Enteric neuropathy associated to diabetes mellitus
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ABSTRACT
Diabetes mellitus (DM) is a group of diseases highly prevalent nowadays. Its different types produce very similar symptoms with acute and chronic complications. Amongst these, gastrointestinal (GI) dysmotility, associated with the development of neuropathy in the enteric nervous system (ENS) is recognized. The objective is to review the current knowledge on GI dysmotility and enteric neuropathy associated to diabetes mellitus. The different functional and structural alterations within the digestive tract in diabetic patients and animal models are described. Finally, the therapeutic and preventive strategies tested so far in the context of enteric diabetic neuropathy are briefly summarized.

In conclusion, amongst the alterations described in DM, the loss of inhibitory intrinsic innervation of the gut is most remarkable. Different therapeutic and/or preventive strategies, including the use of insulin, nerve growth factor or antioxidants, as well as myenteric neuron transplantation, are proposed.

Key words: Diabetes mellitus. Gastrointestinal dysmotility. Gastroparesia. Enteric neuropathy. Myenteric plexus. Enteric nervous system.

ABBREVIATIONS

INTRODUCTION
Diabetes mellitus (DM) is one of the chronic diseases more prevalent in western societies, and is quickly spreading to developing countries as well. In 2013, 382 million people were diabetic in the world and it has been estimated that this number will reach 592 million in 2035; therefore, DM is considered a major public health problem (1). The most frequent categories of this disease are type 1 (DM1), characterized by the destruction of pancreatic β cells and an absolute deficiency of insulin, and type 2 (DM2), in which insulin levels are more variable but characterized by a frank resistance to its effects (2). The different types of diabetes display a hyperglycemic state that at high levels produces polyuria, polyfagia and polydipsia. The acute complications (hypoglycaemia, ketoacidosis, non-ketotic hyperosmolar coma…) are due to the long-term maintenance of the hyperglycaemic state (with glycaemias higher than 200-250 mg/dl, which overflow renal reabsorption) and the oxidative and inflammatory phenomena caused by this state (3). Thus, DM induces structural and functional alterations in cells, tissues and organs in the whole body, including the gastrointestinal (GI) tract and its innervation.

AIM
The aim is to review the current knowledge of the alterations induced by diabetes in the digestive tract and, particularly, on the enteric neurons responsible for controlling its functions. To this aim, we will describe the functional and
structural/histological alterations that are encountered in the diabetic gut, the animal models developed for its study, and the possible therapeutic and preventative strategies.

**MOTOR CONTROL OF THE DIGESTIVE SYSTEM**

Control of digestive functions depends, mainly, on its innervation, both intrinsic and extrinsic. The enteric nervous system (ENS) is a nervous network spreading from the oesophagus up to the internal anal sphincter, and includes the myenteric or Auerbach’s plexus (which controls motility and is located between the muscle layers), and the submucous or Meissner’s plexus (which regulates microcirculation and epithelial function, and is located underneath the mucosa). Neurons in the enteric nervous system are accompanied by the glial cells and may function independently of the extrinsic control, modulating peristalsis, secretion, pain perception and inflammation (4).

The basic unit of motor function in the GI tract is the peristaltic reflex, which is produced in the myenteric plexus in response to the luminal contents (4). It has, amongst others, sensitive neurons, interneurons and excitatory and inhibitory motor neurons to both muscle layers. The electrophysiological, morphological, immunohistochemical and retrograde labelling studies have established the differences amongst the neuronal subpopulations (4,5) (Fig. 1). Further, the interstitial cells of Cajal (ICC), considered as pacemaker cells and located amongst the myenteric neurons and the muscle fibres, coordinate the contractile responses (6).

The extrinsic nervous system modulates the function of the ENS and encompasses the parasympathetic pathways of the vagus and sacral nerves, which produce excitatory responses of the muscle and inhibition of the sphincters, and the thoracolumbar sympathetic nervous system, with opposite effects. The myenteric plexus in the stomach and proximal duodenum is densely innervated by the vagus nerve. Distally to the duodenum, the number of myenteric ganglia innervated by the vagus nerve decreases rapidly, but the parasympathetic innervation turns out to be important again in the caecum and proximal colon (4). Thus, after ingestion, fundus relaxation activated by the vagus nerve allows accommodation of the stomach. Peristaltic contractions of the antrum push the gastric contents towards the pylorus, which eventually allows little amounts of food to reach the duodenum upon pylorus opening. The arrival of nutrients to the distal part of the small intestine slows down gastric emptying (enterogastric reflex), whilst postprandial gastric distension promotes the flux of luminal contents towards ileum and caecum (gastrocolic reflex). Finally, the GI hormones contribute also to regulate motility of the digestive tract, either facilitating gastric distension (ghrelin), inhibiting gastric emptying (GPL-1, colecstokinin and secretin), or facilitating motility (gastrin, motilin). Interestingly, some of these peptides (GPL-1, colecstokinin, gastrin, ghrelin) are known mediators of glucose metabolism and induce effects such as insulin synthesis, β cell proliferation or glucagon release (7).
DIABETIC GASTROENTEROPATHY: FUNCTIONAL ALTERATIONS

In up to 75% patients DM is associated to GI symptoms like nausea, bloating, abdominal pain, diarrhoea, constipation, and slowed gastric emptying. Moreover, motility alterations enable microflora overgrowth, which aggravates the problem, because it may induce bloating, diarrhoea or abdominal pain, as well as malabsorption, and even alter both macro and microscopic intestinal morphologic structure (8). Amongst all the alterations associated to diabetes in GI function, gastroparesia is best known (9). It is found both in DM1 and DM2, in patients with and without complications associated to diabetes, and may occur in up to 50% of patients (10).

The best test to assess patients with gastroparesis is scintigraphy, using food labelled with technetium 99m. However, it presents some limitations, like its high cost, exposure to radiation and need for specialized equipment, and therefore, new technologies have been developed to improve diagnostics, such as the endoscopic capsule, 3D ultrasound, MRI, SPECT or the breath test using Spirulina platensis labelled with $^{13}$C (9). Physical evaluation is limited to nutritional status and signs of diabetic neuropathy (8).

DM is the most common cause of autonomic neuropathy and contributes to the GI symptoms, including abnormal intestinal motility (11,12). Diagnosis of GI autonomic neuropathy is not easy because it may affect both the intrinsic innervation (enteric neuropathy associated to DM), which is difficult to assess (it requires samples including part of the enteric plexuses), and the extrinsic innervation, and in this case, the cardiovascular alterations are of low value in the prediction of motor alterations of the GI tract (11). In fact, a lot of information available on this neuropathy has been obtained from animal models of diabetes. Thus, it is convenient to briefly review the studies performed in these models.

ANIMAL MODELS, IN VITRO AND IN VIVO STUDIES

Many different models of diabetic neuropathy have been developed, although the alterations of the ENS have been evaluated in all of them (13,14). For DM1, although genetic models like the inbreeding BB rats do exist, the most frequent model is that induced by streptozotocin (STZ) administration. STZ is a toxin capable of destroying pancreatic β cells (15-17). At common doses, STZ itself may induce neurotoxicity (18), contributing to the neuropathy observed in these models. However, lower but repeated doses do not produce toxicity to the myenteric plexus or the ICC in control mice but in transgenic RIP-I-hIFNβ diabetic mice, these low repeated doses of STZ allowed to evaluate the changes in digestive motility due to the pathology itself and those adaptive changes induced by polyfagia, polydipsia, etc. (19).

The growing prevalence of DM2 in the last few years has provoked a great amount of studies in models of obesity and diabetes induced by high-calorie and/or high-fat diets, both in mice (20,21) and rats (22). There are also genetic models of obesity and DM2 in which GI motility alterations and/or enteric neuropathy have been evaluated, including ob/ob, db/db, KK and KKAY mice (20-24), and Otsuka Long-Evans Tokushima Fatty (OLEFT) and Zucker Diabetic Fatty (ZDF) (25,26) rats.

In vitro studies performed in these models include contractility studies and pharmacological responses in isolated GI tissues (19), as well as cultures of enteric neuronal lines or of myenteric ganglia dissected away from the tissue using a laser (22). Regarding in vivo studies, invasive procedures have been used: Intragastric administration of markers and analysis of their progression along the digestive tract (19,21), electromyography of the external muscle after the insertion of electrodes to the serosa (27), and non-invasive studies. These non-invasive studies allow the evaluation of functional changes occurring throughout time, along the development of the disease. Thus, breath tests using octanoic acid labelled with $^{13}$C have been used to test for gastroparesia (28), and tests evaluating the expulsion of artificial faecal pellets inserted in the colon have been used to determine colonic dysmotility (21). It is also remarkable the use of radiopaque markers allowing to determine the alterations in transit and morphology in each GI region (26) (Fig. 2).

DIABETIC GASTROENTEROPATHY: STRUCTURAL ALTERATIONS

Both in rats after STZ administration (29) and in RIP-I/hIFNβ diabetic mice (19), the GI tract shows a larger length. Apart from these macroscopic alterations, the damage induced by DM in the gut wall and the specific populations of enteric neurons has been analysed using conventional histology, immunohistochemistry and electronic microscopy, as we will show below.

Mucosa

In diabetes, mucosa of the small intestine suffers changes that alter transit of the food bolus, the secretion of enteric juices and the absorption of the digestion products (30). Acute hyperglycaemia in jejunum of rats did not induce any alterations in the mitotic index or mucosal morphometry. However, chronic hyperglycaemia in the rat occurs with hyperplasia and hypertrophy of the intestinal mucosa (30). The increase in the mitotic index may be related with an adaptation of the mucosal layer to chronic pathology.
On the other hand, an increase of goblet cells in the intestinal villi has been found in the small intestine of rats with acute hyperglycaemia (31) and in the crypts of the small intestine in diabetic mice (32). The maintenance of this cell population allows the preservation of the mucosal layer as a morpho-functional adaptation to chronic diabetes (33).

**Smooth muscle**

The alterations in the width of the muscle layers depend on the region considered and the moment in which the analysis is made along the development of the disease (33). The cause of these regional differences is not clear and, in fact, their intrinsic function might not be affected. Thus, in organ bath studies with ileal longitudinal and colonic circular smooth muscle preparations from RIP-I/hIFNβ diabetic mice, the contractile responses were similar to those from control animals, suggesting that the function of the smooth muscle layers might get adapted to the damage associated to diabetes (19).

**Interstitial cells of Cajal**

DM may have an influence also in the pacemaker cells of the GI tract. Degenerative changes and/or loss of ICC have been observed throughout the digestive tract of patients and animal models (34,35). As in the diabetic mice, DM2 patients with gastroparesia, have less ICC in the stomach (34). Furthermore, ICC loses their common association with enteric neurons, which has been related to the decrease of electrical activity of the antral smooth muscle (10).

The possible alteration in intestinal ICC is less clear. Thus, in RIP-I/hIFNβ diabetic mice, the frequency of spontaneous contractions decreased in the small intestine, but the network of ICC within the myenteric plexus (ICC-MP) was intact. In contrast, in the colon the frequency of these contractions was similar to that in control animals (19). Nevertheless, areas with scarce ICC-MP were found in the ileum and colon of db/db mice (36) and in the smooth muscle layer of the colon in diabetic patients (37).

**Extrinsic innervation**

It has been considered that an autonomous visceral neuropathy or changes in the vagal innervation are responsible for the alterations of gastric motor control in diabetes (38). In diabetic BB rats with alterations in jejunal motility, the presence of cardiac autonomic neuropathy was not demonstrated (27). However, the expression of neuronal nitric oxide synthase (NOS) in the rat jejunum is independent of the vagus nerve (39), and therefore the vagal neuropathy could not underlie the alterations in motility further away from duodenum. It does not seem that hyperglycaemia is essential in these cases either because in animals with different levels of glycaemic control similar changes were apparent (27).

With regards to the sympathetic innervation, the levels of noradrenaline (NA) decrease in the ileum, increase in the proximal colon and do not change in the distal colon. Furthermore, the decrease of NA in the ileum of diabetic rats occurs simultaneously with the occurrence of inflamed varicosities in the nerve fibres (40).

**Intrinsic innervation: Enteric neuropathy associated to diabetes mellitus**

Changes in the number of neurons and the expression of neurotransmitters within the myenteric plexus have been described, together with the loss of myenteric neurons in several areas of the digestive tract in rodents with DM (41), and morphologic alterations in particular subtypes of neurons in the stomach of patients with DM (34,35). The different subpopulations of enteric neurons respond in a different manner to DM, and the extension of changes seems to be specific to the segment of intestine, model and duration of DM (17). In rat and mice, both the selective loss of neuronal populations and neurodegeneration of the myenteric plexus have been demonstrated (18,41).

Electric stimulation is used to generate muscle responses dependent on neuronal activity. In these studies, the main response of the longitudinal muscle in control animals is
excitatory, mediated by cholinergic contractions, and the incubation with atropine unmasks the presence of inhibitory functional co-transmission followed by a non-cholinergic contraction sensitive to tetrodotoxin. On the contrary, in the circular smooth muscle, the main response under non-cholinergic non-adrenergic conditions is inhibitory, both in rats and humans (42,43). In diabetic mice a slight increase of cholinergic contractions together with a failure in inhibitory neurotransmission is observed in the ileum. In addition, a decrease in the non-cholinergic contractions is found in the ileum and is even more intense in the colon. These results show that both components (nitricergic and purinergic) of the relaxation evoked by electric stimulation are inhibited. Moreover, the damage of the nitricergic inhibition in the colon is evidenced also by the decrease in the contractile activity in the presence of NOS inhibitors or tetrodotoxin (19).

Regarding the causes of neurodegeneration, the contribution of the inflammatory reaction activated by DM must be considered (27). In this case, there is inflammation in the stomach, the small intestine and the colon in BB rats, and this is accompanied by damage to the myenteric and submucous plexuses and limitation of motor function (44). The cause of the inflammatory infiltration is not clear; it might mirror an alteration in the intestinal barrier (microflora overgrowth would contribute to this), a decrease in the neuronal NOS (nNOS) regulation through inducible NOS (iNOS) induction, producing high amounts of NO (45) or apoptosis-dependent death. The changes in immunoreactivity to nNOS persisted after inflammation resolution (27). Another possibility would be that the intestinal inflammation has an autoimmune origin (46). Studies in humans (47) seem to confirm a similar situation to that found in the rat.

Histopathologic and experimental studies in vivo have demonstrated that the enteric glia play a key role in the pathologies associated to dysfunction in the intestinal barrier and motor alterations, such as constipation, diverticular occurrence and idiopathic megacolon, reduction of intestinal motility and slow gastric emptying (48). In fact, the normal activity of glial aspartate-cyclase (ASPA) is essential in maintaining neuronal health because it is an important inductor of neurodegeneration, and it is elevated in the duodenum of diabetic mice. Therefore, hyperglycaemia alters ASPA activity and may contribute to the neuropathy observed in DM2 (49).

On the other hand, it has been suggested that the changes in vascularization contribute to the enteric neuropathy associated to DM as well. Permeability of the big mesenteric blood vessels is altered in diabetes (50), but the effect in those capillaries lying within the gut wall is not well known. The excess of capillary permeability may be pathologic to the enteric neurons and lead to neuropathy in particular intestinal regions (51). Thus, the alterations in the capillary walls were pronounced in ileum and colon, but not in duodenum. The recovery of insulin levels avoid-
ed the structural alterations in the capillary wall in the ileum, but not in the colon. In the duodenum an up-regulation of caveolin-1 and NOS of the endothelial cells (eNOS) was observed. This agrees with the hypothesis that the microvessels close to the myenteric plexus are targets of damage in the DM (52).

The susceptibility of microvessels to hyperglycaemia is related to the prevalence of bacteria in different parts of the GI tract because they induce an anaerobic state in the distal colon (53). The structural damage in the colon is irreversible, in the ileum it may be reverted with insulin supply, and in the duodenum the mechanism regulating vascular permeability is active. Hypoxia mediates the up-regulation of lipooxygenase, responsible for the compromised function of the basal layer barrier in diabetic retinopathy (54). In the colon, it would determine an irreversible increase in the width of the basal layer after the first hyperglycaemic shock. These studies demonstrate the need to evaluate the participation of the antioxidant systems, intestinal microflora composition and the regulation of the expression of lipooxygenase in the different intestinal segments in diabetes (52).

**Innervation of the mucosa**

It is convenient to mention the studies on mucosal innervation because the initial work on the vagus nerve in DM are contradictory (55,56), and because, although the subjects with DM2 and gastroparesis have less ICC in samples of stomach and less inhibitory neurons in the myenteric plexus (34), these alterations are not observable in conventional endoscopic biopsies (57). Thus, it has been histologically confirmed that the autonomous neuropathy alters the nerves of the gastric mucosa in DM1 patients: A decrease in the density of mucosal nerve fibres (MNF) occurs, as well as an alteration in the pattern and morphology of innervation. The density of MNF in the antrum was correlated in an inverse manner with the duration of diabetes, and amongst the symptoms of gastroparesis, abdominal bloating tended to be associated with MNF. However, none of the measurements of gastric symptoms, or the density of skin fibre nerves predicted the deficit in MNF. The severity of the alterations found in the autonomous cardiorespiratory reflex tests was not correlated either with the severity in the loss of MNF (57). Therefore, it has been proposed that the density of gastric mucosa innervation might be a good biomarker of diabetic autonomous neuropathy (57).

**STRATEGIES OF TREATMENT AND PREVENTION**

Optimal management of the diabetic patient requires a multidisciplinary approach and should first be directed to
alleviate the GI symptoms, as well as to improve the nutritional status and the control of glycaemia, which might improve gastric motor function per se (58,59). Vomit and nausea may reduce weight and cause dehydration and electrolytic dysregulations that should not be forgotten in spite of the possible initial overweight of some diabetic patients. There are no definite guidelines on how to address the nutritional status although it is thought that frequent and small meals, decreasing fat and fibre ingestion (both fat and fibre reduce gastric emptying), and adjusting carbohydrate intake according to medication, may improve symptoms and glycaemic control (60). The presence of pain complicates the problem because many analgesics further worsen GI motor function. In some patients gastroparesis may even force surgical approaches, including jejunostomy (in order to achieve proper nutrition), endoscopy to administer botulinum toxin into the pylorus (so that the sphincter is relaxed and gastric emptying is improved) or even bariatric surgery (9,61-63).

The most common strategy to improve GI symptoms in DM is the use of prokinetic drugs. These drugs increase digestive motility favouring transit of food and intestinal secretions through the different segments of the gut. Depending on their main mechanism of action, prokinetics may be divided into different categories, namely drugs with a dopaminergic, serotonergic or a cholinergic action or motilin agonists (64). The main drugs of this kind are metoclopramide and domperidone; both are dopaminergic D2 antagonists and, in addition to improving gastric emptying, reduce nausea and emesis, although domperidone shows less central and more cardiovascular effects (65). Cisapride, a serotonergic 5HT4 agonist, was proselytically used in the past, but its proarythmogenic effects have forced its withdrawal from the market some years ago (66). Erythromycin, a macrolide antibiotic, stimulates motilin receptors and thus also cholinergic neurons (65,67,68). Ghrelin and the agonists of its receptor (TZP-101) also facilitate gastric emptying (68), as well as do tetrahydrobiopterin or its precursor sepiapterin (70). Dual agents like levosulpiride (a dopaminergic D2 antagonist and serotonergic 5HT4 agonist) and cinitapride (5HT4 agonist and 5HT2 antagonist) are also useful and safe (64,69). The use of antiemetics is equally important in order to avoid one of the most debilitating symptoms in these patients. In addition to the dopaminergic antagonists already mentioned, those antiemetics more frequently used are phenotyazines like prochlorperazine, which act upon cholinergic and dopaminergic receptors, although it may induce undesirable effects like dyskinesia, and not necessarily improve gastric emptying (63,65,67). To the same aim, electrical stimulation (gastric pacemaker) is being used and has demonstrated to be able to improve quality of life, to relieve symptoms, nutritional alterations and even has improved control of diabetes, although it is reserved for patients with refractory symptoms, particularly nausea and vomiting (65,67).

Regarding the loss of ICC, and due to the high oxidative stress level generated in diabetic conditions, it has been shown that hemin increases NOS expression and improves diabetic gastroparesis in mice (35). It is important to note that hemin also increases oxygenase activity in humans (67). Finally, regenerative therapy with stem cells could also be useful to restore the ICC population (71).

Amongst the different mechanisms underlying the development of the enteric neuropathy associated to diabetes (13,14,52), the following are included: Endothelial and microvessels dysfunction; direct cellular effects of hyperglycaemia; mitochondrial dysfunction and osmotic or oxidative stress; altered metabolism of fatty acids; changes in nerve growth factors and altered brain – GI tract interactions; increase in neuronal apoptosis. As a consequence, some of the strategies assessed and addressed specifically to preserve or restore innervation within the digestive tract and, therefore, to avoid enteric neuropathy have been:

- **Insulin:** in animal models of DM1, like those generated after treatment with STZ, is capable of reverting the initial (but not the secondary) loss of nitrergic neurons (16), as well as glial cells (72).
- **Nervous growth factors:** GDNF (glial-derived nerve factor) reverted functional and morphological neuronal damage induced by hyperglycaemia (41).
- **Antioxidants** protected enteric glial cells and neurons:
  - Lipoic acid, onagra oil or a combination of both (29); oleic acid (22).
  - Ascorbic acid (73), which is also an aldose reductase inhibitor.
  - Vitamin E (33), Gingko biloba extract (75), quercetin (76).
  - L-glutamine (77); reduced glutathione (78).
- **Modulation of nitrergic signalling** (modulation of S-nitrosothiol availability) (79).

Finally, another possibility that has been considered is enteric neuron transplantation (79).

**CONCLUSIONS**

Numerous studies do exist, both in patients and animal models, demonstrating the development of neuropathy in the enteric nervous system throughout diabetes progression. This neuropathy, which affects mainly nitrergic inhibitory neurons, underlies and contributes to GI complications that interfere importantly with quality of life of diabetic patients. Although diagnosis of GI dysmotility is relatively simple using functional tests, enteric neuropathy associated to diabetes is not so easy to demonstrate and only recently the possibility to evaluate the density of nerve fibres in mucosal biopsies has been suggested. The strategies of treatment and prevention of GI dysmotility and the underlying enteric neuropathy include the adequate control of glycaemia, but additional measures are proposed, such as the use of antioxidants or nerve growth...
factors. Other more sophisticated strategies, like myenteric neuron transplantation, could also be adopted in the future.

REFERENCES


