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The Effects of Early Diabetes on Duodenal Alterations in the Rats

Efectos de la Diabetes Temprana sobre las Alteraciones Duodenales en Ratas

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SUMMARY: Diabetes mellitus (DM) mainly affects functional changes in the duodenum, which plays an important role in the digestion and absorption of food. The impairment of duodenal function contributes to malnutrition, abdominal bloating and pain in diabetic patients. Thus, this study aimed to investigate the histological alterations and quantitative measurements of duodenal structures in the early stage of streptozotocin (STZ)-induced diabetic rats. Eight male Sprague–Dawley rats were divided into three control and five diabetic rats. Diabetes was induced by a single intraperitoneal dose of 60 mg/kg STZ. After four weeks of diabetic induction, the duodenum was prepared for histological study and morphometric analysis. In diabetic rats, there were deformed villi with disrupted surface epithelium and mildly distorted shapes of crypts, together with an increase in villus height and crypt depth. The epithelial cells detached from their underlying basement membrane. The goblet cells decreased in number, whereas an increased number of Cellula panethensis (Paneth cells) with pale-stained eosinophilic granules occurred in the DM group. A diabetic thickened submucosal layer was observed as enhanced duodenal glands (Brunner's glands) hypertrophy and collagen accumulation. These findings indicated that histopathologic lesions of the duodenum developed in the early stage of diabetes. The destruction of villi, crypts, and epithelium may affect digestion and absorption. The structural changes in goblet and Cellula panethensis and duodenal glands may be associated with malfunction to protect duodenal mucosa from bacteria and stomach acid. These conditions can worsen the quality of life in diabetic individuals, leading to complications such as maldigestion, malabsorption, and duodenal ulcer.

KEY WORDS: Duodenum; Early diabetes; Streptozotocin.

INTRODUCTION

The small intestine is a major organ involved in the digestion of food and absorption of nutrients and minerals. The duodenum, which is the first part of the small intestine and connects to the stomach, is a primary site in the digestion and absorption of food. In the epithelium of the duodenal mucosa, enterocytes or columnar absorptive cells mainly function in food digestion and absorption (Jaladanki & Wang, 2011). To protect the mucosa of the duodenum from bacteria and stomach acid, goblet cells are present in the epithelium of intestinal villi and crypts, Cellula panethensis at the base of crypts, and duodenal glands (Brunner's glands) in the submucosa. Indeed, goblet cells secrete mucin to lubricate the luminal surface of the duodenum (Kim & Khan, 2013), and Cellula panethensis produce lysozyme to kill bacteria (Yu *et al*., 2016). Moreover, duodenal glands secrete mucus and alkaline (bicarbonate ion) to neutralize acid from the stomach (Collaco *et al*., 2013).

Diabetes mellitus (DM), a chronic metabolic disease with hyperglycemia, is associated with abnormalities in structures and functions in the gastrointestinal tract, especially the small intestine (Krishnan *et al*., 2013). Alterations in the microvasculature in the small intestinal villi, including a decreased diameter of blood vessels and a reduced number of endothelial fenestrae, have been found in diabetic rats (Tahara & Yamamoto, 1988). Furthermore, DM causes villus edema and mucosal ulcers of the small intestine with abdominal bleeding and pain in diabetic

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patients (Zhong *et al*., 2016). Finally, the abnormal structures of the small intestine lead to impairments in digestion, absorption of food, and protection of the epithelium, which contribute to malnutrition and intestinal ulcers, particularly duodenal ulcers in diabetic patients (Zhao *et al*., 2017; Kalaichelvi & Iyyadurai, 2018). Nevertheless, the effect of DM on duodenal histology is still unclear. Therefore, the histomorphology and quantitative analyses of the height of villi, depth of crypts, numbers of goblet and Cellula panethensis, and thicknesses of duodenal glands layers in the duodenum were also investigated in the early diabetic duration.

MATERIAL AND METHOD

Animal preparation, diabetic induction and histological study. Eight male Sprague–Dawley rats (5-8 weeks old, 200-270 g) were used and obtained from the National Laboratory Animal Center, Mahidol University, Thailand. All experimental processes were approved by the Siriraj Animal Care and Use Protocol, Mahidol University, Thailand (COA No. 001/2564), and performed in compliance with the Guide for the Care and Use of Laboratory Animals. Five diabetic rats were intraperitoneally injected with a single dose of 60 mg/kg body weight streptozotocin (STZ) in citrate buffer. Three control rats were injected with the buffer alone. After STZ or buffer administration, the rats were sacrificed at 4 weeks as early diabetes (Chookliang *et al*., 2021). Then, the small intestine was removed between the pyloric sphincter of the stomach and the ileocecal junction. The duodenum was obtained as the first 10 cm from the pyloric sphincter of the stomach (Kararli, 1995). Thereafter, the specimen was fixed in Bouin's solution and underwent the histological procedure. The sections (6 μm thick) were stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) for goblet cell staining, and Masson's trichrome for collagen detection in the mucosa and submucosa of the duodenum. Light micrographs were captured under an Olympus Bx43 microscope, equipped with a DP73 digital camera and cellSens Standard software (Olympus Optical, Co. Ltd., Tokyo, Japan).

Height of villi and depth of crypts. Fifty cross-sectional sections of the duodenum were chosen to study the mean value of duodenal villi and crypts at 4X magnification. The length of villi was measured between the tip of villi and the junction crypt- villi, whereas the crypt depth was the extension between the junction crypt- villi and the crypt base. These measurements were randomly investigated in 5 well-oriented villi and crypts per tissue section. The villus height and crypt depth were measured by using the ImageJ software tool (National Institute of Mental Health, Bethesda, Maryland, USA).

Numbers of goblet cells. A total of ten sections of every five sections in the serial cross-sectional duodenum were selected to study the numbers of PAS-positive (PAS+) goblet cells per villus and crypt at 20X magnification. These cells were randomly counted on 5 well-oriented villus-crypt units per tissue section. The criteria of goblet cells were a wine glass shape with purple-stained mucin on its apical distended cytoplasm and a round to oval shaped nucleus.

Numbers of Cellula panethensis (Paneth cells). In the cross sections of the duodenum, fifty crypts of every five sections were chosen to analyze the mean numbers of Cellula panethensis per duodenal crypt at 40X magnification. The selected Cellula panethensis were pyramidal in shape with a round nucleus and presented acidophilic secretory granules in the apical cytoplasm.

Thicknesses of duodenal glands (Brunner's glands) layers in submucosa. Fifty sections in the serial cross-sectional duodenum were used to measure the mean thickness of duodenal glands layer in the submucosa at 20X magnification. In each cross section of the duodenum, pictures were captured at the 3, 6, 9, and 12 o'clock positions. Every image was measured 5 times in the thickness of duodenal glands layer from muscularis mucosae to muscular layer by using the ImageJ software tool (National Institute of Mental Health, Bethesda, Maryland, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). The body weights of the rats were compared by using the Mann–Whitney U test. Moreover, the villus height, crypt depth, number of PAS+ goblet cells and Cellula panethensis, and duodenal glands layer thickness were analyzed by using independent t-test (SPSS 18.0 software). The significance level was set at p value < 0.05.

RESULTS

Administration of STZ to induce diabetes caused abnormal increases in blood glucose (≥ 300 mg/dL) and urine glucose (≥ 500 mg/dL) levels. Diabetic rats had significantly lower mean body weights (239.50 ± 13.86 g) than the controls (405 ± 38.50 g).

Histological findings. The normal duodenum was made up of four layers: mucosa, submucosa, muscular layer, and serosa. The mucosa comprised the epithelium, lamina propria, and muscularis mucosae. The duodenal mucosa had two main portions: a villus and a crypt. The duodenal villi were fingerlike or leaflike structures, which were evaginations of the epithelium and lamina propria protruding

into the lumen. At the junction between two adjacent villi, the intestinal gland or crypt of Lieberkühn was an invagination of epithelium with lamina propria, deeply extending into muscularis mucosae (Fig. 1A). In early DM, there were deformed villi with disrupted surface epithelium and mildly distorted shapes of crypts (Fig. 1B) together with significant increases in villus heights (Fig. 1C) and crypt depths (Fig. 1D) compared to the controls.

The duodenal epithelium consisted of enterocytes and goblet cells. The enterocytes, a major cell type of duodenal epithelium, were simple columnar cells lining throughout the villi and crypts. The cell contained a single round to oval basal nucleus and apical cytoplasmic protrusion, called the brush border. The goblet cells were interspersed among enterocytes in the duodenal villi and crypts. Their morphology resembled a wineglass in shape with clear mucus secretory granules at the apical cytoplasm and contained a round to oval nucleus at the bottom of the cell (Fig. 2A). In the diabetic group, the epithelial cells detached from their underlying basement membrane and represented densely packed cells with unclear cell boundaries. Moreover, partial loss of the brush border on the apical cytoplasm in diabetic enterocytes was found (Figs. 2B, 2D). Lymphoid cell accumulation in the lamina propria can be seen in DM (Fig. 2B). In addition, the PAS+ goblet cells of both groups showed purple-stained mucin in their apically distended portion (Figs. 2C, 2D). Significantly decreased numbers of PAS+ goblet cells were observed in the DM (Fig. 2E).

Moreover, the pyramidal cellula panethensis in the lowest part of the crypts had densely packed eosinophilic secretory granules in their apical cytoplasm (Fig. 3A). Diabetic cellula panethensis were filled with pale-stained eosinophilic secretory granules compared to densely packed eosinophilic secretory granules in the control (Fig. 3B). Additionally, there were significantly increased Paneth cell numbers in DM (Fig. 3C).

There were duodenal glands in the submucosa between the muscularis mucosae and muscular layer. These glands were tubuloalveolar type and consisted of simple columnar to simple cuboidal cells with basally positioned round or oval nuclei (Fig. 4A). Duodenal glands were larger in size and surrounded by abundant collagen fibers in the submucosa of the diabetic duodenum (Fig. 4B). These alterations caused a significant increase in submucosal or duodenal glands layer thickness (Fig. 4C).

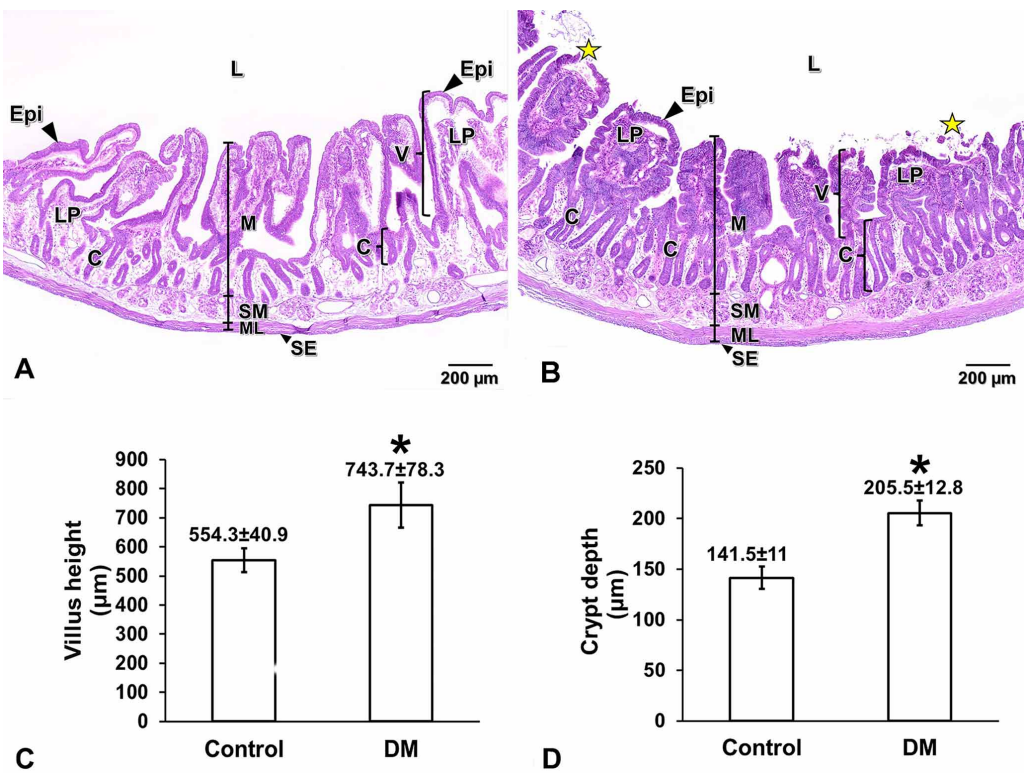

Fig. 1. Micrographs with H&E staining showed four layers of duodenal wall in cross sections of the control (1A) and diabetic (1B) rats: M= mucosa; S= submucosa; ML= muscular layer; SE= serosa; L= lumen; V= villus; C= crypt; Epi= epithelium; LP= lamina propria; yellow stars= disrupted surface epithelium. 4X magnification. Graphs presented comparisons between control and DM rats in terms of the duodenal villus heights (1C) and crypt depths (1D), *p < 0.05, compared with the control.

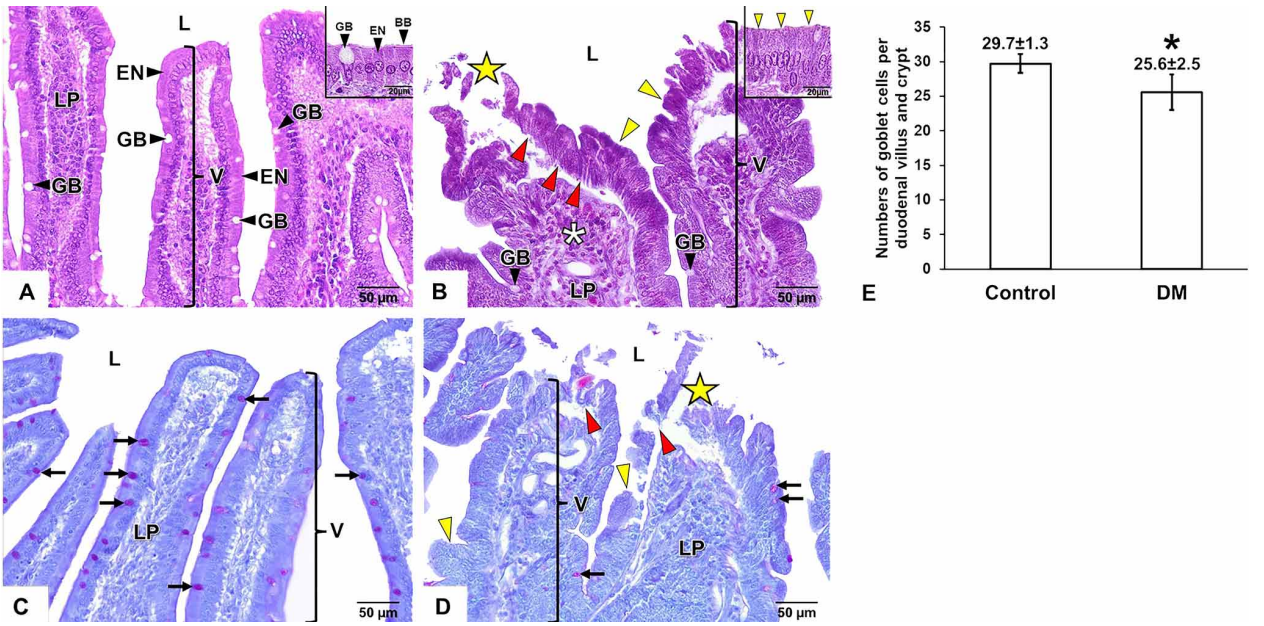

Fig. 2. H&E staining of the villi in the duodenum of the control (2A) and diabetic (2B) rats. 20X magnification. Insets: high magnifications of epithelial cells in duodenal villi. 60X magnification: L= lumen; V= villus; LP= lamina propria; EN= enterocyte; GB= goblet cell; BB= brush border; yellow stars= disrupted surface epithelium; red arrowheads= the detachment of epithelial cells from the basement membrane; yellow arrowheads= densely packed epithelial cells with unclear cell boundaries and partial loss of brush border in apical cytoplasm of enterocytes; white asterisk= abundant lymphoid cells in lamina propria. Moreover, PAS staining of the PAS+ in the apical portion of goblet cells in the control (2C) and diabetic (2D) duodenum: black arrows= PAS+ goblet cells. 20X magnification. Quantification of the PAS+ goblet cell numbers per duodenal villus and crypt (2E), *p < 0.05, compared with the control.

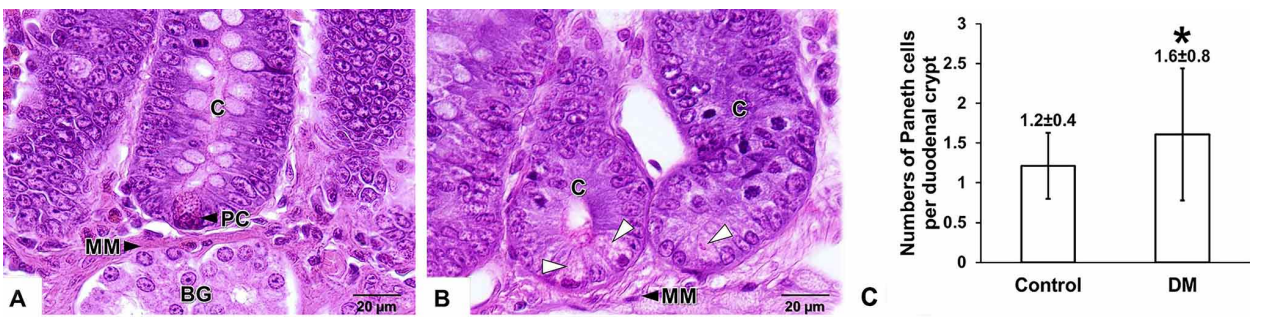

Fig. 3. H&E staining presented crypts of Lieberkühn in the duodenum of the control (3A) and diabetic (3B) rats: C= crypt; PC= Paneth cell; MM= muscularis mucosae; BG= Brunner's gland; white arrowheads= pale-stained eosinophilic secretory granules at apical cytoplasm of Paneth cells. 60X magnification. Comparison of the Paneth cell numbers at the base of crypts (3C), *p < 0.05, compared with the control.

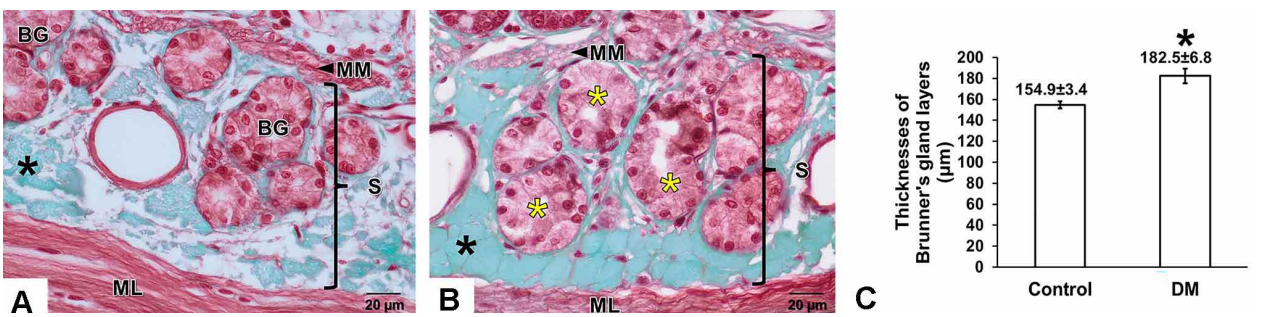

Fig. 4. Masson's trichrome staining of Brunner's glands in the submucosa of the duodenum in the control (4A) and diabetic (4B) rats: MM= muscularis mucosae; S= submucosa; ML= muscular layer; BG= Brunner's gland; black asterisks= collagen deposition; yellow asterisks= Brunner's gland hypertrophy. 40X magnification. Quantification of the Brunner's gland layer in submucosa (4C), *p < 0.05, compared with the control.

DISCUSSION

In early DM, the villus height and crypt depth significantly increased, similar to the previous examination (Min *et al*., 2014). Indeed, intestinal stem cells (ISCs) are characterized by their ability to self-renew and to differentiate into specialized cell types of the intestinal epithelium. It has been reported that hyperglycemia enhances the proliferation and differentiation of intestinal epithelial cells, regulated by Wnt/b-catenin and Notch signaling pathways in ISCs at the bottom of crypts (Dorfman *et al*., 2015). Thus, intestinal epithelial cell proliferation and differentiation in DM may cause increases in villus height and crypt depth. Additionally, disruption of the surface epithelium and detachment of epithelial cells were found in the diabetic duodenum. These observations may be involved in the loss of epithelial cell interlocking. During hyperglycemia, wider intervals between intestinal epithelial cells and damaged tight junction proteins, including zona occludens-1 and occludin, are observed. Ultimately, these results lead to loss of cell integrity and disrupted intestinal mucosal barrier (Min *et al*.).

A reduced number of PAS+ goblet cells was observed in the diabetic duodenum. Min *et al*. found decreased expression of Tff3 (a goblet cell marker) and a lower number of goblet cells in diabetic intestinal epithelium. Moreover, several studies have indicated that a decrease in the mucin content of goblet cells is related to reduced goblet cell numbers in the intestine of diabetic rats (Mantle *et al*., 1989; Barman & Srinivasan, 2019). The decreased number of goblet cells during diabetes can be explained by the extrinsic apoptosis pathway. In the diabetic intestine, intestinal macrophages secrete tumor necrosis factor alpha, which binds to its receptor on the cell membrane of goblet cells, finally resulting in active caspase-8 (Ding & Lund, 2011; Kawano *et al*., 2016). Then, active caspase-8 activates caspase-3, which induces apoptotic goblet cells, leading to a reduced number of goblet cells in DM (Ramachandran *et al*., 2000; Lau *et al*., 2012).

There were elevated Paneth cell numbers in the diabetic duodenum. It has been observed that diabetes causes a high level of Lyz1 expression (a Paneth cell marker), and it is possible that the increased Lyz1 might reflect the elevated Cellula panethensis of the diabetic duodenum in this study (Min *et al*.). Even though the numbers of Cellula panethensis increased in the DM, they were filled with pale-stained eosinophilic secretory granules. Normally, abundant eosinophilic secretory granules in the apical cytoplasm of Cellula panethensis contain numerous antimicrobial peptides (Shanahan *et al*., 2014). Thus, the pale-stained eosinophilic secretory granules in diabetic Cellula panethensis might be related to reduced antimicrobial peptides. Yu *et al*. found that diabetes causes decreased expression of Paneth cell-derived antimicrobial peptides, although the Paneth cell numbers increase. This evidence suggests that diabetes leads to the impairment of antimicrobial function in Cellula panethensis to kill bacteria, causing impaired protection of the intestinal mucosa (Yu *et al*.).

Duodenal glands hypertrophy was shown in the diabetic duodenum. In the polyol pathway, hyperglycemia is changed to augment intracellular sorbitol, resulting in water influx into duodenal glands (Tang *et al*., 2012). Moreover, the conversion process of sorbitol to fructose causes excess NADH, which is the substrate for NADH oxidase to generate reactive oxygen species (ROS) (Ntimbane *et al*., 2009; Tang *et al*.). Then, ROS disrupt the electron transport chain to cause mitochondrial dysfunction, leading to ATP depletion. The reduced ATP impairs sodium-potassium ATPase to promote high levels of sodium ions (Na+) in the cells. In addition, ROS induce endoplasmic reticulum stress, causing elevated intracellular calcium ions (Ca2+) in the cells (Ntimbane *et al*.). As a result, the retention of Na+ and Ca2+ enhances osmolarity to increase water influx into duodenal glands. Therefore, all of the mechanisms induce the hypertrophy of duodenal glands in diabetes. Remarkably, duodenal glands secrete bicarbonate ions (HCO3-) to neutralize the acidic chyme. HCO3- secretion from duodenal glands involves intrinsic and extrinsic factors (Collaco *et al*.). With respect to the intrinsic factor, HCO3- in the blood vessels is imported into the acinar cells of duodenal glands via sodium-bicarbonate cotransporter 1 (NBCe1). Moreover, intracellular HCO3- in duodenal glands is exported to the lumen through cystic fibrosis transmembrane conductance regulator (CFTR) (Collaco *et al*.). In the pancreatic acinar cells of diabetes, HCO3- in the blood vessels cannot move into the acinar cells via NBCe1 due to Na+ retention (Futakuchi *et al*., 2009). Furthermore, Ca2+ retention in pancreatic acinar cells can inhibit CFTR from secreting HCO3- into the lumen of the duct (Ntimbane *et al*.). Thus, these mechanisms in diabetic acinar cells of the pancreas may occur in diabetic duodenal glands (Futakuchi *et al*.; Ntimbane *et al*.). With respect to the extrinsic factor, HCO3- secretion from duodenal glands can be controlled by hormonal and neuronal pathways. In the hormonal pathway, gastric acid induces enteroendocrine S cells in the duodenal crypt to secrete secretin, which stimulates the release of HCO3- in duodenal glands (Collaco *et al*.). It has been reported that hyperglycemia is involved in low levels of plasma secretin (Futakuchi *et al*.) and that HCO3- output decreases in the diabetic duodenum (Takehara *et al*., 1997). In the neuronal pathway, HCO3- secretion from duodenal glands is regulated by vagal parasympathetic fibers of the enteric nervous system (Collaco *et al*.). During

hyperglycemia, ROS cause the vagal neuropathy, which leads to dysfunction of the vagal-neuronal pathway to reduce HCO3- secretion in the duodenum (Takehara *et al*.). Therefore, hormonal and neuronal dysfunction in diabetes might be related to lower HCO3- secretion in duodenal glands. All of the evidence indicates that the lack of HCO3- for mucosal protection against gastric acid in the duodenum can enhance duodenal ulcers in diabetic patients (Takehara *et al*.). Furthermore, collagen accumulation was visible in the submucosa of the diabetic duodenum. Diabetes has been shown to cause the upregulated mRNA expression of fibronectin and type- IV collagen, leading to intestinal wall fibrosis and thickening in the diabetic small intestine (Barman & Srinivasan). Consequently, both hypertrophy of duodenal glands and abundant collagen deposition caused increased layer thicknesses of the duodenal submucosa in diabetes.

Conclusively, early diabetes caused structural changes in the duodenum. These changes result from hyperglycemia-induced intestinal epithelial cell proliferation, which was found to increase villus height and crypt depth and to breakdown tight junction proteins. These changes are strongly related to the development of maldigestion and malabsorption. Furthermore, the reduced goblet cells, increased Cellula panethensis, and duodenal glands hypertrophy may correlate with the impaired protection of duodenal mucosa from gastric chyme and bacterial pathogens. Therefore, the impairment of duodenal structures can lead to malnutrition and duodenal ulcers in diabetic patients. This basic knowledge will be beneficial for the early diagnosis and prevention of progressive complications in patients with diabetes.

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RESUMEN: La diabetes mellitus (DM) afecta principalmente a cambios funcionales en el duodeno, que juega un papel importante en la digestión y absorción de los alimentos. El deterioro de la función duodenal contribuye a la desnutrición, distensión abdominal y dolor en pacientes diabéticos. Por lo tanto, este estudio tuvo como objetivo estudiar las alteraciones histológicas y determinar las mediciones cuantitativas de las estructuras duodenales en la etapa temprana de ratas diabéticas inducidas por estreptozotocina (STZ). Ocho ratas macho Sprague-Dawley fueron distribuidas en dos grupos: tres ratas control y cinco diabéticas. La diabetes se indujo mediante una dosis intraperitoneal única de 60 mg/kg de STZ. Después de cuatro semanas de inducción, se preparó el duodeno para estudio histológico y análisis morfométrico. En ratas diabéticas, había vellosidades deformadas con epitelio superficial destruido y formas ligeramente distorsionadas de las criptas, junto con un aumento en la altura de las vellosidades y la profundidad de las criptas. Las células epiteliales se encontraban separadas de la membrana basal subyacente. Las células caliciformes habían disminuido en número, mientras que en el grupo DM se produjo un aumento en el número de *Cellula panethensis* (células de Paneth) con gránulos eosinofílicos teñidos pálidos. Se observó una capa submucosa engrosada con aumento de la hipertrofia de las glándulas duodenales (glándulas de Brunner) y acumulación de colágeno. Estos hallazgos indican que las lesiones histopatológicas del duodeno se desarrollaron en la etapa temprana de la diabetes. La destrucción de vellosidades, criptas y epitelio puede afectar la digestión y la absorción. Los cambios estructurales en *Cellula panethensis* y glándulas duodenales pueden estar asociados con un mal funcionamiento en la protección de la mucosa duodenal tanto de las bacterias como del ácido gástrico. Estas condiciones pueden empeorar la calidad de vida de las personas diabéticas y provocar complicaciones como mala digestión, malabsorción y úlcera duodenal.

PALABRAS CLAVE: Duodeno; Diabetes temprana; estreptozotocina.

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