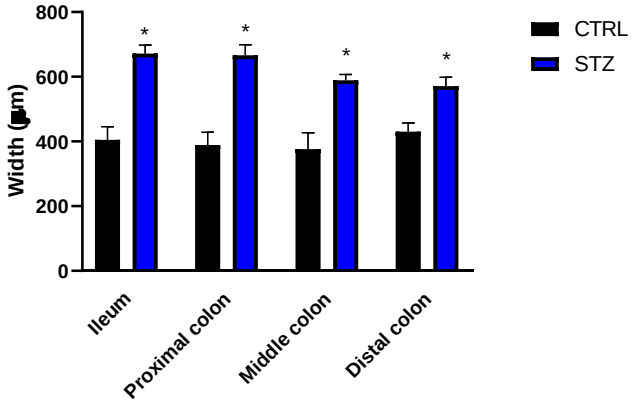

*Figure 10 - Wall thickness (µm) of the intestinal segments (ileum, proximal colon, middle colon and distal colon) of control and STZ-induced animals. Values are mean±SEM; * Statistical difference (p<0,05).*

Also, we noticed that the increased thickness was present in all the intestinal layers of the four segments, except on the submucosa of the proximal and distal colon and on the mucosa of distal colon (Figure 11). The 2-way ANOVA showed an interaction between the experimental group (control vs STZ) and the intestinal layers (longitudinal muscle, circular muscle, submucosa and mucosa) for the ileum (p=0.0058), proximal colon (p=0.0002), middle colon (p=0.0027) but not for the distal colon (p=0.1109).

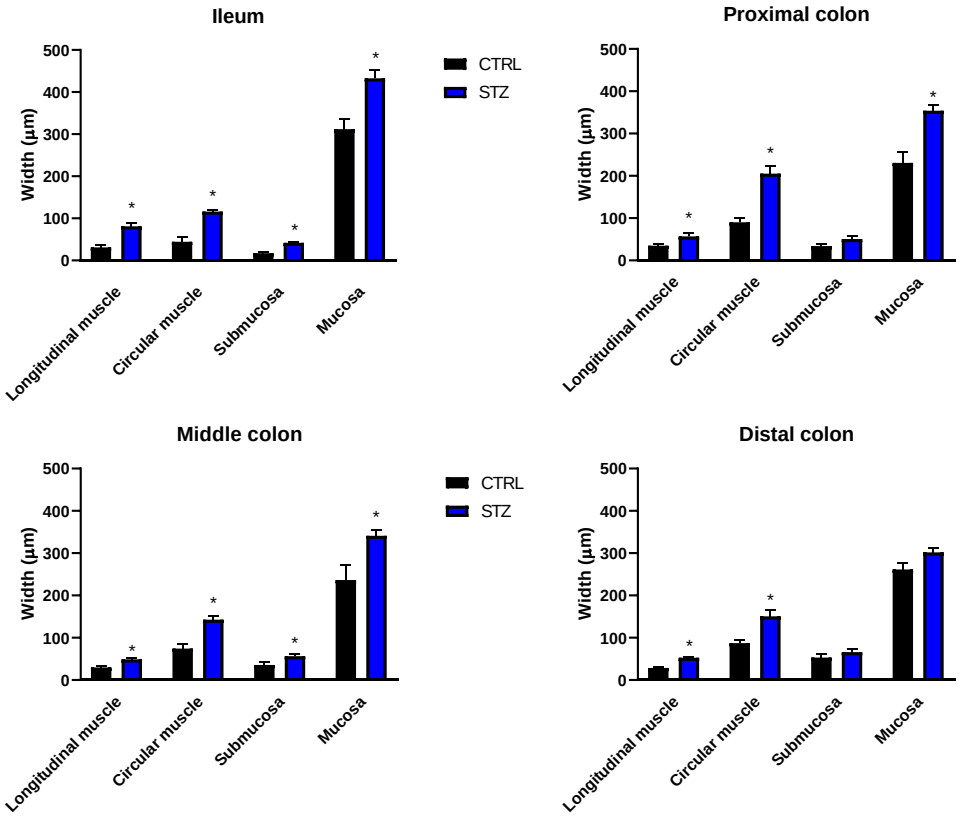

Figure 11 - Wall thickness (µm) of the different layers of the intestinal wall (longitudinal muscle, circular muscle, submucosa and mucosa) of ileum, proximal colon, middle colon and distal colon of control and STZ-induced rats. Values are mean±SEM; Statistical difference p<0.05 vs correspondent control.

Our study uncovers several early structural alterations in the intestines of STZ-induced rats. Indeed, there are no previous histopathological data on the colon of STZ-induced rats just 2 weeks after induction. A previous study, carried out with STZ-induced rats, showed similar results to those that we now present, but only in the ileum. In that study, 2 weeks after diabetes induction, the ileum presented increased villus length, crypt depth, mucosa (layer with the highest increase), submucosa, muscle layer and total wall thickness[66]. The same authors also studied the histological characteristics of the middle colon, but 4 and 8 weeks after induction, when they report an increase in the thickness of the mucosa, submucosa and muscle layers of the intestine of diabetic animals compared to the control group[67]. Another study with the same animal model, but 56 days after induction, also found an increase in the thickness of the ileum layers, but no statistical difference in the thickness of the colon[42]. The fact that the difference between diabetic and control animals is gradually less preeminent in the distal direction is also compatible to what was described by Fregonesi *et al*..They showed that there is a differential effect of diabetes in the GI tract, with the distal segments being affected last, which appeared to be directly dependent on food consumption. In a group of diabetic rats feed *at libitum*, the ileum mucosa was significantly thicker than the controls, but this difference was not found in the diabetic group with restricted feeding, as thickness of the mucosa was actually reduced compared to controls, suggesting that hyperphagia is essential to mucosal proliferation. One of the mechanisms responsible for increased thickness is suppression of apoptosis. Apoptosis of the intestinal mucosa seems to decrease in the first week after STZ injection, returning to normal 3 weeks after, suggesting a transient effect. However, the suppression of apoptosis in the early stages associated with the gradual increase of food intake may be an important contributing factor to initial intestinal mucosa increase[89]. As described earlier, GLP-2 seems to have a trophic action on the intestinal epithelium, an effect enhanced in diabetic animals. In addition, GLP-2 also has the ability to inhibit apoptosis in small intestine[105], thus contributing to its enlargement. In a different study, insulin administration prevented the marked increase in the ileal epithelial cells proliferation rate of diabetic rats, resulting in reduced intestinal mucosal growth compared to non-treated diabetic animals[96]. Part of the insulin effect could be related to glycemia control, and subsequent AGE/RAGE normalization. As explained earlier, AGE/RAGE accumulation in the mucosa may be related to increased mucosa thickness[42], besides affecting digestive function by changing the properties of the intestinal epithelial cells and digestive enzymes activity[106]. Regarding the muscular layers analyzed, previous work demonstrated an extensive remodeling of the diabetic distal colon associated with an increased production and accumulation of collagen type I and AGE. The collagen fibers accumulate mostly around and between SMC, causing stiffening of the diabetic colon and decreased resting compliance. In addition to extracellular matrix remodeling, authors also found SMC hypertrophy, with increased number of contractile

protein actin and myosin[68]. Increased production of AGE and RAGE was also found in ileum muscular layers, an observation that was related to its increase in thickness[42]. So, the increase in the thickness of the diabetic intestinal wall seems to be mainly related with: a) mucosa proliferation (due to increased food intake, increased expression of GLP-2, accumulation of AGE and suppression of apoptosis and b) increased muscle layers (due to AGE and collagen type I accumulation and SMC hypertrophy).

Functional study

During peristaltic contractions the intestine is physiologically subjected to dimensional changes that allow its content to be propelled[94]. The morphological alterations observed in the intestinal wall of STZ-induced rats are certainly associated with changes in mechanical properties that may modify contractile function. The stiffening and modified stress distribution of the intestinal wall affects elastic properties of the GI tract, changing tissue distension while filling and shortening while emptying[97]. Also caused by the stiffening of the matrix and wall remodeling, SMC and neurons compression may affect cellular mechanotransduction mechanisms[68], resulting in different perception and motility. However, the association between the remodeling and motility disorders detected in diabetic patients is not completely understood[67].

The functional study that we conducted initially suggested that there were no changes in the intestinal function of STZ-induced rats just 2 weeks after induction, since the contractile response to 125 mM KCl was similar between control and diabetic rats, in the four intestinal segments studied (Figure 12).

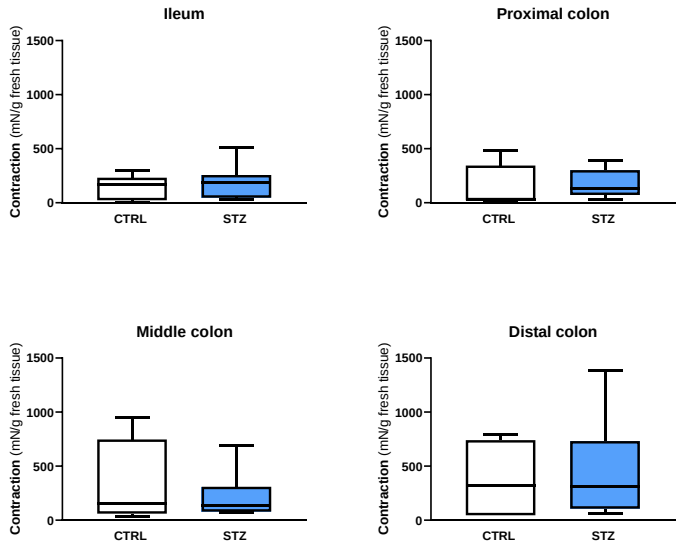

Figure 12 - Contractile response (mN/g) to KCl 125 mM in the ileum, proximal colon, middle colon and distal colon of control and STZ-induced rats. Values are median and 95% confidence limits.

Reactivity to acethylcholine

Acetylcholine is the most common neurotransmitter of the ENS being responsible for smooth muscle contraction and enteric motility[44,49]. In order to determine possible alterations in the cholinergic system, we decided to study the responsiveness of the 4 different portions of the intestine (from ileum to distal colon) to exogenous acetylcholine in an early phase of STZ-induced diabetes. In all portions, ACh caused a similar concentration-dependent contraction in both control and STZ-induced animals (Figure 13). The contractile response to ACh between control and STZ animals had similar E_{max} and EC_{50} values either when expressed as mN of force *per* gram of fresh tissue or as % of the correspondent contraction to 125 mM KCl (Table 1). The only difference noted was a lower E_{max} for ACh in the ileum of STZ-induced animals (Table 1).

Previous studies using rat ileum have shown a depressed contractile response to exogenous acetylcholine after 30 days [62,107] and 6 months of diabetes induction by STZ [62]. This change does not appear to be related to damage in the cholinergic innervation or alteration in acetylcholinesterase activity, since the expression of cholinergic nerves and the acetylcholinesterase histochemistry showed similar results in diabetics (20 weeks after STZ induction) and control animals[93]. Concerning the colon, only one study has previously evaluated the colonic response to exogenous ACh, and the authors did not find differences between control and STZ-induced rats 30 days after diabetes induction[107]. However, in a genetic model of DM, after a longer period of disease (60 weeks) the contractile response to carbacol in the proximal colon was lower than that of controls, while the response in the distal colon appeared to be unaffected[48]. Thus, it seems that cholinergic activity in the GI tract may depend on the duration of diabetes, but according to some studies, even after a long period of illness (6 months after STZ induction in rats) the exogenous ACh response showed only a lower E_{max}, with no change on the EC50[62]. So, the same authors suggested that alterations in diabetic intestinal motility are probably related to changes in smooth muscle layers and non-cholinergic innervation, since they were unable to find signs of cholinergic denervation. This hypothesis is also consistent with the observations of Cuervas-Mon and colleagues[93], who revealed no alteration in the density of cholinergic nerves 20 weeks after STZ induction, but a reduction in norepinephrine-containing nerves.

In our study, two weeks after induction of diabetes with STZ, the colonic response to exogenous ACh was similar between controls and STZ-induced animals. On the other hand, in the ileum we observed a decrease in the response when results were expressed as % of the correspondent contraction to 125 mM KCl. This finding may be related to the fact that the damage to the ENS seems to occur first in the proximal segments of the GI tract[56].

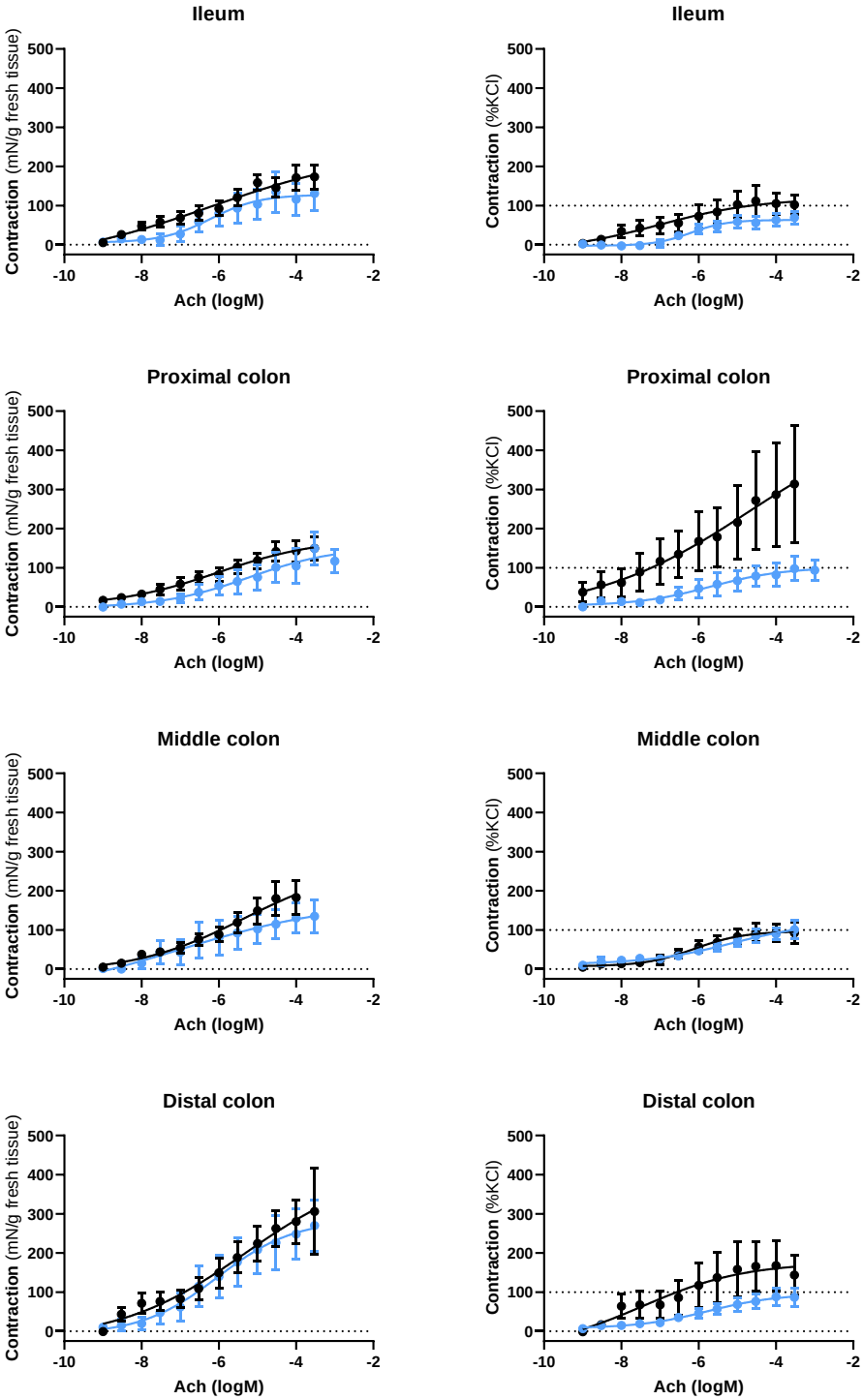

Figure 13 – Concentration-response curves to Ach in the ileum, proximal colon, middle colon and distal colon of control (n=6-7) and STZ-induced rats (n=10). Data is expressed as mN of force per g of fresh tissue (left) or % of the response to KCl 125 mM (right). Values are mean±SEM.

Table 1 - Effect of ACh evaluated by the E_{max} and EC_{50} values in the ileum, proximal colon, middle colon and distal colon of control (n=6-7) and STZ-induced animals (n=10).

	Ileum	Proximal colon	Middle colon	Distal colon
Control				
E_{max} (mN/g)	165.9 [116.4-216.0]	141.0 [116.7-278.5]	184.7 [68.95-378.8]	313.4 [176.2-823.1]
E_{max} (%KCl)	223.6 [70.67-1302.0]	38.49 [34.13-134.8]	106.8 [50.69-129.3]	81.21 [56.07-404.5]
EC_{50} (µM)	0.85 [0.32-3.53]	1.15 [0.22-14.70]	3.41 [1.1-4.8]	2.74 [0.94-7.47]
STZ				
E_{max} (mN/g)	79.06 [34.65-338.9]	158.0 [75.0-569.5]	143.6 [86.56-411.3]	271.7 [163.6-370.9]
E_{max} (%KCl)	63.49 [19.83-113.7]*	99.95 [66.06-248.6]	89.85 [57.86-143.2]	69.22 [26.93-199.3]
EC_{50} (µM)	0.82 [0.27-1.87]	114.0 [8.31-3408]	18.96 [0.87-75.7]	2.94 [0.28-142.0]

Values are median[95% confidence limits]; * p<0.05 vs correspondent control.

Reactivity to angiotensin II

There is evidence for the involvement of the RAS in GI fluid and electrolyte homeostasis, smooth muscle activity and inflammation, so the manipulation of this system can be beneficial in the control of various GI pathologies[72]. As there are no previous studies on the RAS function in the diabetic GI tract, we decided to evaluate the effect of Ang II in the colon of STZ-induced rats, in order to understand if there are any changes compared to controls.

In all the three portions of colon studied, Ang II caused a concentration-dependent contraction, both in control and STZ animals (Figure 14). The contractile response to Ang II was lower (but with the same EC_{50}) in the proximal and middle colon of STZ-induced animals, when the contraction was normalized to the tissue weight (Table 2). Interestingly, the maximum response in the distal colon was similar between control and STZ-induced animals, but the distal colon of diabetic animals was more sensible than that of controls (Table 2).

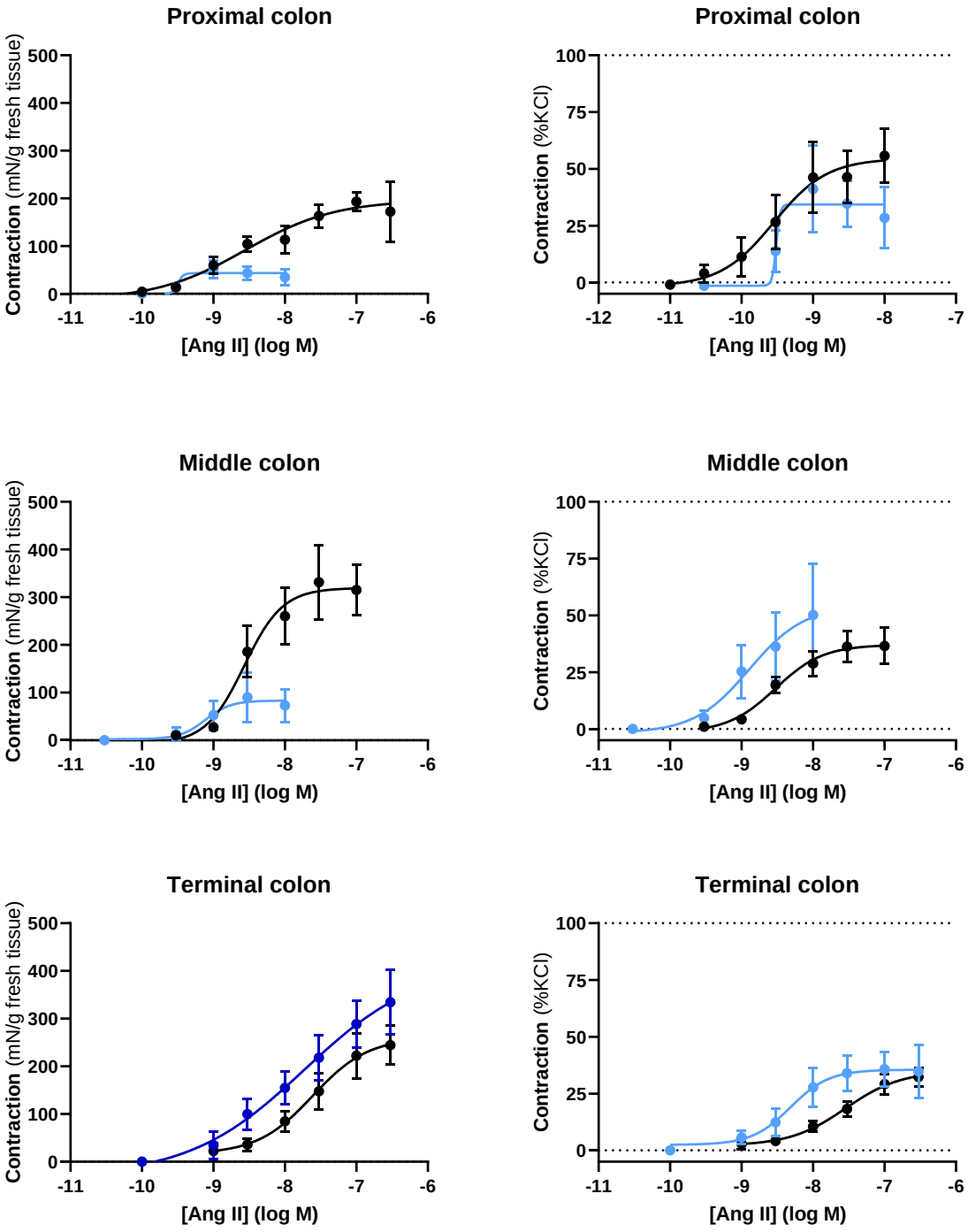

Figure 14 – Concentration-response curves to AngII in the ileum, proximal colon, middle colon and distal colon of control (n=5-8) and STZ-induced rats (n=5). Data is expressed as mN of force per g of fresh tissue (left) or % of the response to KCl 125 mM (right). Values are mean±SEM.

Table 2 - Effect of Ang II evaluated by the E_{max} and EC_{50} values in the proximal colon, middle colon and distal colon of control (n=5-8) and STZ-induced animals (n=5).

	Proximal colon	Middle colon	Distal colon
Control			
E_{max} (mN/g)	181.5 [136.0-297.0]	276.6 [246.4-451.1]	344.4 [222.4-433.5]
E_{max} (%KCl)	97.28 [31.96-117.1]	32.99 [22.97-57.12]	38.30 [24.21-54.52]
EC_{50} (nM)	1.10 [0.36-2.12]	3.80 [1.95-4.76]	40.50 [17.08-309.3]
STZ			
E_{max} (mN/g)	50.46 [15.32-78.15]*	100.6 [22.86-163.5]*	263.5 [165.0-415.9]
E_{max} (%KCl)	33.13 [20.46-56.57]	41.21 [18.88-113.2]	40.29 [18.37-61.39]
EC_{50} (nM)	0.59 [0.35-14.93]	2.60 [0.89-7.81]	4.17 [0.84-8.38]*

Values are median [95% confidence limits]; * p<0.05 vs correspondent control.

Knowing that the differences observed in the contractile response to Ang II could result from an imbalance between AT_1R and AT_2R responses, we further characterized the response to Ang II in both control and STZ-induced rats.

The contractile response to Ang II was antagonized by Candesartan (10 nM), an AT_1R antagonist, in the three colonic segments of both control and STZ-induced rats (Figure 15). Differently, in control animals, PD123319 (AT_2R antagonist, 100nM) increased the response to AngII in all colonic segments (Figure 15). However, in STZ-induced animals the AT_2R antagonist decreased the response to Ang II in the proximal colon but did not modify the contraction to Ang II in the middle or distal colon (Figure 15).

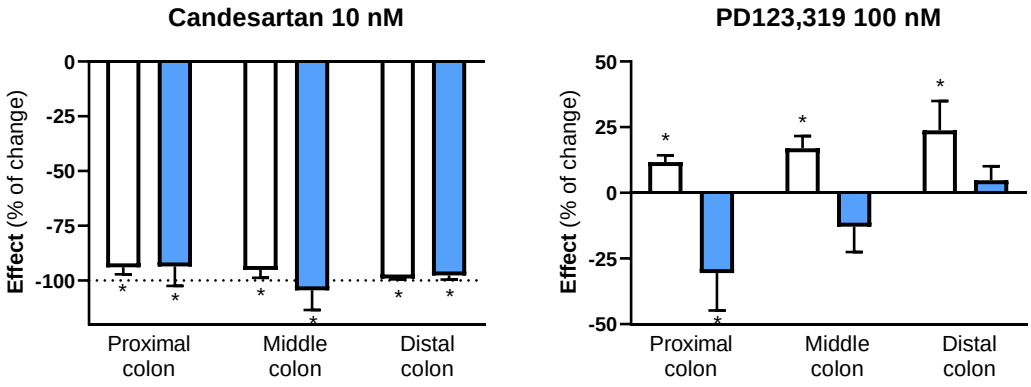

Figure 15 - Effect of candesartan (AT_1R antagonist, 10 nM, left), and of PD123,319 (AT_2R antagonist, 100 nM, right) on the response to Ang II in the proximal, middle and distal colon of control (white bars, n=5-7) and STZ-induced (blue bars, n=4-6) rats. Values are mean±SEM. p<0.05 vs the correspondent response to Ang II in the absence of the antagonist.*

To our knowledge, this is the first time that the response to Ang II is studied in the colon of diabetic animals. In this protocol, Ang II was always able to induce contractile responses mediated by AT_1R activation, a response that was already expected since it was previous demonstrated in the intestine by our research group[75,77] and others[108] and the AT1R is the main effector of the contractile response to Ang II in several tissues[108]. The maximum response in control animals was similar to that found in another study, where they described the concentration close to log 10^{-7} M as being responsible for the maximum effect on the colon[109] and small intestine[78]. They also described that with higher concentrations of Ang II, the response tends to fade, which was also observed in our study, probably due to receptors desensitization[78]. In order to avoid this, we washed our tissues for 1h between each concentration of Ang II tested, and we stopped performing the concentration-response curve when we got 2 similar responses or a lower response to a higher concentration than the previous one.

As in previous studies, in the experiments that we now report, the effect of AngII was antagonized by candesartan in all parts of the colon, thus confirming that the contractile response is mediated by AT_1R[74,75,77,78]. Regarding AT_2R activation, these receptors are known to counterbalance Ang II-mediated AT_1R effects, and that was observed in colonic segments from control rats. But, in the MC and DC of STZ-induced rats, AT_2R antagonism with PD123319 had no effect on Ang II-mediated contraction, suggesting that in the STZ-diabetic rat middle and distal colon AT_2R are not functionally opposing AT_1R-mediated contraction. This data is in line with data from our group with a rat experimental model of colitis, where we found that AT_2R-mediated Ang II-induced relaxation was also blunted[75]. However, in that previous study, rats with experimental colitis showed marked signs of inflammation that does not seem to be the case in the present study, since our STZ-induced diabetic rats do not present any signs of inflammation 2 weeks after induction. Interestingly, the PC of STZ rats seems to behave differently than the MC and DC. In that proximal colonic portion, the contractile effect of Ang II was decreased in the presence of PD123319, pointing to a contractile effect mediated by the AT_2R. The AT_2R-mediated contractile effect is not common, but has previously been described in a vascular study[110]. However, another study did not find a detectable expression of AT_2R in the PC in mouse colon using RT-PCR, but the authors proposed that some pathological conditions (such as inflammation) could modify the tissue localization of Ang II receptors[74]. This same idea was reinforced by other authors, who consider it possible that the distribution and action of Ang II receptors may change whenever GI functional disorders and other pathophysiological conditions are present, but further investigation in needed to understand how this affects Ang II response[73,78,74,109]. Another intriguingly point is the merge of our data from the concentration-response protocol with that of the receptor blockade protocol. Indeed, if in the STZ-diabetic rats the AT_2R is either nonfunctional or mediating contraction, the overall

response to Ang II in the colon of STZ-induced rats should be higher than the correspondent effect in controls. Even more unexpectedly, the difference in Ang II-mediated contraction between controls and STZ-induced rats is more marked in the PC (28% of the control response) than in the MD or DC (36% and 77% of the control response). With this in mind, one would be expecting a more relevant relaxant role of the AT_2R in the PC of STZ-induced rats, which was not observed at all. We have no explanation for these results so far but future experiments can be designed to ascertain this issue.

So, the results presented in this functional study suggest a loss of contractile force in the PC and MC (but not in the DC) of STZ-induced rats, in response to Ang II. In the rat colon, Ang II receptors can be found mostly in the muscularis (in the SMCs), but appear to exist in the mucosa in a small percentage. It was previously described that AT_1R and AT_2R were also located on the myenteric nerves in human colon while in the guinea pig they only found AT_1R[73,109]. The predominant receptor is AT_1R, but a small number of AT_2R were also observed[111]. Considering receptor location, the altered AngII response seen in STZ rats could be associated with the structural alterations observed, loss of neurons, mostly in the myenteric plexus, and alterations in the mechanistic pathways involving Ang II contraction, being DAN the main cause of this loss. Ang II activates both receptors in the smooth muscle cells but also presynaptic receptors in other cells crucial for colonic function[75,108,112] and this intricate network has been reported to be altered in the diseased colon[75,112,113]. The maintenance of the response of the DC can be related to the fact that the distal segments of the GI tract are the last ones to be affected by diabetic complications[56].

Conclusions

This study shows, for the first time, that as early as 2 weeks after induction of DM with STZ, the induced rats exhibit higher fecal excretion and marked structural differences such as distension, increased length and circumferential perimeter of the colon, corroborated by increased thickness along the intestinal wall. The well-known typical signs of T1DM (polydipsia, polyuria, polyphagia and loss of body weight) were also present. Furthermore, our study shows that early STZ-induced diabetes is also associated with functional alterations that differentially affect the regulatory mechanisms of colonic contractility. Although at this early stage there were no differences in the response to KCl or Ach between control and diabetic rats, it was observed a lower contractile force in the PC and MC (but not in the DC) of STZ-induced rats, in response to Ang II. These alterations in the reactivity to Ang II are associated with an imbalance between the function of AT_1R and AT_2R although other mechanisms seem to play a role. The structural differences and altered reactivity observed could underline the enteric dysmotility seen in diabetic patients and should be taken into consideration in future studies.

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