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ORIGINAL ARTICLE

Reticular Groove of the Domestic Ruminants: Histochemical and Immunocytochemical Study

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Summary

The reticular groove mucosa of adult cattle, buffalo and sheep was investigated by histochemical and immunocytochemical techniques. Intense NADPH-d staining was observed in the folds of the epithelium mucosa and at the bottom of the reticular groove in all domestic ruminants studied. The NADPH-d staining showed that the innervations of the tunica muscularis of the reticular groove lip were composed of nerve corpuscles, nerve fibres and nerve cells of the mucosa epithelium. SEM analysis showed an intense nitric oxide synthase (NOS) I immunoreactivity in deep and medium cellular layers. It is interesting to note that the same morphologies were observed in samples of the mucosa epithelium, and of the tunica muscularis processed by NADPH-d and in those processed by immunogold techniques. This study has demonstrated that nitric oxide (NO) is involved in the rumination activity and that it plays a double role in this activity in the reticular groove of all domestic ruminants studied: (1) NO plays a role similar to the one it has in the mucosa epithelium of all the other compartments of the ruminant forestomach, (2) The lip sections of the reticular groove has shown abundant innervations that may indirectly coordinate and control the forestomach motility through the direct activation of the nitrergic (nitroxidergic) nerve cells and nerve fibres.

Introduction

The reticular groove (*Sulcus reticuli*) is a groove along the upper margin of the forestomach of domestic ruminants, which can be closed into a channel conducting liquid foodstuffs past the fermentation chamber directly into the regions where digestion takes place. The reticular groove extends from the cardiac orifice to the reticulo-omasal orifice along the inside of the lesser curvature. In neonatal ruminants, only ingested fluids pass through the reticular groove, whereas in adult ruminants, both fluids and solids pass through. In particular in the reticular groove of adult ruminants, there is a mechanism that actuates the reflex function necessary for the digestion of solids. Many studies have been carried out regarding the morpho-functional characteristics of reticular groove, which reinforce the hypothesis that the reticular groove plays a fundamental role in the digestion of fluids and solids.

Habel (1956), Morrison and Habel (1964) and more recently Chiocchetti et al. (2003) have studied the morphology of the innervation of the ruminant stomach and hypothesized on the basis of their observations that the myenteric plexus coordinates the movements of the entire stomach in response to vagal impulses received in the region of the reticular groove. They hypothesized that the reticular groove is the coordinating centre for all the movements of all the compartments of the forestomach. This latter hypothesis is partially supported by the research of Paino et al. (1976) regarding the innervation and their pattern of distribution in the reticular groove of ruminants. In their morphological study of the lamina propria in the reticular groove of cattle and buffalo, these authors observed free arabesque-like nerve terminations and Ruffini's corpuscles. Successive histochemical studies of the forestomach of cattle (Kitamura et al., 1986, 1987), sheep (Whatuta, 1986) and goat (Yamamoto et al., 1994)

found biologically active peptides in the forestomach innervation, thus adding support to the above hypothesis. Ruckebusch (1989) reported that in the ruminant forestomach, motility is strongly influenced by parasympathetic and sympathetic nerves. Moreover, for Kitamura et al. (1986) and Pfannkuche et al. (2002a), the motility of forestomach ruminants is also controlled by myenteric neurons, based on the release of the specific combinations of excitatory and inhibitory neurotransmitters. The basis for this modulation is circuits of cholinergic and nitrergic neurons within the myenteric plexus (Brookes, 2001a). In addition, another immunohistochemical study of the intrinsic innervations of the myenteric neurons in the sheep forestomach (Pfannkuche et al., 2003) showed the presence of a nitrergic neuron population in both the reticulum and the reticular groove. In fact, these neurons immunoreactive for nitric oxide synthase (NOS) suggested that this specific neurochemical code is combined with a distinct morphology.

Recently, Lalatta-Costerbosa et al. (2011) reported the presence of NOS and some neurochemical markers on the enteric nervous system along the entire length of the RG of suckling lambs.

Moreover, Scala et al. (2011) in the forestomach mucosa in adult cattle, buffalo and sheep employing NADPH-d and NOS I detected the presence of the nitric oxide (NO), an important signalling molecule in the innervation of mammals that serves to facilitate communication between neighbouring cells (Toda and Okamura, 2003).

Then, the aim of the present study is to investigate the above hypothesis by studying the presence and the distribution of nicotinamide dinucleotide phosphate diaphorase (NADPH-d) and neuronal NOS I in various zones of the reticular groove in adult cattle, buffalo and sheep using histochemical and immunocytochemical techniques.

Materials and Methods

Animals

The reticular grooves were collected immediately after slaughter from six adult cattles (*Bos taurus*), six adult buffalos (*Bubalus bubalis*) and six adult sheeps (*Ovis aries*) of both sexes. The reticular grooves were divided into three parts: proximal third, middle third and distal third. The specimens of each three parts were studied using different techniques.

Histochemistry

The specimens were washed in 0.1 M PBS, transferred into a graded series of saccharose (10, 20, 30%), immersed in Tissue Teck OCT compound (Sigma Chem. Co, St. Louis, MO, USA), frozen in liquid nitrogen and sectioned by a cryostat. To measure NADPH-d activity, sections were incubated with 0.25 mg/ml nitro blue tetrazolium, 1 mg/ml NADPH and 0.5% Triton X-100 in 0.1 M Tris–HCl buffer, pH 7.3, at 37°C for 10–15 min in a dark box or at room temperature for 30 min. The reaction was stopped by sample immersion in 0.1 M Tris–HCl buffer. Finally, sections were mounted on cover slips, examined under a light microscope (Orthoplan; Leitz GmbH, Wetzlar, Germany) and photographed. Control sections included incubation in media in which substrate was omitted, and preincubation with the sulphydryl inhibitor, 5.5'-dithiobis-2-nitrobenzoic acid. Neither of these controls produced positive immunosignals.

Immunogold-labelling SEM analysis

For the immunogold-labelling SEM analysis, the specimens were immediately immersed in PBS for 1 h. The samples were incubated for 2 h with a solution containing normal goat serum (X 0907; Dako Italia s.p.a., Milan, Italy) diluted 1:10 in PBS, and next with a primary polyclonal antibody directed towards NOS I (AB5380; Chemicon, Temecula, CA, USA), diluted 1:1500 in PBS, overnight at 4°C. After washing in PBS, the samples were incubated with gold-conjugated goat anti-rabbit IgG (E.M.GAR10; Agar Scientific Limited, Stansted, UK) diluted 1:200 in PBS, for 1 h at room temperature. The secondary antibody was conjugated with gold particles of different sizes, namely 5 and 15 nm. After washings in PBS, samples were fixed by 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing $CaCl_2$, pH 7.2, for 30 min. After the fixation step and washings with distilled water, samples were subjected to silver enhancement (British BioCell International, Cardiff, Wales, UK). The enhancement process enables the use of antibodies conjugated to smaller (1–5 nm) gold particles, preserving the advantage of faster penetration and higher labelling efficiency (Owen et al., 2002). Next, samples were dehydrated through an ethanol series and dried to the critical point. The specimens, mounted on stubs, were examined under a LEO 435 VP scanning electron microscope at variable pressure (80–120 Pa) in the backscattered electron mode, which allows the detection of gold particles associated with cells even if they are located intracellularly (Richards et al., 2001). The samples were not coated with gold–palladium, so that the only conjugated gold derived from immnunocytochemical reaction was observed under SEM and photographed. No immunoreactivity was observed in reticular groove samples treated with PBS substituting the primary antibody (negative control).

Western blotting

The specimens were homogenized by an Ultraturrax L-407 at 4°C with 5 ml/1.5 g tissue of buffer containing 50 nM Tris–HCl, pH 7.4 150 nM NaCl, 1 nM ethylenediaminetetraacetic acid, 10 nM NaF, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulphate (SDS), 1% nonidet P-40, 1 Nm phenylmethylsulfonyl fluoride, 0.1 U/ml aprotinin, 10 mg/ml leupeptin and 1 mM Na_3VO_4. Homogenates were centrifuged at 15 000 ***g*** for 20 min at 4°C. Supernatants were divided into small aliquots and stored at −80° C until used. The amount of total proteins in each sample was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples containing equal amount of proteins were boiled for 5 min in SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 5% b-mercaptoethanol) and run on a 10% SDS/polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose using a Mini Trans-Blot apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions. Membranes were blocked for 1 h at room temperature with TBS-T buffer (150 mM NaCl, 20 mM Tris–HCl, pH 7.4, 0.1% Tween-20) containing 5% milk. The blots were incubated overnight with a rabbit polyclonal antibody raised against a peptide mapping the 724 –739 amino acid sequence of human brain NOS I (AB1632; Chemicon International Inc.) diluted 1:1000 in TBS-T containing 2.5% milk. After incubation, the membranes were washed three times with TBS-T and incubated for 1 h with horseradish per-oxidase-conjugated anti-(rabbit IgG) Ig (Sigma Chemical).

All nomenclature in this work was adopted from *Nomina Anatomica Veterinaria*, *Nomina Histologica* and *Nomina Embryologica Veterinaria* (World Association of Veterinary Anatomists, 2005).

Results

Histochemistry (LM)

Intense NADPH-d staining was observed in mucosa epithelium and in tunica muscularis in each of the three parts (proximal, middle and distal) of the reticular groove examined. More specifically, intense staining was observed in the cells of the medium and deep layers of the mucosa epithelium, and in the endothelium of the lamina propria subepithelial blood capillaries. The cells of the medium and deep layers were stained in the cytoplasm, but not in the nucleus. There was no difference in staining intensity or distribution among the animals studied.

In all domestic ruminants studied, the same intensity of NADPH-d staining was observed in the folds of the epithelium mucosa and at the bottom of the reticular groove (Fig. 1). In addition, the NADPH-d staining showed that the innervation of the tunica muscularis of the reticular groove lip was composed of single and double nerve corpuscles, single and double nerve fibres, and neurons (nerve cells). The single and double nerve corpuscles were always wrapped around a muscle fibre. The double corpuscles were connected to each other by a nerve fibre (Fig. 2a,b).

In the connective tissue, all the single nerve fibres formed an endomysium throughout the muscle tissue, and all the double nerve fibres formed individual bundles in the smooth muscle cells (Fig. 2c). Bipolar neurons were found in the fibres of the loose connective tissue

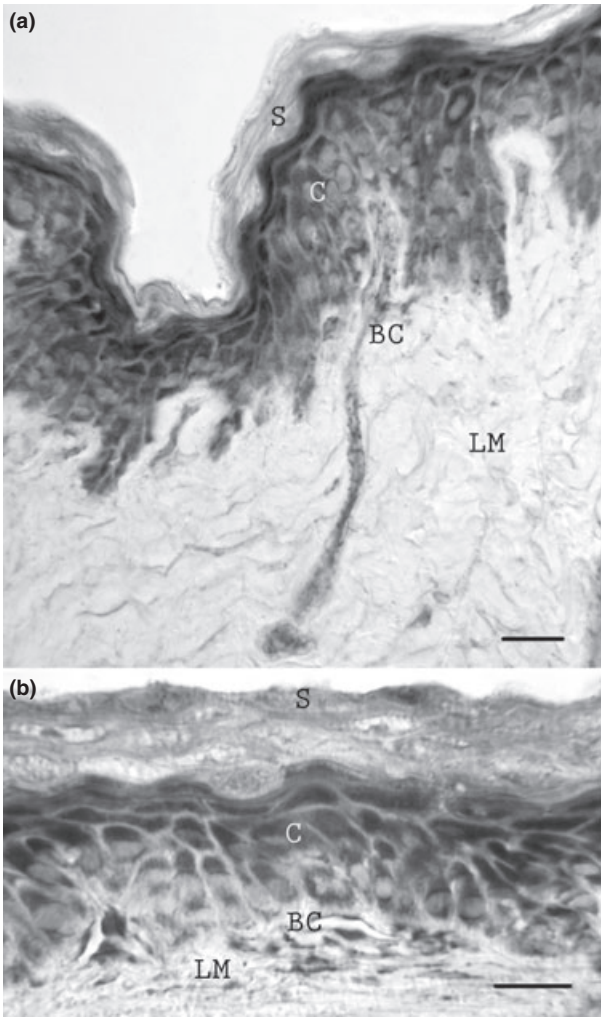

Fig. 1. Light microscopy (LM) NADPH-d of the reticular groove mucosa epithelium of the cattle: (a) Fold mucosa; (b) bottom mucosa. BC, blood capillary; C, epithelial cells of medium layer; LM, lamina propria of the mucosa; S, squamous cells (*stratum corneum*). Scale bars = 10 μm.

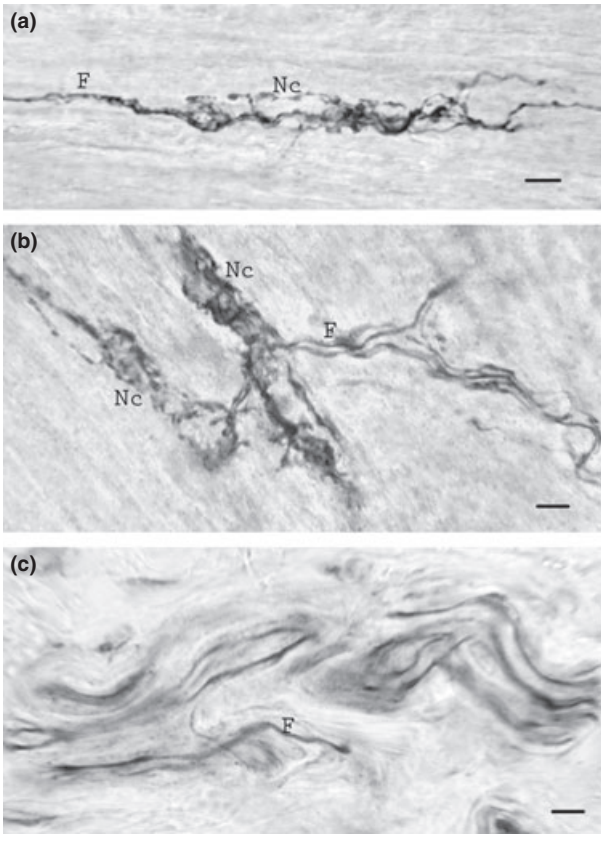

Fig. 2. Light microscopy (LM) NADPH-d of the nerve corpuscles and nerve fibres contained in the *tunica muscularis* of the reticular groove lip. (a) Single elongated nerve corpuscle in cattle reticular groove; (b) Double elongated nerve corpuscles in buffalo reticular groove; (c) Nerve fibres in sheep reticular groove. F, nerve fibres; Nc, nerve corpuscle. Scale bars = 10 μm.

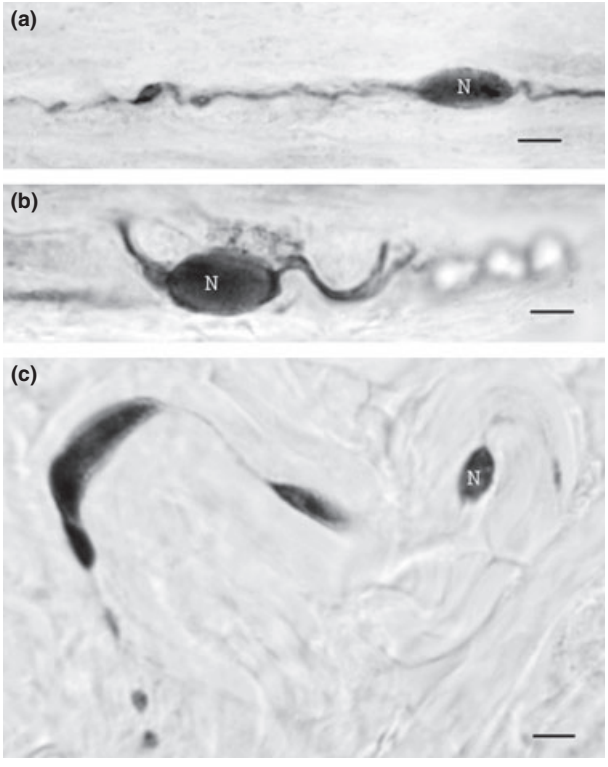

Fig. 3. Light microscopy (LM) NADPH-d of the neurons contained in the loose connective tissue surrounds a group of muscle cells in the *tunica muscularis* of the reticular groove. (a) Bipolar neuron in cattle reticular groove; (b) bipolar neuron in buffalo reticular groove; (c) rosary bead neurons in the sheep reticular groove. N, neuron. Scale bars = 5 μm.

that separated the bundles of smooth muscle cells. Each neuron had an ovoid cell body and two processes, which emerged from the cell body at opposite ends. Frequently, these processes made contact with the blood capillaries of the smooth muscle cells. Sometimes, the bipolar neurons formed rosary beads. In addition, a small cell body was sometimes present along a process of a bipolar neuron (Fig. 3).The muscle cells showed an intense staining of the sarcolemma.

Immunogold-labelling SEM analysis

Intense NOS I immunoreactivity was detected in the reticular groove of all domestic ruminants studied with the immunogold-labelling SEM technique. The epithelium that covered the mucosa showed an intense and uneven NOS I immunoreactivity in deep and medium cellular layers, whereas the sarcolemma of the lip muscle cells showed a moderate and uniform NOS I immunoreactivity

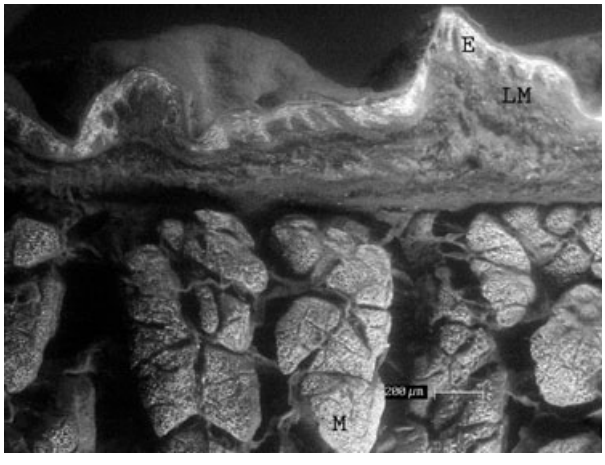

Fig. 4. Scanning electron microscopy (SEM) immunogold of the cattle reticular groove. Intense nitric oxide synthase I (NOS I) immunoreactivity located in the mucosa and in the muscle cells of the left lip (*Labium sinistrum*). E, Epithelium of mucosa; LM, lamina propria; M, muscle cells of the *tunica muscularis*.

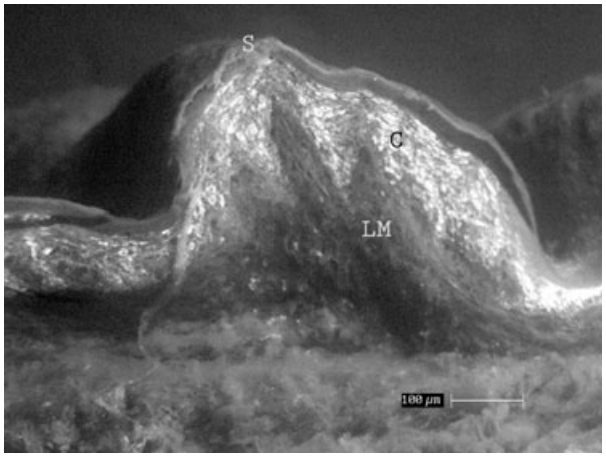

Fig. 5. Scanning electron microscopy (SEM) immunogold of the cattle reticular groove. Nitric oxide synthase I (NOS I) immunopositivity located in the mocosa epithelium. C, cells of the medium and deep layers of the mucosa epithelium; LM, lamina propria; S, *stratum corneum* of the mucosa epithelium.

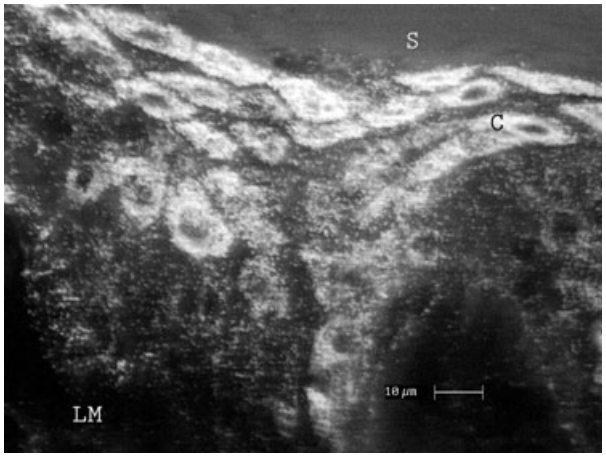

Fig. 6. Scanning electron microscopy (SEM) immunogold of the buffalo reticular groove mucosa. Intense nitric oxide synthase I (NOS I) immunopositivity located in the cells of the medium and deep layers of the mucosa epithelium; C, cells of the medium and deep layers of the mucosa epithelium; LM, lamina propria; S, *stratum corneum* of the mucosa epithelium.

(Fig. 4). A few areas of the mucosa epithelium showed an intense immunoreactivity characterized by a positive cytoplasm labelling in the cells of the medium layer. No positivity was observed in the nucleus of these cells.

The cytoplasm of the cells located in the deep and medium layers of the lip folds showed an immunoreactivity composed of dense gold particles (Figs 5–7).

It is interesting to note that the same morphologic characteristics were observed in samples of the mucosa epithelium processed by NADPH-d, and in those processed by immunogold techniques (Fig. 8).

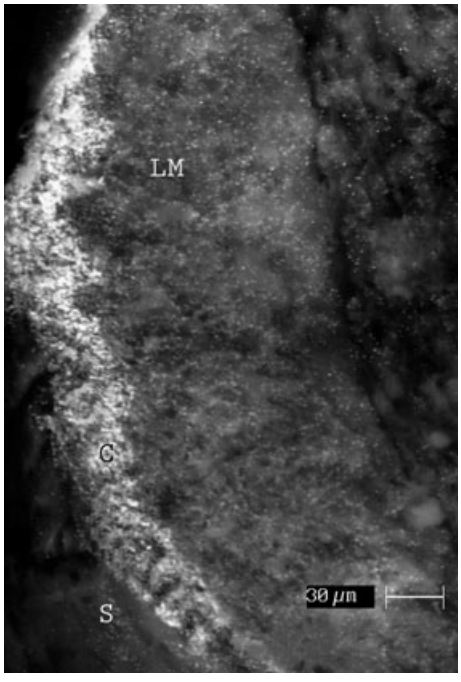

Fig. 7. Scanning electron microscopy (SEM) immunogold of the sheep reticular groove mucosa. Intense NOS I immunoreactivity located in the mucosa epithelium of the left lip. C, cells of the medium and deep layers of the mucosa epithelium; LM, lamina propria; S, *stratum corneum* of the mucosa epithelium.

The expression of NOS I in the reticular groove was confirmed by Western blotting (see Scala et al., 2011).

Discussion

The present study utilizing NADPH-d histochemical, immunogold and Western blotting techniques has demonstrated that NO is involved in the rumination activity in the reticular groove of the domestic ruminants studied. In particular, NO plays a double role:

1. In the mucosa epithelium of the reticular groove, NO plays a role similar to the role it has in the mucosa epithelium of all the other compartments of the ruminant forestomach (Scala et al., 2011). In short, NO modulates oxygen consumption in the mitochondria of the basal cells present in the mucosa of the entire forestomach epithelium. NO accomplishes this concentration by inhibiting the cytochrome *c* oxidase, which is the terminal haem-containing enzyme in the mitochondrial respiratory chain. The fact that the modulation activity carried out by NO is inversely dependent upon the concentration of oxygen in the mucosa epithelium suggests that NO plays a crucial role in the generation of cellular energy and in

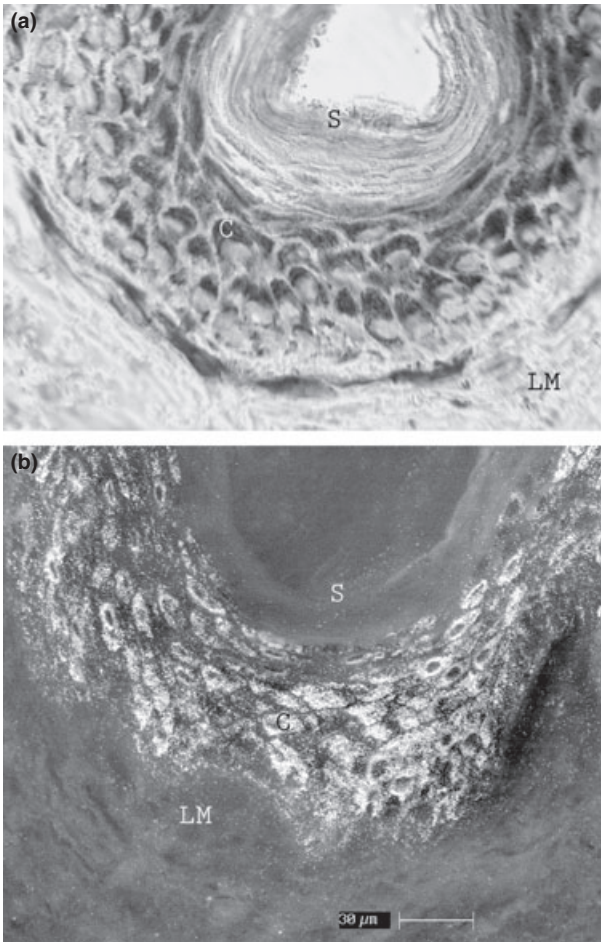

Fig. 8. Epithelium of mucosa fold in buffalo reticular groove. Comparison between light microscopy NAPDH-d and scanning electron microscopy NOS I. (a) Light microscopy NAPDH-d mucosa; (b) scanning electron microscopy NOS I. C, Cells of the medium and deep layers of the mucosa epithelium; LM, lamina propria; S, *stratum corneum* of the mucosa epithelium.

the mediation of apoptosis (Beltran et al., 2000; Castello et al., 2006, 2008).

2. NO has been shown to be a signalling molecule involved in the regulation of the central nervous system (CNS) and the peripheral nervous system (PNS). In the PNS, NO is involved not only in signalling, but also in the release of a combination of messenger chemicals depending on factors such as physical stimuli and innervated tissue. The present study has shown that the lip sections of the reticular groove contained an abundant innervation composed of single and double nerve corpuscles, single and double nerve fibres, and neurons. This innervation may indirectly coordinate and control the forestomach motility through the direct activation of the nitrergic (nitroxidergic) nerve cells and nerve fibres, that is, the lip movements of the reticular groove. NO plays an important role as an inhibitory neurotransmitter of the peripheral non-adrenergic, non-cholinergic (NANC) nerves, namely the peripheral nitrergic nerves. These nerves are widely distributed in the viscera and are particularly important because they produce a relaxation of the smooth muscle in the gastrointestinal tract.

Many researchers have confirmed the signalling role played by NO: (1) several morpho-functional studies have shown that free NO is the transmitter substance released by nitrergic nerves (Lilley and Gibson, 1997) and (2) several functional studies have shown that neuronal production of NO is involved in many physiological and pathophysiological reflexes that correlate to changes in muscle relaxation of the gastrointestinal tract (Barrachina et al., 2001). A further negative confirmation of the signalling role of NO is shown by the fact that the dysfunction of the inhibitory NANC nerves in the lower oesophageal sphincter result in a motility disorder (Mearin et al., 1993), and the said dysfunction is probably involved in oesophageal spasms and related primary motor disorders in the oesophageal body (Yamato et al., 1992).

The biochemistry of the NO activity in playing these roles is complex. NO in the PNS is the intercellular messenger that mediates the increase in the cyclic guanosin monophosphate (cGMP) level that follows the activation of glutamate receptors (Garthwaite et al., 1988). Once inside target cells, NO binds the haem iron contained within the active site of soluble guanylyl cyclase and activates this enzyme to form the small cellular mediator cyclic guanosine monophosphate (cGMP). The cGMP activates a cGMP-dependent protein series (kinase, phosphodiesterase and some membrane channels). Both cGMP and cGMP-dependent protein kinase have been found within the cytoplasm of the reticular groove mucosa epithelial cells. Moreover, the activation of cGMP-dependent protein kinase controls Ca++ homoeostasis, which in turn influences the NO/ Ca++ ratio. This NO–Ca++ interaction is very important in the regulation of the vascular tone: in fact, an increase in the Ca++ concentration stimulates the activation of the kinase in the light-chain myosin of the cytosol and brings on the contractions of the vascular smooth muscle (Moncada and Higgs, 2006; Burkard et al., 2007). Thus, NO plays a significant role in the inhibitory phase of the forestomach motility, and the reticular groove plays a central role in the coordination of the gastric movements (Vittoria et al., 2000).

With the exception of that observed in the domestic ruminants forestomach, the reticular groove in these species shows intense NOS I immunoreactivity. Moreover, the muscle cells of the tunica muscularis present an

evident NADPH-d staining and a moderate NOS I immunoreactivity. Therefore, the NO plays an important role in not only delaying the onset of mucosa epithelial cells but also regulating the nitrergic innervations of the reticular groove smooth musculature (Scala et al., 2011).

In conclusion, according to Habel and more recently Chiocchetti et al. (2003), the reticular groove not only coordinates the rumination activity in the central and peripheral regions of the forestomach, but also might better orchestrate the control of all rumination motility throughout the entire forestomach, similar to the way a director of orchestra directs all his/her musicians to produce a work of beauty.

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