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Original contribution

Counting myenteric ganglion cells in histologic sections: an empirical approach☆

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Summary An abnormal density of myenteric neurons is a putative cause of intestinal pseudo-obstruction. Quantification of myenteric ganglion cells may be necessary to establish hyper- or hypoganglionosis, but published norms are very discordant. We investigated how observer bias and tissue sampling affect the accuracy and reproducibility of myenteric neuron counts obtained from histologic sections immunostained for HuC/D, a neuronal cell body-specific antigen. Despite a collective effort to standardize neuronal identification criteria, significant discrepancies were found between the counts obtained by different observers. In contrast, counts by a single observer, over a period of several months, revealed excellent reproducibility. To investigate effects of tissue sampling on the accuracy of ganglion cell density estimates, one observer counted immunoreactive neurons in 22 full-circumference rectal sections from the same paraffin block. The mean number of neurons per circumference from all 22 sections was considered a target, against which estimates from smaller samples were compared. To ensure an accurate estimate of the circumferential density (within 10% of the target value), counts had to be averaged from at least 5 sections of nearly the full circumference. Examinations of fewer sections or less than two thirds of the circumference were prone to errors. Application of these principles to sections from the transitional zone in Hirschsprung disease validated the approach and discriminated relatively subtle changes in neuronal density. We conclude that neuronal counts are best performed by individuals using their own normative data for reference and that biopsies of small portions of the circumference may not resolve potentially significant hypo- or hyperganglionosis.

1. Introduction

Intestinal pseudo-obstruction is characterized by symptoms of mechanical obstruction without evidence of luminal occlusion. It is a result of defective gut motility, which can be categorized as either myopathic (abnormalities of smooth muscle) or neuropathic (abnormalities of the enteric nervous system) [1-3]. The best understood neuropathic form is Hirschsprung disease (HD), a congenital malformation characterized by absent ganglion cells in the myenteric and submucosal nerve plexuses of the terminal rectum and a variable length of contiguous intestine. Other forms of enteric neuropathology include alterations in neuronal density, that is, hypo- or hyperganglionosis [4-6]. The latter can be challenging histopathologic diagnoses, which should be based on rigorous and well-controlled quantitative data,

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but often rest on subjective impressions with uncertain reliability. One context in which ganglion cell density may be particularly important is the transitional zone (TZ) of HD, a hypoganglionic length of bowel that lies proximal to the segment of aganglionosis and varies from a few millimeters to several centimeters in length [7-11]. Some studies suggest that failure to resect the TZ along with the aganglionic segment may account for the postoperative obstructive symptoms observed in many HD patients [10,12,13], but conflicting data exist [14]. Isolated hypoganglionosis (independent from HD) is also a putative cause of pseudo-obstruction [15-20].

Several investigators have counted myenteric neurons and established norms for human intestinal samples. However, published data are very discordant (Table 1). Some of the discrepancy stems from methodological differences, but even seemingly identical procedures have yielded different results. Hematoxylin and eosin (H&E)–stained sections have been used to count ganglion cells, which are typically recognized by their large nuclei with prominent nucleoli, surrounded by a granular, amphoteric neuronal cytoplasm [21]. However, such descriptive criteria for neuron identification are often difficult to apply, particularly when differences in neuronal maturation, section quality, varied planes of section through a cell body, autolysis, and various handling artifacts are considered. For histologic sections, a widely adopted alternative to H&E-based counts is immunohistochemistry (IHC) with antibodies that recognize one or more neural antigen. In particular, immunolocalization of the RNA-binding proteins, HuC and HuD, has been advocated as an excellent method for neuron quantitation because Hu appears to be expressed in the cell soma of virtually every enteric ganglion cell [28,29].

Table 1 Selected examples of neuronal counts from histologic sections of "normal" colons [a]

Reference	Staining method	Mean number of ganglion cells per cm [b]
Meier-Ruge et al, 1970[21]	LDH histochemistry	756
Meier-Ruge et al, 1999[15]	LDH histochemistry	149
Wedel et al, 2001[18]	PGP9.5 IHC	127
Scharli & Sossai, 1998[19]	LDH histochemistry	100
Smith, 1993[22]	H&E	70
Csendes et al, 1992[23]	H&E	50
Aldridge and Campbell, 1968[24]	H&E	46
Yu et al, 2002[25]	Neurofilament IHC	34
Iwase et al, 2005[26]	H&E	30
Yu et al, 2002[25]	H&E	27
Faussone-Pellegrini et al, 1999[27]	H&E	25
Krishnamurthy et al, 1993[20]	H&E	5

Abbreviations: LDH, lactate dehydrogenase; PGP, protein gene product.
[a] Specific part of colon was not stated in every study. For studies of more than one portion of colon, data correspond to rectum or most distal colonic area.
[b] Includes data from wide range of ages.

Other variables that affect estimates of neuronal density include section thickness, specimen size, natural variation in the circumferential distribution of ganglion cells, bowel distension, and the number of sections analyzed. Potential variation in the circumferential distribution of neurons has important implications for choosing the biopsy size and site. In the TZ of HD, for example, the distribution of ganglion cells around the circumference is uneven, with irregularities in the "leading edge" of ganglion cells that extends into the aganglionic distal bowel [10,30,31]. Irregularity in the circumferential distribution of ganglion cells may be important in other contexts as well, and it is unclear whether biopsies that sample a portion of the circumference can be used to accurately estimate the full circumferential density of neurons in normal or diseased bowel.

Observer bias can also affect neuronal counts. This may account in part for differences in published norms. Criteria used to identify individual neuronal cell bodies (eg, presence/absence of a nucleus, cell boundaries, intensity of staining or immunoreactivity) may be interpreted differently by different observers. The type of microscope used and the experience of a pathologist may influence perceptions. Ideally, criteria for counting ganglion cells should be easily learned by pathologists and yield results with minimal inter- and intraobserver variability. Although some criteria have been proposed, none has been rigorously evaluated to assess its reproducibility by multiple observers.

The current study is an effort to develop and test a standardized approach to quantitate myenteric neurons with minimal inter- and intraobserver variability. In this investigation, we counted all of the myenteric neurons present in each of multiple step sections cut from a single paraffin block that comprised the full circumference of the bowel wall. The mean number of ganglion cells calculated from all of the ganglion cells in all of the sections was considered the target value, against which estimates from smaller samples were compared. Our goal was to find an approach by which any estimate of the full-circumference ganglion cell number would fall within 10% of the target value, and thereby determine the optimum number of sections and portion of the circumference that need to be assessed to obtain an accurate estimate.

2. Materials and methods

2.1. Study design

An overview of the study design is presented in Fig. 1. The research project was approved by the Institutional