Histologic and immunohistochemical evaluation of intestinal innervation in dogs with and without intussusception

Leda M. Oliveira-Barros, DVM, MS; Thais A. Costa-Casagrande, DVM; Bruno Cogliati, DVM; Lilian R. M. Sá, DVM, PhD; Julia M. Matera, DVM, PhD

Objective—To assess viability of innervation in bowel segments appearing macroscopically viable from dogs with intussusception.

Animals—7 dogs without gastrointestinal dysfunction that had been euthanized for reasons unrelated to the study (control dogs) and 13 dogs with intussusception that underwent enterectomy and intestinal anastomosis (affected dogs).

Procedures—A total of 31 samples of intestinal tissue were obtained from the control dogs; 28 samples were obtained from affected dogs during surgery. Samples were histologically and immunohistochemically prepared and subjectively scored for degree of vacuolization and staining, respectively. Other data collected included mean muscle cell density of circular and longitudinal muscular layers, ratio between areas of muscular layers, mean number of myenteric plexuses, mean ganglion cell density of myenteric plexuses, and degree of degeneration in neuronal plexuses as estimated through synaptophysin and neuron-specific enolase (NSE) immunoreactivity.

Results—Mean muscle cell density of longitudinal muscular layers, ratio between areas of muscular layers, and synaptophysin immunoreactivity did not differ significantly between affected and control dogs; values of all other variables did. Correlations were evident between mean ganglion cell density in myenteric plexuses and mean muscle cell density in circular muscular layers, degree of neuronal degeneration in myenteric plexuses and NSE immunoreactivity, and degree of neuronal degeneration in myenteric plexuses and mean ganglion cell density of myenteric plexuses.

Conclusions and Clinical Relevance—Innervation may be impaired in bowel segments that appear macroscopically viable. Therefore, careful evaluation of preserved surgical margins during enterectomy and enteroanastomosis and monitoring of digestive function after surgery are important. (Am J Vet Res 2010;71:636–642)

Small bowel (ie, duodenum, jejunum, and ileum) obstruction is a common clinical condition in small animals and can result from mechanical or functional causes.1 Intestinal occlusion can lead to obstructed ingesta passage, intestinal wall injury, and severe distension proximal to the site of the obstruction. In chronically affected animals, morphometric remodeling such as hypertrophy of the muscular tunica, particularly in the inner circular layer, can occur.2-4

In healthy intestines, motility depends on the preservation of muscle function, neuromuscular junctions, and neuroendocrine control of movement. Dysfunction of one or more of these components may lead to abnormal intestinal motility, which yields various clinical manifestations.3 The myenteric plexus (plexus of Auerbach) plays a major role in the regulation of intestinal motility, whereas the submucous plexus (Meissner plexus) ensures regulation of intestinal secretion and absorption.6

Intraoperative assessment of intestinal viability continues to be a major challenge in gastrointestinal surgery.7 Surgeons presently assess bowel viability on the basis of traditional and subjective criteria such as bowel color, presence of peristalsis, and blood vessel pulse. However, the wrong interpretation of these variables can result in the preservation of damaged tissue or in unnecessary bowel resection.7,8 Several studies have

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From the Department of Surgery and Department of Pathology, School of Veterinary Medicine, University of Sao Paulo, Sao Paulo, 05508-270, Brazil.
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Address correspondence to Dr. Oliveira-Barros (ledamobarros@gmail.com).

Abbreviations

NSE Neuron-specific enolase
SY38 Monoclonal mouse anti-synaptophysin clone 38
been conducted to objectively and qualitatively assess the degree of intestinal damage, yet there is no consensus on the best method. Preservation of an injured segment may cause dysfunction in intestinal motility and future clinical manifestations of that dysfunction. 

Histologic and immunohistochemical techniques have been used to evaluate patterns of intestinal innervation in pigs, horses, rats, mice, chicken, guinea pigs, dogs, and humans. 

In the related studies, quantitative and morphologic aspects of myenteric and submucous plexuses were evaluated, as well as cell density of the plexus and cell density of the muscular layers, degree of neuronal degeneration, and plexus immunoreactivity.

Intussusception is defined as the invagination of 1 portion of the gastrointestinal tract into the lumen of an adjoining segment and is a common type of intestinal obstruction in dogs. 

The ileocolic junction is the most commonly reported site of intussusception, and the incidence of this condition is higher in puppies than in adult dogs. 

Predisposing causes include changes of diet, enteritis, intestinal parasitism, foreign body, previous surgery, neoplasia, and carbamate intoxication. 

The obstruction that results from intussusception may be partial or complete and may result in intestinal dilatation, bacterial overgrowth, ischemia, and necrosis as well as peritonitis and septic shock. Often, treatment involves intestinal resection and anastomosis.

The purpose of the study reported here was to assess viability of innervation in bowel segments appearing macroscopically viable from dogs with intussusception that underwent enterectomy and intestinal anastomosis. A secondary aim was to determine whether findings were associated with digestive function as assessed 6 months after surgery.

Materials and Methods

Animals—This prospective study was performed at the Veterinary Hospital of the School of Veterinary Medicine, University of São Paulo, Brazil, between July 2006 and January 2008. Twenty-eight samples of intestinal tissue were obtained during enterectomy and intestinal anastomosis from 13 dogs (9 males and 4 females) with a clinical and ultrasonographic diagnosis of intestinal intussusception (affected group). The intestinal segment from which samples were obtained depended on the site of intussusception. Samples from the affected group were divided into 3 subgroups based on quality of life and digestive function as follows: dogs that died in the early postoperative period and dogs with and without clinical signs in the postoperative period. Another 31 intestinal samples were obtained from 7 dogs that had been euthanized for reasons unrelated to the study and had no clinical signs of gastrointestinal dysfunction. Five dogs were healthy dogs obtained from the zoosis control center, and 2 had been used in an ophthalmologic study. The study protocol was approved by the Bioethics Commission of the School of Veterinary Medicine of University of São Paulo (protocol No. 915/2006). All owners received and signed a form of informed consent prior to the start of surgery.

Processing of intestinal tissue samples—Samples of intestinal tissue were fixed in phosphate-buffered 10% formalin (pH 7.4) for at least 24 hours. Histologic and immunohistochemical evaluations were performed on paraffin-embedded tissue cross sections. Tissue sections (5 µm thick) were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin wax.

Histologic evaluation—For histologic evaluation, slides of tissue cross sections were stained with H&E. The degree of innervation within intestinal samples was evaluated by use of a computer system to capture images of areas and count the number of plexuses and cells therein. Slides were examined by means of light microscopy, and the following variables were used to characterize innervation patterns: mean muscle cell density of circular and longitudinal muscular layers (determined by manual count of 3 selected fields magnified with a 4× objective lens), mean number of myenteric plexuses (determined by manual count of 10 consecutive fields magnified with a 10× objective lens), mean ganglion cell density of myenteric plexuses (measured in 10 myenteric plexuses), and ratio between areas of muscular layers (measured in 3 randomly selected fields by use of a line grid). A semiquantitative score ranging from 0 to 3 was used to quantify degree of vacuolization of submucous and myenteric ganglion cells as an indirect method for determining the degree of degeneration of submucous and myenteric plexuses. Scores were assigned as follows: 0, no vacuolization; 1, rare vacuolization; 2, mild vacuolization; and 3, intense vacuolization.

Immunohistochemical staining and assessment—Formalin-fixed tissues sections were prepared for immunohistochemical evaluation by incubation for 30 minutes in methanol supplemented with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Nonspecific antibody binding was subsequently blocked by incubation of sections in PBS solution supplemented with 5% nonfat dry milk. Then, sections were incubated with monoclonal mouse anti-synaptophysin clone SY38 antibody and a labeled streptavidin-biotin kit with diaminobenzidine, in accordance with the manufacturer’s instructions. The degree of immunohistochemical staining in myenteric plexuses was assessed by an observer who was blinded to dog identity and who used the following scoring system: 0, no staining; 1, light staining; 2, moderate staining; and 3, abundant staining.

Quality of life and digestive function—Six months after surgery, follow-up examinations of affected dogs were performed. At that time for each dog, quality of life and digestive function (fecal consistency, frequency of defecation, and episodes of acute intermittent diarrhea) were evaluated by use of a standard questionnaire administered by veterinary staff to the dog owners.

Statistical analysis—Continuous data such as cell density in tissue sections are reported as mean ± SD.
Descriptive data (age, sex, number of dogs with predisposing causes, scores for vacuolization in ganglion cells of myenteric and submucous plexuses, and scores for staining for NSE and synaptophysin in ganglion cells of myenteric plexus) are reported as absolute (n) and relative (%) frequencies. Associations among descriptive data were evaluated by use of a likelihood ratio test. Differences between affected and control dogs with respect to quantitative data that were confirmed to normally distributed were evaluated by use of a t test. Pearson product-moment correlation coefficients (r) were calculated to assess the following correlations: mean cell density of the myenteric plexus versus mean cell density of the circular muscular layer, degree of neuronal degeneration versus degree of NSE immunoreactivity, and degree of neuronal degeneration versus mean cell density of the myenteric plexus. Values of P < 0.05 were considered significant for all analyses.

Results

Affected dogs—Age of dogs with intussusception ranged from 4 to 84 months (mean age, 8 months). No sex predilection toward development of intussusception was apparent. Enteritis, parasitism, gastroenteritis, and carbamate intoxication were identified as predisposing causes in 11 affected dogs. Jejunum and ileum was represented by 86% (n = 24) of samples, whereas colon and cecum represented only 14% (4). The mean ± SD interval between surgery and first defecation was 1.7 ± 1.1 days, and the mean duration of medical treatment was 13.3 ± 1.9 days. Three affected dogs died during the early postoperative period.

Histologic evaluation—Mean muscle cell density in the circular muscular layer of intestinal sections was

<table>
<thead>
<tr>
<th>Group</th>
<th>Myenteric plexus vacuolization</th>
<th>Submucous plexus vacuolization</th>
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<tr>
<td></td>
<td>0 1 2 3</td>
<td>0 1 2 3</td>
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<tr>
<td>Aged</td>
<td>29 14 21 36</td>
<td>43 21 18 18</td>
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<tr>
<td>Control</td>
<td>68 26 6 0</td>
<td>97 0 3 0</td>
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A semiquantitative score ranging from 0 to 3 was used to quantify degree of vacuolization of submucous and myenteric plexuses as an indirect method for determining the degree of degeneration of submucous and myenteric plexuses. Scores were assigned as follows: 0, no vacuolization; 1, rare vacuolization; 2, mild vacuolization; and 3, intense vacuolization. The distribution of scores differed significantly (P < 0.05) between groups.
significantly \( (P = 0.001; t \text{ test}) \) lower in affected dogs \( (1,184 \pm 507 \text{ cells/mm}^2) \) than in control dogs \( (1,631 \pm 488 \text{ cells/mm}^2) \). Mean muscle cell density in longitudinal muscular layers in affected dogs appeared lower than in control dogs; however, the difference between groups was not significant \( (P = 0.368; t \text{ test}) \). The ratio between the muscular layers area appeared greater in affected dogs than in control dogs; however, again, the difference was not significant \( (P = 0.293; t \text{ test}) \). The mean number of myenteric plexuses and the mean ganglion cell density of myenteric plexuses were significantly \( (P = 0.028 \text{ and } P = 0.043, \text{ respectively}; t \text{ test}) \) lower in dogs with intussusception \( (8.9 \pm 2.1 \text{ plexuses/10 fields} \text{ and } 5,419 \pm 1,197 \text{ cells/mm}^2, \text{ respectively}) \) than in control dogs \( (10.2 \pm 2.3 \text{ plexuses/10 fields} \text{ and } 6,054 \pm 1,154 \text{ cells/mm}^2, \text{ respectively}) \). A significant difference was detected when the degree of vacuolization of the submucous \( (P < 0.001; \text{ likelihood ratio test}) \) and myenteric plexus \( (P = 0.001; \text{ likelihood ratio test}) \) of affected dogs was compared with vacuolization in tissues from the control group (Figure 1; Table 1).

**Immunohistochemical evaluation**—The distribution of scores for degree of immunohistochemical staining appeared to differ between affected and control dogs for markers NSE and synaptophysin (Table 2). However, only staining for NSE differed significantly between the groups. Only 36% of samples from affected dogs were classified as abundantly stained, compared with 87% of samples from control dogs. The intensity of staining obtained for marker NSE was better than that for synaptophysin (Figures 2 and 3).

**Correlation analysis**—Mean cell density of myenteric plexuses was positively correlated with mean cell density of the circular muscular layer \( (r = 0.578; P < 0.001) \). Degree of neuronal degeneration was negatively correlated with NSE immunoreactivity \( (r = -0.465; P \)
Quality of life and digestive function—Ten dogs in the affected group were evaluated 6 months after surgery for intussusception. At that time, 3 of the dogs had signs of digestive dysfunction including an increase in defecation frequency, abnormal fecal consistency, and episodes of acute intermittent diarrhea. The remaining 7 dogs had no signs of gastrointestinal dysfunction, nor were any surgical complications reported.

On the basis of quality of life and digestive function in the postoperative period, samples were compared in relation to histologic and immunohistochemical variables. Groups compared included samples from dogs that died in the early postoperative period (n = 8), samples from dogs with clinical signs in the postoperative period (7), and samples from dogs without clinical signs in the same period (13). The mean muscle cell density of circular muscular layers in dogs that died (1,449.33 ± 593.79 cells/mm²) was greater than that in dogs with and without clinical signs (1,077.12 ± 502.81 cells/mm² and 1,080.03 ± 423.37 cells/mm², respectively); however, the difference between groups was not significant (P = 0.224; t test). The ratio between areas of the muscular layers was also not significant (P = 0.504; t test).

The mean number of myenteric plexuses and the mean ganglion cell density of myenteric plexus did not differ significantly (P = 0.537 and P = 0.351, respectively; t test) among dogs that died (9.5 ± 2.1 plexuses/10 fields and 5,857 ± 1,630 cells/mm², respectively), dogs with clinical signs (8.2 ± 2.1 plexuses/10 fields and 5,541 ± 1,044 cells/mm², respectively), and dogs without clinical signs (8.9 ± 2.0 plexuses/10 fields and 5,083 ± 932 cells/mm², respectively) in the postoperative period. When degree of vacuolization of plexuses was compared among groups, the difference was not significant (P = 0.216; likelihood ratio test). The same was true for the distribution of scores for degree of immunohistochemical staining for markers NSE and synaptophysin (P = 0.056 and P = 0.324, respectively; likelihood ratio test). The mean muscle cell density of longitudinal muscular layers was the only value that differed significantly (P = 0.037; t test) between dogs that died and dogs with and without clinical signs.

Discussion

The study reported here was designed to assess the viability of intestinal innervation in intestinal tissue samples obtained from dogs with intussusception.
that subsequently underwent enterotomy and intestinal anastomosis. Whereas maintenance of nonviable bowel segments can be a consequence of underestimation of the extent of damaged bowel, overestimation of damage can result in extensive resection and development of so-called short intestine syndrome.\(^7,8\) In our study, we observed lower muscular density in dogs with intussusception in longitudinal and circular muscular layers, but, similar to results of other studies\(^2,4,22\) in rats, guinea pigs, and humans, the difference achieved significance only in the circular inner layer. Unlike those studies, in our study, hypertrophy of tissue segments from sites proximal to the obstruction was not more pronounced than hypertrophy of segments from sites distal to the obstruction. We believe that the changes in the distal segments were caused by ischemia and the release of free radicals, as has been described in humans.\(^23\)

In the present study, the number of myenteric plexuses and the cell density of those plexuses were lower in dogs with an intussusception versus dogs with no gastrointestinal dysfunction. These results are consistent with those of other investigators who also observed a decrease in these measurements in horses\(^5\) and chick embryos\(^5\) with small bowel obstruction.

Degeneration of neuronal plexuses can manifest in various manners, including lack of neurons, loss of neuronal nuclei, glomerodermal reaction, hypereosinophilic amorphous degeneration of neurons, shrinkage of degenerated neuron yielding empty spaces, and vacuolization and microvesiculation of neuronal cytoplasm.\(^8\) We used neuronal vacuolization as an indirect means of evaluating neuronal degeneration, and the scoring system used revealed a significant difference in degree of degeneration between dogs with and without intussusception.

Synaptophysin and NSE have been used as markers to evaluate intestinal innervation in small animals.\(^23,26\) Synaptophysin is a glycoprotein present in small synaptic vesicles in the terminal portion of almost all neurons. Also a glycoprotein, NSE catalyses the interconversion of 2-phosphoglycerate and phosphoenolpyruvate. This enzyme is localized primarily within the neuronal cytoplasm. Despite the fact that both antigens are cytoplasmic markers, the intensity of NSE staining was better than that of synaptophysin (Figures 2 and 3). These observations are consistent with a previous report\(^25\) in which this phenomenon was attributed to a higher quantity of NSE within the ganglion cell and axonal extension, compared with the quantity of synaptophysin. According to our results and those in the other report, synaptophysin should not be used as an exclusive marker for quantifying degree of innervation.

Six months after surgery to correct intussusception, 3 dogs in our study reportedly had gastrointestinal dysfunction that included altered fecal consistency and frequency of defeation as well as episodes of acute intermittent diarrhea. However, we were unable to con firm that these alterations were attributable to impaired innervation. The length and location of the portion of the intestine that was removed must be taken into account. In the situation of these 3 dogs, the ileocolic segment (including the valve) had been resected in 2. Furthermore, postsurgical histologic analysis of the resected segments resulted in the diagnosis of lymphoplasmacytic enteritis in 1 dog and neutrophilic enteritis in the other 2 dogs.

With the exception of mean muscle cell density of longitudinal muscular layers, no other variable was significant when compared among dogs that died, dogs with postoperative dysfunctions, and dogs without intestinal disorders in the same period. The authors believe that this finding may have been spurious given the number of hypotheses tested.

Results of the present study indicated that bowel segments considered viable under macroscopic inspection had less innervation than those of the control group. However, no considerable differences were detected in intestinal innervation between dogs with or without clinical signs in the postoperative period. The apparent lack of clinical manifestation could be explained by a bowel adaptation or enteric nervous system plasticity and regenerative capacity.

Mean cell density of the myenteric plexus was correlated with mean cell density of the circular muscular layer in intestinal samples in the present study. It might therefore be possible to estimate the amount of hypertrophy in the circular muscular layer on the basis of the cell density of the myenteric plexus. Likewise, there was an association between mean density of myenteric cells and the degree of neuronal degeneration. Moreover, the degree of neuronal degeneration was associated with the degree of NSE immunoreactivity. Given these findings, use of several techniques including immunoreactivity to different antibodies may be unnecessary for estimating viability of intestinal tissues. The main finding was that bowel segments considered viable under macroscopic inspection may, in fact, have impaired innervation, which could eventually manifest clinically. Therefore, careful determination of the extent of abnormal bowel for surgical correction is recommended when treating dogs with intussusception, as it is monitoring of digestive function after surgery.

References


