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Histological and immunohistochemical studies of changes in myenteric plexuses and in interstitial cells of Cajal associated with equine colic

S. Pavone [a,*], R. Gialletti [b], M. Pepe [b], A. Onofri [c], M.T. Mandara [a]

[a] Department of Biopathological Science and Hygiene of Animal and Food Productions, Faculty of Veterinary Medicine, University of Perugia, Perugia, Italy
[b] Department of Veterinary Pathology, Diagnostic and Clinic, Faculty of Veterinary Medicine, University of Perugia, Perugia, Italy
[c] Department of Agricultural and Environmental Sciences, Faculty of Agriculture, University of Perugia, Perugia, Italy

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ABSTRACT

In this study we investigated the histological changes of the myenteric plexuses and interstitial cells of Cajal (ICC) in gut samples from horses with colic to try to find results useful in the prognostic evaluation of enteric lesions.

A morphologic and quantitative study of myenteric ganglia, ganglion cells and neuronal chromatolytic and necrotic changes of 24 horses with colic was performed. For ganglion cells, enteroglial cells and ICC immunolabeling was also performed to identify cell functional disorders.

A significant increase of neuronal chromatolysis and necrosis occurred in horses suffering from colic throughout the gut. The neuron-specific enolase (NSE) and synaptophysin immunoreaction quantified with image analysis showed a significant loss of neuronal activity in all intestinal tracts of the animals under study associated with a significant loss of ICC immunoreactivity.

The results supports immunohistochemical evaluation of ENS and ICC as a useful tool along with morphometric investigations in the evaluation of gut lesions produced during colic syndrome.

1. Introduction

Gastrointestinal motility disorders constitute a significant cause of morbidity and mortality in the horse (Tinker et al., 1997). For these reasons research is focussing on the aetiology, clinical signs, diagnostic and treatment procedures and preventive measures to reduce colic incidence in this species. The majority of these cases reflects poor management practices: inappropriate parasite prevention programs, abrupt changes or low quality in nutrition, insufficient water supply, poor trailering techniques, infrequent dental care or pasturing in sandy paddocks (Burns et al., 1990). However, sometimes well-nurtured horses with a history of chronic impaction colic are presented to the equine practitioner. In some cases, spontaneous remission of the colic syndrome or resolution by medical treatment prevents a definitive diagnosis to be made (Burns et al., 1990; Fintl et al., 2004). Moreover, also for horses undergoing exploratory surgery the cause of the lesion may not be evident at surgery. Indeed, while some strangulating conditions in the small intestine recognize well-defined physical causes, such as a pedunculated lipoma, a number of conditions, such as large colon displacements or torsions, simple pseudo-obstructions and impactions, abnormal motility patterns remain without an evident proved organic disorder (Fintl et al., 2004). This is why it is often difficult to formulate post-surgery prognosis. Even though clinical and surgical investigations can contribute to making a prognosis in horses suffering from colic, they often fail to establish the actual ability of the intestine to completely recover its motility after pharmacological and surgical treatments.

Data in the literature are somewhat incomplete and insufficient as to the exact role played by the Enteric Nervous System (ENS) in the pathogenesis of equine colic syndrome.

Previously published data report a statistically significant reduction in the number of myenteric ganglia in horses with colic caused by strangulation of the large intestine (Schusser and White, 1997), or a decrease of neuronal cells in horses with chronic recurrent caecal impaction (Schusser et al., 2000). In some cases a significant decreased number of myenteric and submucosal ganglia, and of neuronal cells have been reported associated with an increase of the chromatolysis process in small intestine and small colon of horses affected by grass sickness (Doxey et al., 1995). Chronic idiopathic intestinal pseudo-obstruction (CIIP) with loss of myenteric ganglia and neuronal cells with severe central chromatolysis and cytoplasmatic vacuolization (Burns et al., 1990), and aganglionosis in the progeny of overo spotted horses have also been observed (Hultgren, 1982; Vonderfecht et al., 1983).

In the present study we investigated the histological changes of the myenteric plexuses and interstitial cells of Cajal (ICC) applying

* Corresponding author. Address: Department of Biopathological Science and Hygiene of Animal and Food Productions, Faculty of Veterinary Medicine, Via S. Costanzo No. 4, 06126 Perugia, Italy. Tel.: +39 075 5857771; fax: +39 075 5857738.
E-mail address: pavone.silvia@libero.it (S. Pavone).

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morphometric and immunohistochemical evaluations in gut samples from horses with colic to try to find results usable as a platform for further studies on prognostic evaluations of equine enteric lesions.

2. Material and methods

2.1. Animals

Five regularly slaughtered horses, from 8 months to 10 years of age, were used as controls. Ante mortem inspection was performed and inspector passed it as suitable for slaughter. Post-mortem gross examination of gastrointestinal tract excluded any macroscopic intestinal lesions.

Sixteen horses with colic, from 2 to 18 years of age, were submitted to a bioptic sampling during surgery. Four of these horses were affected by obstructive disorders of the small intestine, including Epiploic Foramen Entrapment (EFE) ($n = 2$), inguinoscrotal hernia ($n = 1$), ileocaecal intussusception ($n = 1$). Three horses were affected by an inflammatory small intestine disease reported as Proximal Enteritis (PE) ($n = 2$) and Granulomatous Enteritis (GE) ($n = 1$). The remaining cases consisted in Large Colon Accident (LCA) corresponding to displacement and/or torsion of the large colon ($n = 9$).

Finally, a group of eight horses with colic, from 4 months to 23 years of age, were euthanized due to unsuccessful medical and surgery treatment and submitted to a necropsy sampling. Four of them suffered from obstructive disorders of the small intestine including ileocaecal intussusception ($n = 1$), EFE ($n = 2$), and Meckel's Diverticulum (MD) ($n = 1$). Only one case of inflammatory small intestine disease was reported as parasitic enteritis caused by Ascaridae family. A case of inflammatory large intestine disease consisted in Granulomatous Colitis (GC) caused by *Rhodococcus equi*. Lastly, a case of LCA and a case of Primary Large Colon Impaction (PLCA) were also included in this study.

The clinical course of the colic syndromes ranged from 2 to 72 h.

2.2. Sample collection and preparation

After gross gastrointestinal examination, tissue samples from animals in the control group were collected within 30 min after slaughter from nine gut segments, including jejunum, ileum, ileocaecal junction, caecal body, right ventral colon, left ventral colon, pelvic flexure, left dorsal colon, right dorsal colon, and descending colon. All samples, $15 \times 10\ cm^2$ in size (longitudinal $\times$ transverse measurements), were taken from the antimesenteric area, in the middle of each segment, excluding taenial bands.

In the eight horses with colic submitted to euthanasia, tissue samples were collected within 4 h after death from the same areas. Finally, in the 16 horses with colic submitted to surgery, after colon decompression, full-thickness gut biopsy samples, 3.5×2.5 cm in size, were collected from the pelvic flexure, except for the ileocaecal intussusception case which was submitted to a double sampling from ileum and jejunum.

All tissue samples were rinsed in running water and placed immediately in 10% neutral buffered formalin for 72 h. For routine paraffin embedding, the tissue samples were oriented to ensure the circular muscle layer of the muscularis externa was cut parallel to the fibre direction. This orientation allowed better visualisation of ICC as well as standardising the method as previously used (Pavone and Mandara, 2010). From each sample, 5-μm serial paraffin embedded sections were prepared. The first 10 sections were retained, the next five discarded, the next 10 retained, and so on until there were three groups of 10 consecutive sections. Within each group, one section was selected at random for each histochemistry staining and immunohistochemistry labeling while two sections were left unstained. HE was performed to confirm the absence of histopathological lesions in the control horses and to evaluate histopathological lesions in horses suffering from colic. To perform evaluations on specific elements of ENS, cresyl violet stain was also used. Van Gieson trichrome stain was used to reveal perigangliar fibrotic event. In addition, Giemsa staining was used to evaluate the presence and localization of metachromatic mast cells in all the sampled intestinal tracts. Immunolabeling was performed with avidin–biotin complex (ABC) method to evaluate the neuronal functionality and enteroglial and ICC density, as previously standardized (Pavone and Mandara 2010). Consequently, primary antibodies anti-neuron-specific enolase (NSE, 1:200), anti-synaptophysin (1:30), anti-glial fibrillary acidic protein (GFAP, 1:500), and anti-CD117 (c-Kit, 1:200) were used as previously reported in horses (Sisó et al., 2003; Hilbe et al., 2005; Porter et al., 2007; Muravnick et al., 2009; Pavone and Mandara, 2010) (Table 1). After deparaffinization and rehydration, antigen retrieval was performed by microwave for 20 min in Tris–EDTA buffer solution (10 mmol/L Tris Base, 1 mmol/L EDTA, pH 9.0) for antibodies against CD117 and synaptophysin, in sodium citrate buffer (10 mmol/L Sodium Citrate, pH 6.0) for antibodies against GFAP and NSE. Endogenous peroxidase was blocked using 3% hydrogen peroxide in water for 5 min at room temperature. Afterwards, the slides were covered with primary antibodies for 1 h in a humidified chamber at room temperature. Immunoreactivity was revealed by the avidin–biotin method (LSAB+/System-HRP, Dakocymation Glostrup, Denmark) using aminoetile-carbazole substrate (AEC + Substrate-Chromogen Ready-to-use, Dakocymation). Carazzi's haematoxylin was used as a counterstain. Faramount Mounting Medium (Dakocymation) was used to mount coverslips on slides. Negative controls were performed in the same manner, omitting the primary antibody. This procedure led to the complete absence of immunolabeling.

2.3. Histologic evaluation

Myenteric plexuses were identified as neurons clustered in round to oval structures between circular and longitudinal muscle layers with distinct borders surrounded by dense intermuscular connective tissue.

Neuronal cells were identified as large cells with abundant basophilic granular cytoplasm (Nissl substance) with a large round to oval nucleus and prominent nucleolus in HE staining slides.

Table 1
Antibodies used to immunostain Enteric Nervous System elements and interstitial cells of Cajal.

Antibody	Company	Clone	Code No.	Immunogen	Dilution
Polyclonal rabbit anti-human CD117, c-Kit	DakoCytomation	–	A 4502	Cytoplasmic C-terminal part of c-Kit	1:200
Polyclonal rabbit anti-glial fibrillary acidic protein	DakoCytomation	–	Z 0334	GFAP isolated from cow spinal cord	1:500
Monoclonal mouse anti-synaptophysin	DakoCytomation	SY38	M 0776	Crude fraction of coated vesicles from bovine brain	1:30
Monoclonal mouse anti-human human Neuron-Specific Enolase (NSE)	DakoCytomation	BBS/NC/VI-H14	M 0873	γγ-enolase purified from human brain	1:100