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ORIGINAL ARTICLE

Hirschsprung's disease: the "Swiss roll" technique revisited

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Abstract
Purpose During pull-through for Hirschsprung's disease (HSCR), the assessment of innervation is mainly based on the presence of ganglion cells when conventional Hematoxylin and Eosin (HE) staining is used. In hypoganglionosis, the evaluation is difficult. We adapted a standardized methodology for the examination of resected bowel after HSCR surgery, using the technique described by Moolenbeek on rodent intestine and later by Meier-Ruge in children. We have analysed the entire innervation of surgically resected bowels and compared the results with the follow up of patients.
Methods Three longitudinal strips of colon were harvested from the mesenteric, anti-mesenteric and intermediate part in the whole length of resected colon of six patients with HSCR. Each strip was divided into two parts. One of the contiguous strips was assessed with HE and Hematoxylin–Phloxin–Safran, and the other one with acetylcholinesterase (AChE) histochemistry. We analyzed the distribution of ganglion cells and nerve arrangement along the strips with both techniques and compared the results obtained in the three different regions of the bowel.
Results There was no significant difference in the pattern of innervation circumferentially. There was a correlation between a progressive increase of AChE activity and nerve hypertrophy and a decrease of ganglion cells from the proximal to the distal part of the resected colon in the submucosa and the myenteric plexus. Nerve hypertrophy and AChE-positive reaction in the mucosa were found at the resection border in patients who presented postoperative complications.
Conclusions Simultaneous assessment of nerve cells, nerve fibers and AChE activity is important in the evaluation of the innervation of the bowel segment proximal to the aganglionic zone. The method described is feasible and can be adapted to older children and adults with larger bowels. These results point out the importance of assessing nerve fibers in intraoperative biopsies during pull-through procedures to prevent uncomplete surgical bowel resection.

Keywords Hirschsprung's disease · Enteric nervous system · AChE · Aganglionosis · Pathological technique

Introduction

In spite of the technical innovations in the surgical treatment of Hirschsprung's disease (HSCR), results still remain disappointing in terms of postoperative complications and bowel function [1–3]. The rate of complications has merely changed in the past decade. Enterocolitis, constipation, soiling and strictures are still encountered in more than 15% of patients submitted to surgery for HSCR. Many causes have been mentioned for these complications, the main one being the pull-through (PT) of dysganglionic segments (i.e. aganglionic, hypoganglionic or dysganglionic) [4, 5]. This is mainly due to the method of assessment of intestinal segments proximal to the aganglionic zone (AZ), based solely on the presence or absence of ganglion cells with conventional staining like Hematoxylin

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and Eosin (HE) or Hematoxylin–Phloxin–Safran (HPS). In order to rule out dysganglionosis of pulled-through bowel, Kobayashi [5] proposed the use of a rapid AChE immunohistochemistry for the assessment of intraoperative biopsies during PT procedures for HSCR. Furthermore, some authors have shown uneven circumferential distribution of ganglion cells in the colon, raising questions about where precisely to perform intraoperative biopsies in order to rule out hypoganglionosis and avoid the PT of a transition zone (TZ) [6].

So as to analyze the changes in enteric nervous system (ENS) along with the aganglionic and hypoganglionic bowel, and the adjacent normal segment, and possibly help prevent postoperative complications, we adapted the pathological approach proposed by Moolenbeek [7] in 1981 on rodent intestine and later by Meier-Ruge [8] in children to study both the circular and the longitudinal patterns of innervation of resected bowel in patients submitted to surgery for HSCR.

Materials and methods

Six consecutive patients operated for HSCR were included in this study. After confirmation of HSCR with rectal biopsies [9–12], the level of resection was determined by intraoperative full-thickness biopsies. These biopsies were assessed by HE and HPS. Colon resection was made at least 3 cm proximal to a biopsy presenting a normal distribution of ganglion cells. All patients underwent a modified Duhamel PT (Table 1).

The resected consecutive specimens were received fresh and oriented. The proximal surgical margin was inked. Bowel segments were sectioned longitudinally from the proximal to the distal part into six strips of material (5 mm width). Two contiguous strips were harvested in the mesenteric part, two in the anti-mesenteric region and two strips were obtained in the region in between. One of each strip was frozen and used for AChE histochemistry and the other one was fixed in 10% formalin. Depending on their size (particularly for the longest colon specimens reaching 31 and 19 cm, see Table 1), strips were divided longitudinally in several segments of 3–4 cm of maximal length and coiled, in order to obtain blocks of frozen coiled specimens and fixed coiled material. All blocks were oriented with the proximal part of the segment located in the inner part of the coiled specimen (Fig. 1). The fixed material was then embedded in paraffin, cut into serial 6-μm-thick slices, and stained with HE and HPS for histological analysis. The frozen material was cut into serial slices in order to perform AChE histochemistry according to the method proposed by Meier-Ruge for nerve fibers evaluation [13]. The semi-quantitative histological study consisted in the assessment of the distribution of ganglion cells, the presence of nerve hypertrophy as well as the evaluation of the corresponding AChE histochemistry. Number of ganglia, number of ganglion cells and nerve thickness were measured in each 0.5 cm of the submucosa and myenteric plexus from the resected strips. The AChE activity was classified into three groups: 0 for AChE activity in case of none or rare positive fibers in the mucosa, 1 for AChE activity, when AChE-positive fibers were found in the half inner part of the mucosa and 2 for AChE activity was determined when the whole thickness of the mucosa was occupied by the AChE-positive fibers (Fig. 2). The results were evaluated in two ways. First, a longitudinal evaluation of the innervation of the submucosa as well as of the myenteric plexus was made for each strip, second, a circumferential evaluation was made by comparing the three different areas for each staining at the same level.

Results

Six patients were included in this study (Table 1). All had a neonatal presentation of the disease and abdominal distension with vomiting. Two had a delayed passage of meconium (patients 4 and 5). Five had a chronic constipation (patients 1, 2, 3, 4 and 6). Diagnosis was based on rectal suction biopsies in five patients (patients 1, 2, 3, 5 and 6) and surgical biopsies according to Swenson in one (patient 4). In four patients, biopsies were performed in the first month of life (patients 2, 3, 5 and 6) and in two patients who were referred to our institution from abroad (see Table 1), diagnostic biopsies were only performed at the age of 2 and 6 years, respectively (patients 1 and 4). One patient was referred with a colostomy (patient 4). As secondary diagnosis, one patient (patient 6) had a gastric volvulus. At the time surgery, four patients were aged 1.5–3 months and two were 2 and 6 years old, respectively (see Table 1).

The length of resected colon ranged from 6.5 to 31 cm. Histological evaluation revealed a comparable pattern of distribution of ganglion cells, nerve fibers and AChE pattern, in all three strips harvested in the mesenteric, the anti-mesenteric as well as the area in between. Longitudinally, each strip disclosed a gradual increase of nerve hypertrophy with an increasing pattern of AChE activity in the mucosa from the proximal to the distal part, correlated with a concomitant progressive decrease of ganglion cells in the submucosa as well as a decrease of ganglion cell numbers in the myenteric plexus. We have summarized the results of one strip from each patient in Table 1. The TZ was located in the rectum in one patient, in the rectosigmoid in four, and at the junction between the sigmoid and the

Table 1 Patients treated according to protocols including assessment of innervation after Duhamel procedure containing clinical data of patients and a summary of results of the evaluation of the number of ganglia, ganglion cells and nerve thickness

Patients	1	2	3	4	5	6
Age of first symptoms	Birth	Birth	2 weeks	Birth	Birth	3 days
Symptoms	Vomiting, stool retention	Vomiting, stool retention	Vomiting, stool retention	No meconium for 5 days, stool retention	No meconium for 48 h, stool retention	Vomiting, stool retention
Age at surgery	6 years	2 and ½ months	3 months	2 years	1 and ½ months	2 months
Place of birth	Togo	Switzerland	Switzerland	Togo	Switzerland	Switzerland
Follow-up	Good	Postoperative constipation	Good	Postoperative constipation and enterocolitis	Good	Good
Length of the specimen	31	7.5	10	8.5	19	6.5
Proximal diameter	8	3.5	6	4.2	3.5	7
Distal diameter	6	2.5	6	7.1	2.3	7
Submucosal plexus						
Number of ganglia/2 cm	14, 22, 17, 5, 2, 0, 0, 0, 0, 0, 0, 0, 0, 0.	10, 0, 0, 0	27, 10, 10, 0, 0	35, 14, 19, 4	14, 30, 12, 2, 0, 0, 0, 0	54, 48, 14
Number of cells/2 cm	31, 73, 36, 8, 2, 0, 0, 0, 0, 0, 0, 0, 0, 0.	20, 0, 0, 0	61, 22, 7, 0, 0	148, 84, 35, 63	23, 77, 24, 2, 0, 0, 0, 0, 0	178, 140, 43
AchE activity	0, 0, 0, 0, 0, 0, 0, 0, 0, 0,0, 0, 1, 1.	1, 1, 2, 2	0, 0, 0, 1, 2	1, 1, 1, 1	0, 0, 0, 0, 1, 1, 2, 2	0, 0, 1
Nerve hypertrophy/2 cm	27, 35,28,30,27, 23, 24, 23, 26, 27, 40, 69, 52, 49, 51	36, 41, 47, 48	27, 24, 24, 43, 27	39, 36, 30, 37	27, 25, 32, 23, 22, 24, 27, 38, 39	25, 27, 28
Myenteric plexus						
Number of ganglia/2 cm	18, 14, 18, 17, 25, 16, 12, 4, 3, 1, 0, 0, 0, 0	39, 2, 0, 0	41, 38, 24, 5, 0	39, 27, 38, 27	35, 15, 5, 0, 0, 0, 0, 0	64, 49, 15
Number of cells/2 cm	36, 50, 54, 51, 45, 73, 27, 5, 3, 2, 0, 0, 0, 0	75, 3, 0, 0	146, 70, 56, 6, 0	48, 48, 52, 42	80, 31, 7, 0, 0, 0, 0, 0	225, 128, 58
Nerve hypertrophy/2 cm	50, 44, 35, 38, 36, 25, 29, 26, 33, 47, 34, 64, 95, 104, 74	34, 44, 63, 74	29, 29, 38, 40, 49	50, 39, 43, 47	30, 26, 22, 28, 23, 37, 38, 48, 48	39, 38, 35

These results are shown from proximal to distal and the numbers indicate the total value for every 2 cm of colon

AChE activity: 0, none or rare positive fibers in the mucosa; 1, AChE positive fibers in the half inner part of the mucosa; 2, the whole thickness of the mucosa occupied by the AChE positive fibers

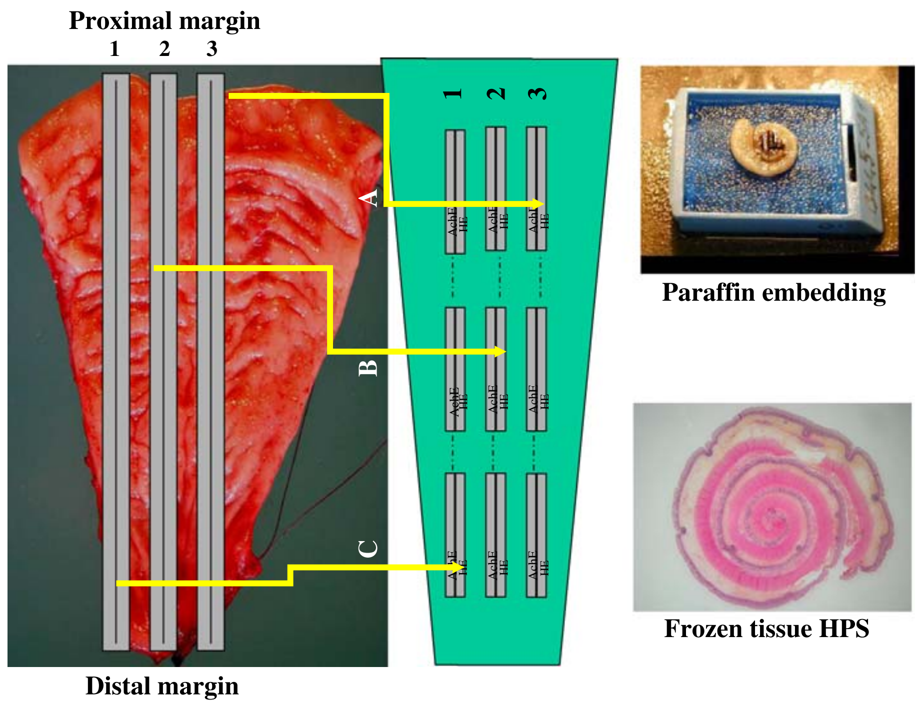

Fig. 1 Preparation of the specimen. The surgical partial colectomies were received fresh and oriented. The proximal surgical margin was inked. Bowel segments were sectioned longitudinally from the proximal to the distal part into six strips of material. Two contiguous strips were harvested in the mesenteric (*1*), two in the anti-mesenteric region (*2*) and two strips were obtained in the region in between (*3*). One of each strip was frozen and used for AChE and the other one was fixed in 10% formalin, paraffin embedded and stained with HE and HPS

descending colon in the last patient. In four patients, the AZ was found in the very distal part of the intestinal resection. In one patient (patient 6), with the shortest resected specimen (6.5 cm), no aganglionosis was found, but nerve hypertrophy and AchE hyperactivity were found in the last 2 distal cm of the specimen. The preliminary biopsies taken at 2, 3 and 4 cm above the dentate line were aganglionic and the patient was considered to have a short segment of HSCR in correlation with the clinical data. In two patients (patients 2 and 4), the proximal edge of resection showed the presence of normal ganglion cells but it was associated with an obvious nerve hypertrophy in the submucosa and AChE positivity in the mucosa. These patients had severe postoperative constipation, one of them with bouts of enterocolitis and both required saline washouts for 3 months. In the remaining four patients, proximal resection margin was normal histologically, with a normal distribution of ganglion cells, no important nerve hypertrophy and no AChE positivity in a minimum length of 3 cm was observed. These four patients had an uneventful follow up of 4 years with normal bowel movements. With a follow-up ranging from 4 to 5 years (mean 4.5 years), all patients have a normal bowel function.

Discussion

The outcome of bowel function after surgery for HSCR still remains challenging. Although the evolution of surgical procedures for HSCR has led to lower mortality and morbidity rates, as well as to shorter hospitalization, postoperative complications such as enterocolitis, constipation or incontinence are still encountered in more than 15% of patients [3].

Complications do not seem to be related to the technique used for PT and the shift towards simpler surgical procedures has only led to the reduction of short-term postoperative complications. This reality is mainly due to the fact that important changes occur in the innervation of bowel segments above TZ in many patients, in a zone previously considered as normal. PT of aganglionic or hypoganglionic segments has been blamed for these complications [1, 2]. Intestinal neuronal dysplasia (IND) above the TZ has been mentioned as another possible cause of bowel dysfunction postoperatively [4, 14]. Some studies have mentioned hypoganglionosis in the proximal pulled-through segment as being the reason for the subsequent chronic constipation [4].

Several procedures have been proposed to assess the enteric nervous system (ENS) at the level or above the zone of resection [15–17]. Authors have proposed to rule out intestinal neuronal dysplasia with serial biopsies of the colon above the TZ [18, 19]. White and Langer [6] have made a morphometric circular study of the innervation at different levels of the colon. This method has the advantage of analyzing the whole circumference of the colon and authors have reported the presence of a distal leading edge containing ganglion cells in contrast to the absence of ganglion cells for most of the remainder of the circumference. This finding has relevance in the interpretation of

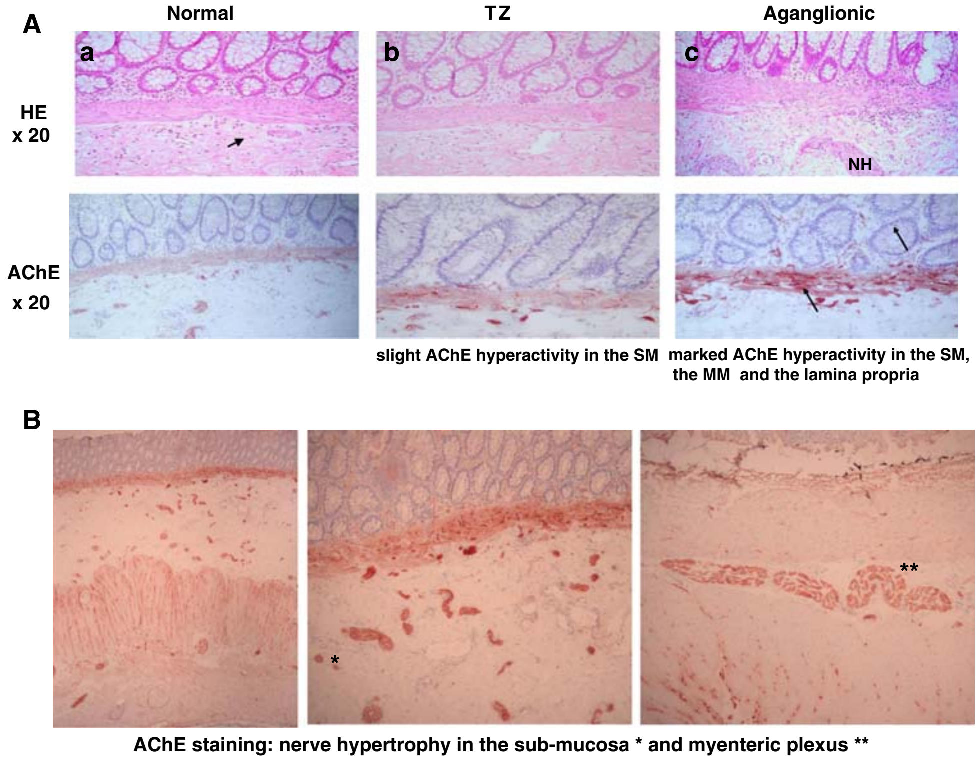

Fig. 2 **a** Conventional HE staining (*a*) and AChE histochemistry (*b*) in normal (1), hypoganglionic (2) and aganglionic (3) colon. The histological evaluation revealed a comparable pattern of distribution of ganglion cells, nerve fibers and AChE activity, in all three strips harvested in the mesenteric, in the anti-mesenteric areas as well as in the region in between. Longitudinally, each strip disclosed a gradual increase of nerve hypertrophy from the proximal to the distal part and a concomitant progressive decrease of ganglion cells as well as an increasing reaction of AChE activity. **b** Abnormal AChE pattern in aganglionic zone with nerve hypertrophy in sub-mucosa and myenteric plexus

intraoperative biopsies at the time of PT surgery and consequently for an accurate surgical resection. Both methods unfortunately have two main disadvantages: they do not offer the possibility of an uninterrupted longitudinal study of the innervation along the resected colon or they are time consuming, therefore not feasible for routine analysis in larger bowel. Meier-Ruge has used a method based on cranio-caudal coiled specimens from the surgical resection [8, 13], a method described in 1981 by Moolenbeck [7] in rodent intestine. This technique allows an accurate longitudinal study of the innervation, but remains time consuming and difficult to apply to large specimens like in older children or adults. Considering the advantages of these different methods and aware of the importance of using a precise method to assess the ENS of bowel at the time of surgery, we refined these techniques for the analysis of ENS in the management of patients during and after surgery for HSCR. This method allows to perform a longitudinal and circumferential cartography of the innervation of the resected bowel.

With this method, we first showed that the proximal edge of the aganglionic and the TZ displayed the same pattern of innervation on the whole circumference in the submucosa as well as in the myenteric plexus. Second, we noticed that there was an inversely parallel increase of AChE positivity and nerve hypertrophy from the proximal to the distal portion of the colon with a decrease of ganglion cells. Correlation between histopathological findings and postoperative course points out the importance of assessing both the aspect of nerve fibers and the ganglion cells in intraoperative biopsies before proceeding to PT. Although careful examination of specimens stained with HE may give important information about the distribution of nerves and ganglion cells, the rapid intraoperative AChE reaction proposed by Kobayashi [5] allows a more precise assessment of the innervation during the PT procedure. Based on these results, this method of assessment disclosed some important features: (1) there was as a steady modification of the neuronal network of the submucosa and the myenteric plexus from the normal bowel to the AZ, (2) the same circular pattern of innervation was found in each studied segment of the bowel, (3) nerve hypertrophy and AChE hyperactivity at the proximal resection margin of the surgical resection was associated with postoperative complications.

In conclusion, the method described is a feasible and useful technique to analyze the innervation of resected bowel in patients with HSCR. It can easily be adapted for the examination of larger bowel specimens such as those found in older children or in adult patients suffering from chronic constipation. This study pointed out the necessity of a careful examination of specimens for nerve hypertrophy in the submucosa as this feature may be the expression of a local intestinal dysfunction and call attention to the importance of performing intraoperative biopsies to inform the surgeon about the level of safe resection and prevent uncomplete bowel surgical resection.

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