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A Study of the Interstitial Cells of Cajal in Aged Donkeys with and without Intestinal Disease

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Summary

Intestinal tissue samples were collected during routine post-mortem examinations from 12 aged donkeys. Six animals were euthanised due to impaction of the pelvic flexure of the large colon, while the remaining six were euthanised for non-enteric reasons such as dental or orthopaedic disease. Immunohistochemical labelling was performed to demonstrate the gastrointestinal pacemaker cells, the interstitial cells of Cajal (ICC), with polyclonal c-Kit antibodies. The distribution and density of the cellular networks were assessed qualitatively and semi-quantitatively. ICC networks are present in the donkey, with distribution similar to that of the horse, and they remain strongly immunoreactive in the older animal. There was no difference in the density and distribution of ICC in animals with or without intestinal disease.

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Keywords: donkey; immunohistochemistry; interstitial cells of Cajal; intestine
Seven animals were euthanised due to colic and five were euthanised due to lameness or advanced dental disease (Table 1). All animals with colic had been given various forms of empirical medical treatments including oral laxatives, oral or intravenous fluid therapy and intravenous non-steroidal anti-inflammatory analgesic drugs. The advanced age and poor physical condition of these animals rendered them poor surgical candidates. Six animals were shown to have pelvic flexure impaction at post-mortem examination. The dental condition of each animal was also assessed and graded as good, moderate or poor depending on the number of teeth missing, the quality and extent of functional occlusal surfaces and the presence of gingival disease.

A segment of ileum level with the midpoint of the ileocaecal fold and a sample of the pelvic flexure from the junction between the left ventral colon and left dorsal colon were collected from all animals apart from donkey 10, from which only a section of pelvic flexure was collected. Following collection, all samples were immediately placed in 10% neutral-buffered formalin and fixed for at least 24 h. The fixed tissue samples were rinsed in running tap water for 1 h prior to placing them into graded sucrose solutions (10% and 30% sucrose in phosphate buffered saline; PBS) for cryoprotection. The samples were then frozen rapidly in isopentane (BDH Laboratory Supplies, Poole, UK) pre-cooled in liquid nitrogen or dry ice and subsequently sectioned (10 and 20 μm). All sections were mounted on slides pre-coated with 3-aminopropyltriethoxysilane (Sigma Aldrich, Poole, UK) and allowed to air-dry overnight. After washing the sections in PBS, they were incubated for 30 min in hydrogen peroxide 0.3% in methanol to quench endogenous peroxidase activity. The sections were then incubated for 1 h in 1% goat serum (Vector Laboratories, Burlingame, California) to block non-specific antibody binding. After subsequent washes with PBS, the sections were incubated overnight at 4°C in a humid chamber with rabbit polyclonal antiserum to c-Kit (Ab-1, Oncogene Research Products, Cambridge, Massachusetts) at a concentration of 1 μg/ml. The tissue sections were then washed with PBS prior to incubation (for 1 h) with biotin-conjugated goat anti-rabbit immunoglobulin (Vector Laboratories) at a concentration of 1 in 200. Immunoreactivity was ‘visualized’ using the avidin–biotin complex method (Vectastain Elite ABC Kit, Vector Laboratories) with 3,3’-diaminobenzidine as substrate (BDH Laboratory Supplies). Sections were dehydrated in ethanol, cleared in xylene and mounted under DPX (Merck, Glasgow, UK).

Primary antibody was replaced by normal rabbit serum for negative control sections. A tissue sample from a normal horse from a previous study (Fintl et al., 2004) that had been shown to have an abundance of c-Kit-immunoreactive ICC was used as a positive control.

The ICC were differentiated from round c-Kit-immunoreactive mast cells (located predominantly in the submucosa) by their morphology (e.g. presence of cellular processes in ICC) or by staining parallel sections with toluidine blue to demonstrate mast cell metachromasia (Hudson et al., 1999).

Two independent observers unaware of the origin of the samples assessed and graded all tissue sections. The grading for each animal was based on scrutiny of at least four adjacent 10 μm sections of tissue (Hudson et al., 2001; Fintl et al., 2004) where c-Kit immunoreactivity was absent (grade 0), sparse (grade 1), moderate (grade 2) or abundant (grade 3). Separate

### Table 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Clinical syndrome</th>
<th>Diagnosis</th>
<th>Dentition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donkey 1</td>
<td>32</td>
<td>F</td>
<td>Lameness</td>
<td>Laminitis</td>
<td>Moderate</td>
</tr>
<tr>
<td>Donkey 2</td>
<td>26</td>
<td>G</td>
<td>Neurological signs</td>
<td>Unknown</td>
<td>Moderate</td>
</tr>
<tr>
<td>Donkey 3</td>
<td>31</td>
<td>G</td>
<td>Dental</td>
<td>Nephrosis</td>
<td>Poor</td>
</tr>
<tr>
<td>Donkey 4</td>
<td>28</td>
<td>F</td>
<td>Renal failure</td>
<td>Nephrosis</td>
<td>Poor</td>
</tr>
<tr>
<td>Donkey 5</td>
<td>26</td>
<td>F</td>
<td>Respiratory signs</td>
<td>Pneumonia</td>
<td>Moderate</td>
</tr>
<tr>
<td>Donkey 6</td>
<td>26</td>
<td>F</td>
<td>Colic</td>
<td>CLF</td>
<td>Poor</td>
</tr>
<tr>
<td>Colic group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donkey 7</td>
<td>27</td>
<td>G</td>
<td>Weight loss</td>
<td>PFI</td>
<td>Poor</td>
</tr>
<tr>
<td>Donkey 8</td>
<td>35</td>
<td>G</td>
<td>Colic</td>
<td>PFI</td>
<td>Poor</td>
</tr>
<tr>
<td>Donkey 9</td>
<td>33</td>
<td>M</td>
<td>Suspected liver disease</td>
<td>PFI</td>
<td>Poor</td>
</tr>
<tr>
<td>Donkey 10</td>
<td>32</td>
<td>G</td>
<td>Weight loss</td>
<td>PFI</td>
<td>Poor</td>
</tr>
<tr>
<td>Donkey 11</td>
<td>28</td>
<td>G</td>
<td>Distended abdomen</td>
<td>PFI</td>
<td>Poor</td>
</tr>
<tr>
<td>Donkey 12</td>
<td>27</td>
<td>G</td>
<td>Distended abdomen</td>
<td>PFI</td>
<td>Good</td>
</tr>
</tbody>
</table>

F, female; G, gelding (neutered male); M, male; PFI, pelvic flexure impaction; LI colic, large intestinal colic; CLF, chronic liver fibrosis.
evaluation of the myenteric and circular muscle regions was carried out for all samples. In the event of disagreement of grading between the two observers, a consensus grade was assigned after joint review of the sections. Density grades and inter-observer variability were assessed using the Mann–Whitney test assuming a significance level of 5%.

In addition to immunohistochemical labelling, all processed tissues were stained with haematoxylin and eosin (HE) in order to assess tissue integrity and determine whether reduced or absent immunoreactivity was a primary finding or secondary to a degree of tissue autolysis. Only samples with good tissue integrity (and not worse than a mild degree of mucosal degradation) were included in order to ensure that immunohistochemical grading was reliable. As no significant degree of tissue autolysis was present in any of the samples collected, all were included in the study.

All samples were collected with the permission of, and in collaboration with, The Donkey Sanctuary, Sidmouth, Devon, which also owned the animals included in the study. The age range of the control group was 26–32 years (median 27 years, mean 28.1 years) and this group comprised two geldings and four mares. The age range for the donkeys with colic was 27–35 years (median 27.5 years, mean 30.3 years) and comprised one stallion and five geldings.

Immunohistochemical labelling of the small intestinal samples demonstrated a continuous band of c-Kit-immunoreactive ICC within the myenteric plexus region (Fig. 1), making it difficult to identify the shape of individual ICC. There was little extension of ICC into the outer longitudinal muscle layer. No significant difference in the density of c-Kit immunoreactivity in the myenteric plexus region was observed between the small intestinal samples collected from diseased and control animals ($P = 0.41$). There was no significant difference in the density of c-Kit immunoreactivity between the control and diseased groups in the circular layer of the muscularis ($P = 0.72$). There was a mixture of slender bipolar and stellate-shaped cells with the cellular processes orientated parallel to that of the muscle fibres. In addition, a band of stellate or bipolar cells was frequently visible at the submucosal border of this muscle layer.

In the large intestine, the pattern of immunoreactivity differed slightly from that of the ileum, with a delicate lace-like pattern of ICC observed in the myenteric plexus (Fig. 2) without extension of cell processes into the outer longitudinal muscle layer. There was no significant difference in the degree of c-Kit immunoreactivity of the myenteric plexus region between control and diseased animals ($P = 0.47$). Similarly, there was no significant difference in the degree of c-Kit immunoreactivity in the circular muscle layer of the pelvic flexure samples between control and diseased animals ($P = 0.94$). A mixture of slender bipolar and stellate ICC was observed in this region similar to that observed in the small intestine (Fig. 3). However, the cells were uniformly distributed throughout the muscle layer and it was not possible to observe the band of cells at the submucosal border seen in the ileum.

Inter-observer variability of grades given was also assessed in order to evaluate the reliability of the semi-quantitative assessment. There was no significant difference between the tissue grades allocated to the individual cases by the two independent observers ($P = 0.61$), showing good inter-observer agreement.

Histological examination of tissue sections revealed all samples to be of sufficient quality to be included in

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**Fig. 1.** Myenteric plexus region of the ileum of a control donkey. A continuous band of c-Kit-immunoreactive ICC (arrow) can be observed surrounding the myenteric ganglia. LM, longitudinal muscle; CM, circular muscle layer.

**Fig. 2.** c-kit immunoreactive ICC in the myenteric plexus region of the pelvic flexure of a control donkey. The ICC form a lace-like pattern (arrows) surrounding the myenteric ganglia. CM: circular muscle.
the study. A mild to moderate degree of mucosal and submucosal inflammatory cell infiltration was evident in a number of samples (Fig. 4). This infiltrate was predominantly lymphocytic, but a number of eosinophils was also frequently observed. These inflammatory cells did not appear to extend into the muscularis externa. This finding was present in samples collected from both the small and large intestine in both groups. Although not quantitatively or semi-quantitatively assessed, there did not appear to be a difference between diseased and control animals in the degree of cellular infiltration.

To the authors’ knowledge, this is the first study to demonstrate the interstitial cells of Cajal in the intestinal tract of the donkey. ICC in the myenteric plexus of the small intestine have been demonstrated to be the intestinal pacemaker cells in several mammalian species including man, dog and mouse (Sanders, 1996). The distribution of ICC described in this anatomical region in these previous investigations was similar to that observed in the current study. Similarly, both the density and morphology of c-Kit-immunoreactive ICC in the anatomical areas examined were similar to those previously described in the adult horse (Hudson et al., 1999).

There were, however, some minor differences between the horse and the donkey. In the myenteric plexus region, when compared with the horse (Hudson et al., 1999), the donkey ICC did not appear to branch as extensively into the longitudinal muscle layer of the small intestine. Similarly, although the basic ICC morphology (stellate and bipolar cells) in the donkey was similar to that described for the horse, subtle differences in the ICC morphology were evident in the circular muscle layer of the small intestine. Subjective assessment suggested that donkey ICC were more slender, with finer processes, than their counterparts in the horse.

There also appeared to be a band of ICC at the submucosal border of the circular muscle layer in the small intestine, which was broadly comparable to the ICC observed in the horse (Hudson et al., 1999). However, this band appeared to be closer to this inner border than that observed in the horse, where it was located along the inner third of the circular muscle layer (Hudson et al., 1999). This finding

![Fig. 3. c-Kit immunoreactive ICC (arrows) in the circular muscle of the pelvic flexure in a donkey from the diseased group.](image_url)

![Fig. 4. (a) Mucosa and submucosa of ileum of a control donkey. There is a moderate infiltration of inflammatory cells, including eosinophils. (b) Detail of the boxed area in (a) showing eosinophils (arrows). HE.](image_url)
has not been reported in other species, although there appears to be an increased density of ICC at the deep muscular plexus region in man and the mouse (Sanders, 1996). Ward et al. (2006) demonstrated that the cells in this latter region were mediators of enteric neurotransmission. The role of this band of ICC must still be determined in both the horse and the donkey.

The distribution and density of immunoreactive ICC in the pelvic flexure region of the large colon were again similar to that described in the adult horse (Hudson et al., 1999) with ICC networks in the myenteric plexus region being less dense than those observed in the small intestine. Similarly, immunoreactive ICC were distributed throughout the circular muscle layer but, in contrast to the ileum, the dense band of ICC at the submucosal border was not present.

The mixture of bipolar and stellate cells observed in the pelvic flexure was also similar to that seen in the adult horse (Hudson et al., 1999). However, these cells also appeared to have a more delicate morphology than the ICC observed in the horse (Hudson et al., 1999).

It was not possible to demonstrate a reduction in the density of ICC in animals with intestinal disease compared with those that were euthanised for other reasons. These findings differ from those of a previous study (Fintl et al., 2004) that reported a reduced ICC density in horses with large colon obstructive disorders, including pelvic flexure impactions, compared with normal horses. It is possible that different pathophysiological processes are involved or that ICC in the donkey are more resilient to injury. However, this is difficult to substantiate without further investigations. It is worth recording, however, that as noted in Table 1, all but one of the animals with colic had poor dentition. Cox et al. (2007) identified both old age and dental disease as risk factors for developing impactions and this is supported by the current findings.

In conclusion, it appears that the general distribution and density of ICC in the donkey are very similar to that of the adult horse, consistent with the highly conserved nature of these important enteric cells across mammals. It was not possible to demonstrate changes in the density of ICC in animals with intestinal disease compared with controls. This differs from previously reported findings in horses with intestinal disease and may therefore indicate different pathophysiological processes in the development of intestinal obstruction in the donkey. Furthermore, this study indicates that these cell populations do not markedly decline with increasing age, although clearly the study of samples collected from younger animals are required to confirm this.

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