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Quantification of nitrergic neurons in the myenteric plexus of gastric antrum and ileum of healthy and diabetic dogs

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ABSTRACT

Diabetes mellitus (DM) determines a wide array of severe clinical complications including gastrointestinal motility disorders. The present study investigates the effects of spontaneous DM on the intramural innervation and in particular on nitrergic neurons of the myenteric plexus (MP) of the canine gastric antrum and ileum. Specimens of antrum and ileum from eight control-dogs and five insulin-dependent DM-dogs were collected. MP neurons were immunohistochemically identified with the anti-HuC/HuD antibody, while nitrergic neurons were identified with the antibody anti-neuronal nitric oxide synthase (nNOS). The density of HuC/HuD-immunoreactive (IR) neurons was determined and the nitrergic neurons were quantified as a relative percentage, in consideration of the total number of HuC/HuD-IR neurons. Furthermore, the density of nitrergic fibers in the muscular layers was calculated. Data were expressed as mean ± standard deviation. Compared to control-dogs, no significant differences resulted in the density of HuC/HuD-IR neurons in the antrum and ileum of DM-dogs; however, HuC/HuD-immunolabeling showed nuclear localization and fragmentation in DM-dogs. In the stomachs of control- and DM-dogs, the percentages of nitrergic neurons were 30 ± 6% and 25 ± 2%, respectively ($P = 0.112$). In the ileum of the control-dogs, the percentage of nitrergic neurons was 29 ± 5%, while in the DM-dogs, it was significantly reduced 19 ± 5% ($P = 0.006$). The density of nNOS-IR nervous fibers was meaningful reduced in either the tracts considered. Notably, the ganglia of DM-dogs showed also a thickening of the periganglionic connective tissue. These findings indicate that DM in dogs induce modification of the myenteric neurons and, in particular, of the nitrergic neuronal subpopulation.

1. Introduction

The gastrointestinal functions is mainly under the control of the enteric nervous system (ENS), which consists of millions of neurons harbored in the wall of the digestive system from the esophagus to the inner anal sphincter. Enteric neurons are organized in two ganglionated plexuses: the myenteric plexus (MP) and submucosal plexus (SMP), which interact in coordinating gut functions almost independently from the central nervous system (Furness, 2006). The gastrointestinal peristalsis is triggered by sensory fibers responsive to the radial distension of the lumen or by chemical stimuli. Once excited, the intramural sensory neurons activate ENS excitatory and inhibitory muscle motor neurons. The excitatory neurons release acetylcholine, whereas the inhibitory neurons release nitric oxide (Furness, 2006). Nitrergic neurons and fibers, which are usually immunohistochemically identified by the use of an antibody against the enzyme neuronal nitric oxide synthase (nNOS), have been already characterized in the canine gastrointestinal tract (Berezin et al., 1994; Ward et al., 1994). Nevertheless, none of these studies quantified the percentage of nitrergic neurons.

A wide spectrum of damages affecting the structural and functional integrity of the ENS can be responsible for many gastrointestinal symptoms and dysfunction. Among the secondary enteric neuropathies, i.e. heterogeneous disease in which the primary target of the disease is not the ENS (that results however damaged), diabetes mellitus (DM) is classified as a "predominantly degenerative neuropathy" (Knowles et al., 2013).

DM is a worldwide endocrine disease affecting humans but also domestic mammals, such as dogs and cats (Nelson and Reusch, 2014). The common feature of DM is hyperglycemia, which must be controlled to avoid severe DM complications such as retinopathy, vascular damage, generalized neuropathy, and gastrointestinal motility disorders (i.e.

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vomiting, constipation, diarrhea, and fecal incontinence), in both human and animal models (Rothstein, 1990; Zandecki et al., 2008; Adewoye et al., 2011; Ciobanu and Dumitrascu, 2011). Seemingly, generalized neuropathy and gastrointestinal motility disorders are two strictly correlated complications. As a matter of fact, a growing body of evidence suggests that gastric and intestinal symptoms in human and animal diabetic patients derive from intestinal motility abnormalities related to enteric neuropathy.

A few studies have focused on the gastrointestinal dysfunction in DM dogs (Takeda et al., 2001; Onoma et al., 2008), and to date, no information is available on the effects of DM on canine ENS.

The present research was focused to evaluate whether and how DM affects the dog myenteric neurons and in particular the nitrergic ones, since in other species (mainly rodents) these neurons are susceptible to diabetic neuropathy.

The aims of the present study were to immunohistochemically quantify, in the gastric antrum and ileum of healthy and DM dogs: 1) the density (neurons/ganglionic area) of MP neurons immunoreactive for the pan-neuronal marker HuC/HuD; 2) the percentage of MP nitrergic neurons; 3) the density of nitrergic nervous fibers in the circular (CML) and longitudinal muscle layer (LML).

2. Material and methods

2.1. Animals

Tissues were collected from eight control (CTRL) dogs (none had evident gastrointestinal disorders) (Table 1) and five non-obese DM-affected dogs (Table 2). The weight of DM and CTRL dogs was 18 ± 11 Kg and 25 ± 13 Kg, respectively. Student's *t*-test did not show any difference between the two groups ($P = 0.385$). The age of DM and CTRL dogs was 126 ± 63 months and 110 ± 65 months, respectively. Student's *t*-test did not show any difference between two groups ($P = 0.694$).

DM type I was diagnosed through a documented clinical history and blood biochemical analysis. All animals died spontaneously or were euthanized, then their tissues were collected following owner permissions. According to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, the Italian legislation (D. Lgs. n. 26/2014) does not require any approval by competent Authorities or ethics committees.

2.2. Tissue collection

The gastrointestinal tracts were removed within 2 h after each animal's death. The stomach and ileum were longitudinally cut open respectively along the greater curvature and the mesenteric border. The stomach and intestine of CTRL dogs did not present apparent mucosal hyperemia or inflammatory lesions, whereas the ileum of DM-affected dogs showed severe (two dogs) or mild (three dogs) mucosal hyperemia. The pyloric portions of the stomach and the ileum were treated to obtain wholemount preparations (Chiocchetti et al., 2009) and tangential (to the serosal surface of the tissues) (1.0 cm × 1.0 cm) and longitudinal (2.0 cm × 0.5 cm) cryosections. In the present study, laminar preparations of antrum and ileum were obtained from no-stretched tissues and in absence of L-type calcium channel blockers.

Specimens from all the subjects were processed for immunohistochemistry as described previously (Sadeghinezhad et al., 2013).

Table 1
Clinico-pathological data of the control dogs included in the present research.

Control dogs	Breed	Gender	Age	Cause of death
CTRL1	German shepherd	M [a]	10 yr	Euthanasia due to progressive physical deterioration
CTRL2	German shepherd	M	9 yr, 6 mo	Heart cancer
CTRL3	Boxer	M [a]	8 yr	Cardiovascular disease
CTRL4	German shepherd	M	10 yr	Cardiovascular disease
CTRL5	Siberian Husky	M [a]	16 yr	Neurological (CNS) disorders
CTRL6	English Setter	F	2 yr	Road accident
CTRL7	Chihuahua	F	8 mo	Head trauma
CTRL8	West Highland White Terrier	M	17 yr	Intracranial neoplasia

Abbreviations: M, male; F, female.
[a] Male neutered.

2.3. Histology

Longitudinal cryosections from antrum and ileum of CTRL and DM dogs were stained using hematoxylin and eosin (H&E) and Masson's trichrome for general histological examination.

2.4. Immunohistochemistry

The antibody anti-human neuronal protein (HuC/HuD) was utilized as a pan-neuronal marker to identify all the enteric neurons.

Nitrergic neurons and nervous fibers were immunohistochemically identified by the use of two antibodies against the neuronal nitric oxide synthase (nNOS) enzyme. Table 3 lists the primary and secondary antibodies employed.

2.5. Specificity of the primary antibodies

The antibodies utilized in the present research (mouse anti-HuC/HuD, mouse anti-nNOS, and rabbit anti-nNOS) were tested for their specificity by Western blot (WB) analysis, which indicated that they were specific for the targeted molecules in dogs (Fig. 1 A). Furthermore, the two anti-nNOS antibodies were tested in a double-staining protocol and were co-localized in neuronal cell bodies and fibers (Fig. 1 B).

The specificity of the secondary antibodies was tested as described in a previous work (Sadeghinezhad et al., 2013).

2.6. Western blot

Tissue samples (dog ileum) were collected, frozen in liquid nitrogen, and stored at −80 °C. Tissues were later thawed and homogenized. Total protein content was extracted using *T*-PER tissue protein extraction reagent in the presence of a protease inhibitor cocktail (Thermo Scientific, Italy, Europe) according to the manufacturer's instructions, and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Italy, Europe). Aliquots containing 50 μg of proteins were denatured by heating at 95 °C for 5 min in Laemmli buffer, separated by SDS-PAGE (12.5% to test HuC/HuD and 7.5% to test nNOS specificity) and transferred onto a nitrocellulose membrane (GE Healthcare, UK, Europe). After blocking treatment, the membranes were incubated at 4 °C overnight with the primary antibodies (Table 3) diluted in Tris-buffered saline-T20 (TBS-T20 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% *T*-20). After washes, the blots were incubated with respective peroxidase-conjugated secondary antibodies (Table 3). Immunoreactive bands were visualized using chemiluminescent substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific, Italy, Europe). The intensity of luminescent signal was acquired on a *C*-DiGit Chemiluminescent Western Blot Scanner using Image Studio Digits Software Ver 3.1(LI-COR Biotechnology, UK, Europe).

For HuC/HuD antibody, a two band of ~42 kDa and ~40 kDa (theoretical molecular weight 41,770 kDa for HuD and 39,547 kDa for HuC, respectively) (http://www.uniprot.org/) were present in extracts from the ileum (Fig. 1). The band revealed by the two nNOS antibodies showed a molecular weight of ~155 kDa (theoretical molecular weight 160,970 kDa) in the ileum (Fig. 1 A). The blotting of the monoclonal antibody (mouse anti nNOS) was more clear and defined compared to that