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ORIGINAL ARTICLE

Intrinsic esophageal innervation in esophageal atresia without fistula

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Abstract Esophageal atresia and tracheo-esophageal fistula (EA + TEF) are often associated with malformations of neural crest origin. Esophageal innervation is also derived from the neural crest and it is abnormal in EA + TEF in which there is motor dysfunction. Our aim was to examine the intrinsic esophageal innervation in children with isolated EA in which different embryogenic mechanisms might be involved. Specimens from the proximal and distal esophageal segments of 6/35 patients who had esophageal replacement for isolated EA between 1965 and 2006 were suitable for the study. They were sectioned and immunostained with anti-neurofilament (NF) and anti-S-100 antibodies. The muscle and neural surfaces on each section were measured with the assistance of image processing software. The surface of the ganglia and the number of neurons per ganglion were determined at high power microscopy. The findings were compared with those of six autopsy specimens from newborns dead of other diseases by means of standard statistical tests and a significance threshold of $P < 0.05$. Unmatched age/size of babies in isolated EA and control groups precluded comparison of the relative surfaces occupied by neural elements. Patients with pure EA had denser fibrilar network and larger ganglia than controls. The number of neurons/ganglion were similar in both groups although the cells from EA patients were larger. The findings were similar at both esophageal levels studied. In spite of methodologic biases, it seems that intrinsic esophageal fibrilar network is denser and the intramural ganglia larger with larger cells in patients with pure EA than in controls on both esophageal ends of the organ. These neural anomalies are only in part reminiscent of those described in regular EA/TEF but may as well explain esophageal dysfunction in patients with repaired isolated EA.

Keywords Esophageal atresia · Tracheoesophageal fistula · Gastroesophageal reflux · Esophagus · Intrinsic innervation · Neural crest

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Introduction

Gastroesophageal reflux (GER) is so frequent after neonatal repair of esophageal atresia (EA) [1–5] with tracheoesophageal fistula (TEF), that it became a component of this condition that often requires fundoplication [6–8]. Distorted gastroesophageal anatomy and the neonatal operation account in part for this phenomenon but malformative elements likely contribute to it. EA with TEF results from an imbalance of the development of the dorsal and ventral components of the foregut during tracheoesophageal separation in the embryo [9, 10] shortly after the foregut has been populated by migrating neuroblasts from the cranial neural crest and at the time when the vagus and laryngeal nerves are being patterned [11, 12]. It is therefore not surprising for the extrinsic and intrinsic innervations to be abnormal in both human and experimental animals with EA and TEF. Pure EA without fistula is rare, it entails a

complete lack of most of esophageal length and the absence of communication with the trachea. This type of EA has been difficult to reproduce in experimental animals and has mechanisms that might be at variation from those of the more usual forms. When anastomosis of the distant esophageal segments is successful, esophageal dysfunction is particularly severe probably because of tension, more extensive mobilization and denervation. The purpose of the present study was to examine whether the intrinsic esophageal innervation in babies with this particular type of EA is abnormal and if the patterns are different from those described in regular EA/TEF by this respect.

Material and methods

Between 1965 and 2006, 587 patients with EA were treated at Hospital Universitario La Paz. Sixty-two of them had pure EA without fistula and 35 (including 12 referred from other hospitals) had esophageal replacement. During the same period of time, 162 autopsies of EA stillborns and patients were performed. The material from the four cases of isolated EA dissected in the last 10 years was examined but, unfortunately, they were either stillborns or preterm babies and the sections were not suitable for our study and were discarded. The paraffin blocks from the proximal and distal esophageal segments harvested during the esophageal replacement were the only ones considered suitable and were therefore investigated after approval by the Institutional Research Committee. The age at the time of surgery was recorded. The findings were compared with those of the upper and distal thirds of the esophagus of six autopsies from newborns dead of non-esophageal conditions. For methodological reasons, only unopen, complete and well-preserved esophagi were considered suitable for the study. Material from the proximal and distal segments of the organ were studied separately.

Transversal 3.5 μm-sections of the specimens perpendicular to the longitudinal axis from both esophageal ends were stained with HE, anti-neurofilament (NF) and anti-glial cell marker S-100 antibodies (Dako Cytomation, Glostrup, Denmark) for depicting the fibrilar network and the glial and neuronal elements, respectively. The muscle surface and the surface of immunostained neural tissue in each section were measured at both levels in 2–5 low-power fields with the assistance of an image processing software (Image Pro Plus, version 5.0, Media Cybernetics, Washington, DC, USA). The areas to be measured were contoured on the PC screen with the cursor and the resulting surface was integrated by the software. The immunostained neural tissue was identified and its surface measured. The surface of the ganglia and the number of neurons per ganglion were assessed at high power light microscopy. In the control group, 60 ganglia from the proximal and 78 from the distal esophagus were examined and the corresponding figures for the EA group were 43 and 65. The variables analyzed were: Muscle surface of each section, mean fibrilar surface per section, mean surface of the ganglia and number of neurons per ganglia. The results were expressed as percentages or as means ± SD and both groups were compared by either two way ANOVA or non-parametric Mann–Whitney tests as appropriate with a threshold of significance at $P < 0.05$.

Results

Three out of the six babies with isolated EA were male and three female and the mean age at the time of surgery was 7 ± 3.8 months. The control group consisted of six newborns, of gestational ages of 39 ± 1.9 weeks, who died of unrelated causes without significant malformations. The mean muscular surfaces of the proximal esophageal sections were 31.91 ± 15.73 and 10.11 ± 3.31 mm^2 in the EA and the control group, respectively ($P < 0.05$) and the distal ones of 25.63 ± 6.20 and 10.22 ± 2.14 mm^2 ($P < 0.05$). Any calculation of the neural area related to the muscular surface was therefore inappropriate because of the age mismatch of both groups of babies. However, the raw area of the intermuscular plexus (uncorrected for muscle surface) of each section occupied by fibres was larger in pure EA patients at both esophageal levels and with both immunostainings (Table 1) and the appearance was that of a denser fibrilar network, particularly with NF immunostaining (Fig. 1). The distribution and the morphology of the ganglia of the intermuscular plexus (Auerbach) were similar in pure EA and control babies but they were larger in the former than in the latter (Fig. 2). The number of neurons per ganglion was similar in both groups (Table 1) although the cells from babies with EA were considerably larger. The nuclear and cytoplasmic patterns were similar in both groups. In summary, the fraction of neural elements in the intermuscular plexus of patients with pure EA was larger than in controls both for the density of fibrilar network and for the size of the ganglia. The neuronal population of the ganglia, however, remained unvariable except for the size of the neurons. These findings were almost identical on both ends of the atretic esophagus.

Discussion

Although nowadays most newborns with EA and TEF undergo repair successfully [13–15], they have esophageal dysmotility for life and variable degrees of swallowing

Table 1 Mean fibrilar surface per section, mean ganglion surface and neurons per ganglion in pure EA and controls

Marker		Control (n = 6)		AE (n = 6)	
		Proximal	Distal	Proximal	Distal
NF	Fibrilar surface (μm^2)	55991 ± 10314	87174 ± 38096	109721 ± 68508	119723 ± 41800
NF	Surface of ganglia (μm^2)	5711 ± 6147	5670 ± 4701	15291 ± 14553*	9610 ± 9779*
NF	Neurons/ganglion (n)	5.0 ± 3.5	5.3 ± 3.6	6.0 ± 5.8	4.5 ± 3.5
S100	Fibrilar surface (μm^2)	42574 ± 8048	68291 ± 26005	95314 ± 45775*	80860 ± 4043
S100	Surface of ganglia (μm^2)	5317 ± 6122	6098 ± 4492	13259 ± 13626*	10765 ± 9563*
S100	Neurons/ganglion (n)	4.6 ± 3.2	5.2 ± 3.8	7.1 ± 6.9*	4.9 ± 4.1

* $P < 0.05$ versus control

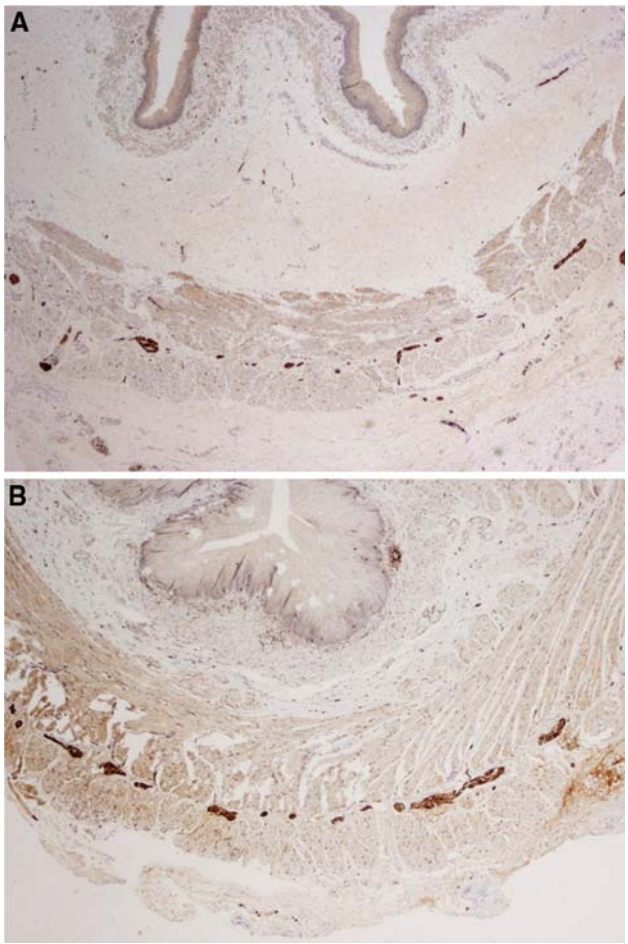

Fig. 1 Transversal sections of the distal esophagus in control (**a**) and isolated EA (**b**) patients. Immunostaining with S-100 (20×). The distribution of the intramural plexus is similar. However, the density of the fibrilar network is denser in **b**. The submucosal plexus is barely visible

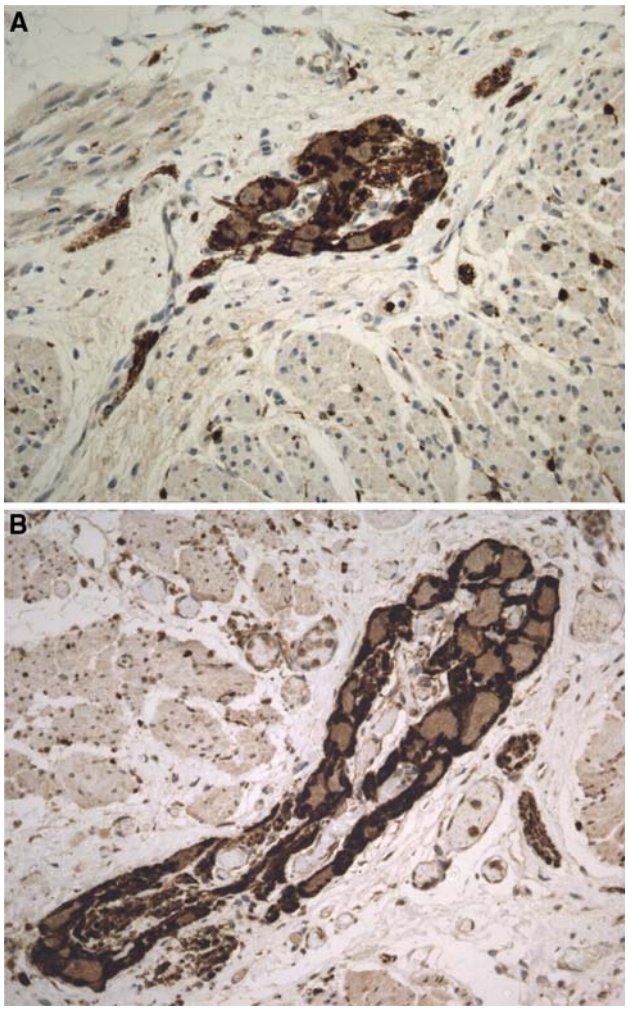

Fig. 2 Details of ganglia in the intermuscular plexus of the distal esophagus of control (**a**) and isolated EA (**b**) patients. Immunostaining with S-100 (400×). Glial cells and fibres are strongly immunostained allowing easy identification of the neurons, which appear in lighter color. The ganglia are larger and so are the neurons in isolated EA. Planimetric measurements revealed that the ganglia were significantly larger in this group

difficulties [5, 16, 17]. They also have a relatively short esophagus with intrathoracic cardia, obtuse angle of His and abnormal lower esophageal sphincter that cause GER with high proportion of esophagitis and in some cases Barrett's esophagus [1, 2, 4, 5, 18–20]. This occurs so often that detection and more or less aggressive treatment of GER become a part of the post-operative work-up during childhood and adolescence [6–8]. The clinical features of GER in this particular group of patients suggested that

peristalsis, the second anti-reflux barrier, must be also severely damaged. This has been demonstrated repeatedly by radiologic, isotopic, pH-metric and manometric studies in survivors to neonatal operations [4, 16, 17, 21] and even in neonates before the operation [22, 23].

This evidence prompted investigation of the esophageal innervation, which is crucial for the regulation of motility. Davies studied in human autopsies the extent of postoperative neural denervation in EA patients [24] and also the malformations of the laryngeal and vagus nerves that take in charge the innervation of the upper and lower esophageal ends in regular unoperated EA/TEF [25]. The structure of the muscle layers and the connective tissue of the esophageal wall was found to be abnormal in EA/TEF [26]. Nakazato et al. who had previously studied the tracheal innervation in EA with TEF [27], investigated the intramural plexuses by microdissection and measured the fraction of neural tissue in the plane of the Auerbach plexus at the upper and lower segments of the esophagus of five unoperated cases of EA/TEF and nine controls. They found that this fraction was significantly reduced particularly in the lower esophagus. In addition, EA patients had larger ganglia with thicker interganglionic fibers in the upper pouch [28]. Recently, other authors addressed this issue in different ways. Boleken et al. investigated immunohistochemically (neurofilament, synaptophysin, S-100 and glial cell-line derived neurotrophic factor—GNDF) the tip of the upper pouch in nine cases of EA/TEF and nine age matched-controls and found marked hypoganglionosis with immature neurons and decreased GNDF, SY and NF immunoreactivity with increased S-100 reactive fibers in the myenteric plexus [29]. Li et al. studied only the upper part of the fistula in 24 patients with EA/TEF and the corresponding level of the esophagus in 10 controls by immunohistochemistry (neuron-specific enolase, NSE; substance P, SP; vasoactive intestinal peptide, VIP and nitric oxide synthase, NOS) and electron-microscopy. They described decreased expression of NSE and SP and increased expression of VIP and NOS together with abnormal mitochondria in the muscle layers of the esophagus of EA/TEF patients that would explain abnormal relaxation of the distal esophageal smooth muscle favorising GER [30]. Although these studies did not address the same anatomical areas and used different methods, they showed that, as expected, intrinsic esophageal innervation is abnormal in EA/TEF patients and concluded that this might be an explanation for the motor dysfunction observed in these patients.

Some of the methodological difficulties of human studies could be to a certain extent circumvented since the adriamycin model of EA/TEF was made available [31]. Studies carried out in this model revealed anomalies of both the extrinsic and the intrinsic esophageal innervation in rats with EA/TEF. The vagus and recurrent laryngeal nerves were absent and/or abnormal upon microscopic assessment of multiple cervico-thoracic sections [32]. The distal esophagus of EA/TEF rats was immunohistochemically studied for various mediators by Cheng et al. who found increased immunoreactivity for S-100 and galanin and to a lesser extent for calcitonin gene-related peptide (CGRP) and SP [33]. These authors also described that the distribution of the immunoreactivity of protein gene product 9.5 (PGP) was abnormal whereas the surface occupied by these elements was similar to that of controls [34]. Qi et al. using whole mount preparation and immunohistochemical staining (NSE, VIP, SP and CGRP) of the distal esophagus of rats with EA/TEF showed that the number of neurons per ganglion and the density of the nerve plexuses were markedly reduced [35]. Nevertheless, despite the close resemblance of the adriamycin rat model of EA/TEF and the human condition, the findings in such model can only cautiously be transpolated to the latter.

Pure or isolated EA is rare (less than 10% of all patients) [36] and has its own peculiarities. The esophagus is reduced to the upper pouch and to a short distal segment that sometimes barely attains the lower mediastinum. The muscle layers are of striated fibres in the upper pouch and of smooth fibres in the distal segment. There is no fistula communicating the trachea and the esophagus and it is difficult to understand the embryogenesis even under the light of the recent investigations in the adriamycin rat model [9, 10]. In our extensive experience on the adriamycin rat model of EA/TEF, we never saw isolated EA and this has only been found in one instance by another group [37, 38]. To the present date, neither the gross anatomy of the nerve supply nor the intramural neural network on both esophageal segments were clinically or experimentally investigated in this form of EA.

The present study is the first to address this issue and confirms in the first place that the intramural innervation of the esophagus is also abnormal in patients with pure EA both at the upper and at the lower segments of the atretic organ. However, the innervation patterns were in part different from those of regular EA/TEF. The scarcity of the neural fraction and the hypoganglionism described at the upper [29] and lower [28] segments of babies and in the fistula of rats [35] with EA/TEF was not confirmed in our investigation of isolated EA. The fibrillar network was apparently denser than in controls at both segments of the atretic esophagus (although only significantly at the proximal one). On the other hand, the markedly larger intramural ganglia without variation of the number of neurons described at the upper pouch of human EA/TEF [28] (but not in the rat model [35]), was also observed on both esophageal segments of our cases of isolated EA. We observed increased expression of S-100 in the neural

elements of both the upper pouch and the lower segment of the atretic esophagus had that might represent a compensatory hypertrophy of the glial components of the neural network as previously suggested [29]. This hyperexpression was also found in the distal fistula in the rat model of EA/TEF [33].

There are some possible explanations for the different innervatory patterns found in regular EA/TEF and in isolated EA: first, neural crest cells migrate along the foregut before tracheoesophageal separation and also before differentiation of the muscle layers occur [39]. Vagal and intrinsic innervation acquire their final pattern after these processes are completed and it is likely that different degrees or mechanisms of abnormal cleavage leading to both types of EA could result in different innervatory patterns as well. Secondly, the lower end of the esophagus in this particular form of EA is likely different from the fistula of regular EA/TEF in which cartilage inclusions and islands of tracheal epithelial ectopia are frequent. The presence of specific respiratory transcription factors and the absence of esophageal ones in the fistula of rats with EA/TEF confirmed its respiratory origin [40, 41]. This might not apply to the lower end of the esophagus in isolated EA. Finally, the patients investigated in the present report were 7 $\pm$ 3.8 months old and both ends of the atretic esophagus were submitted during this period of time to distending intraluminal pressures (swallowing and aspiration at the upper pouch and GER at the lower end) that could have modified the neural network.

The methodological drawbacks of our study should be acknowledged: since the esophageal specimens were recovered during operations for esophageal replacement at variable ages, we were unable to gather an appropriate age-matched (or weight-matched) control group allowing comparison of the relative surfaces of the muscle layers occupied by neural elements and this may have exaggerated the apparently increased density of the fibrillar network. On the other hand, the rarity of this particular form of EA and the astringent conditions of the immunohistologic investigation reduced drastically (we had to discard the four autopsies available) the number of suitable specimens making our conclusions weaker. Finally, the bioptic nature of the material used for quantitative assessment of the intramural network, particularly the tip of the upper pouch, might have introduced by itself some inaccuracies in the results. Our use of image analysis software attempted at reducing subjectivity in all measurements. These drawbacks also apply to the studies carried out in human EA/TEF by other authors, that of Nakazato et al. [28] was the only one in which both ends of the atretic esophagus were examined whereas that of Boleken et al [29] only addressed the tip of the upper pouch and that of Li et al. [30] only examined a short distal fistula segment that can hardly represent the overall histology of the malformed esophagus. Studies in rats involved only the distal esophagus due to the minimal size of the upper pouch [33–35].

The significance of the variable expression of the different neuromediators described in both human and rat individuals with EA/TEF is difficult to discuss because only a few of them were addressed by more than one group. Overall, these findings suggest an imbalance between excitatory and inhibitory signalling that might be an explanation for dysmotility.

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