Distribution of Interstitial Cells of Cajal in the Esophagus of Fetal Rats with Esophageal Atresia

Özefagus Atrezili Rat Fetüs Özefagusunda İnterstisyel Cajal Hücrelerinin Dağılımı

Özefagusda İnterstisyel Cajal Hücrelerinin Dağılımı / Distribution of Interstitial Cells of Cajal in the Esophagus

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Özet

Amaç: İnterstisyel Cajal hücrelerinin (İCH) azalmış olması intestinal motilite bozuklukları ile ilişkilendirilmektedir. Bu çalışmada deneysel özefagus atrezisi (ÖA) rat modelinde, rat fetüs özefaguslarındaki İCH dağılımının değerlendirilmesi amaçlanmıştır. Gereç ve Yöntem: Elde edilen rat fetüsleri üç grupta toplanarak kontrol grubu, özefagus atrezisi eşlik etmeyen ve özefagus atrezisi eşlik eden adriyamisin grupları oluşturuldu. Adriyamisin grubu ratlara 6 ve 9. gebelik günlerinde 4 doz, 2 mg/kg'dan intraperitoneal adriyamisin enjekte edildi. Rat fetüslerinin özefagusunda, immünohistokimyasal yöntemle (c-kit, CD117) İCH varlığı değerlendirildi. Mikroskobik değerlendirme ortalama İCH sayılarına göre, 1'den 3'e kadar olan görsel bir skorlama sistemi kullanılarak yapıldı. Bulgular: Her gruba 7 fetüs dahil edildi. Gruplardaki İCH skor 3 alan fetüslerin sayısı, kontrol grubunda 5 (%72), ÖA eşlik etmeyen adriyamisin grubunda 3 (%42), ÖA eşlik eden adriyamisin grubunda 1 (%14) olarak belirlendi. ÖA'nin eşlik ettiği adriyamisin grubu ile kontrol grubu karşılaştırıldığında, ÖA'nin eşlik ettiği adriyamisin grubunda İCH dağılımında anlamlı bir azalma olduğu belirlendi ($p < 0.05$). Konrol grubu ile ÖA'nin eşlik etmediği adriyamisin grubu karşılaştırıldığında İCH dağılımında anlamlı bir fark olmadığı belirlendi ($p > 0.05$). Tartışma: ÖA'li rat fetüslerin İCH yoğunluğunun ÖA olmayan rat fetüsler ile karşılaştırıldığında daha düşük olduğu görüldü. Bu bulgular ÖA'de İCH dağılımının konjenital olarak anormal olduğunu gösterebilir. Bu durum, ÖA'ne bağlı ameliyat olmuş özefaguslarda görülen dismotilite ile ilişkilendirilebilir.

Anahtar Kelimeler

Özefagus Atrezisi; İnterstisyel Cajal Hücresi; C-Kit

Abstract

Aim: Scarcity of the interstitial cells of Cajal (ICC) is related to motility disorders. In the study, we aimed to evaluate the number and density of ICCs in the fetal rat esophagus in the adriamycin - esophageal atresia (EA) model. Material and Method: Rat fetuses were divided into three groups as a control, adriamycin group without EA and adriamycin group with EA. Four doses of adriamycin, 2 mg/kg each, were injected intraperitoneally to the adriamycin group rats between on 6 and 9 days of gestation. The presence of ICCs in the esophagus of the rat fetuses was determined by using an immunohistochemistry technique (c-kit, CD117). The average numbers of ICCs were calculated with microscopic evaluation by using a visual scoring system (range1 to 3). Results: Seven fetuses were included in each group. The ICCs score 3 distributions of fetuses were 5 (72%) fetuses in the control group, 3 (43%) fetuses in the adriamycin group without EA, 1 (14%) fetus in the adriamycin group with EA. It have been found that there was a marked reduction of ICCs distribution in the adriamycin group with EA compared to control group ($p < 0.05$). There was no significant difference the ICCs distribution between the control group and the adriamycin group without EA ($p > 0.05$). Discussion: ICCs density was significantly decreased in the rat fetuses with EA compared to the fetuses without EA. These findings support the idea that ICCs density may be congenitally abnormal in EA. This may be led to dismotility seen in the operated esophagus due to EA.

Keywords

Esophageal Atresia; Interstitial Cells of Cajal; C-Kit

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Introduction

The current treatment approach for esophageal atresia (EA) and tracheoesophageal fistula (TEF) is esophago-esophagostomy [1]. The motility problems in the patients who underwent an esophageal atresia repair can appear during postoperative period and 60-90% of the patients suffer from dysphagia, vomiting and gastroesophageal reflux disease (GERD) depended on the motility disorder [1-4]. It was determined that the motility disorders seen in the patients with esophageal atresia may result from primary malformation of the neural structures or neural tissue injuries secondary to atresia repair [5-7].

The control of the esophageal motility is provided by the intrinsic and extrinsic innervations of the esophagus that regulate the muscle activity [8,9]. It has been believed that the interstitial cells of Cajal (ICCs) have critical roles in intrinsic innervations of the gastrointestinal system [10]. Distribution and maturation of the intrinsic pacemaker cells are determined as abnormal in some gastrointestinal system diseases related to peristalsis and motility disorders [11-15]. It is likely to be seen a relation between the density of ICCs and esophageal dismotility in the patients operated for EA.

The development of EA/TEF in rat fetuses has been observed experimentally when adriamycin that is known to have a teratogenic effect, was given to pregnant rats [16,17]. In the current study, we aimed to evaluate the number and density of ICCs in the fetal rat esophagus using adriamycin-induced EA rat model.

Material and Method

All procedures involving animals were reviewed and approved by The Institutional Ethics Committee for Animal Experiments (no: 60/2010-24/12/2010). The animal protocol was designed to minimize pain or discomfort to the animals. This study was conducted in the Experimental Animal Department and Pathology Department of Dokuz Eylül University between April and July 2012.

Animal model

Primigravida female Wistar albino rats, weighing 200-250g, kept on standard laboratory chow and with free access drinking water and eating food, were used throughout the experiments. Couples were kept together for 24 hours. After mating, the female rats that had the vaginal plate plaque and sperm in their vaginal smears were accepted as the #0 day of their pregnancy and they were included in the study. The pregnant rats were kept in plastic bathtubs in laboratories in which the humidity and the temperature were controlled. The rats were housed in separate cages with controlled temperature (23°C), humidity (50%) and 12 hour light/dark cycle.

In this study, 2 main groups were created. These are the control group and the adriamycin groups. The control group includes normal rat fetuses that did not undergo any intervention. The adriamycin groups included two subgroups; with EA and without EA groups. Four doses of adriamycin (Adriblastina; Deva, Istanbul, Turkey), 2mg/kg each, were injected intraperitoneally to the adriamycin group rats between the embryologic day (E) E6 and E9 days of gestation.

Tissue Handling

At the E20, all dams were general anesthesia (pentobarbital 50 mg/kg body wt intraperitoneal injection) with ether and the surviving fetuses were harvested by cesarean section, then the dams were euthanized by cervical dislocation and the rat fetuses were killed by decapitation. Then, the fetuses were dissected under the microscope with x10 magnification and the fetuses in the adriamycin group were divided into two groups; fetuses with EA and fetuses without EA. The fetal distal esophagus was dissected starting from the gastroesophageal junction up to the tracheal bifurcation region and harvested. A full-thickness biopsy from the lower one third of the esophageal tissue was obtained from the fetuses of all groups. The study continued until we obtained 7 fetuses in each group.

Tissue preparation and immunohistochemical staining

The 4 µm sections were obtained from the tissues. They were embedded into the paraffin blocks and incubated 48 h in the Bouin's solution for the histopathological examination. The sections were deparaffinized with xylene. The dehydration of the sections was performed with alcohol (1x5 min). The samples were boiled in the microwave oven (56˚C, 5 min) in ethylenediamine-tetraacetic acid (EDTA) buffer in order to expose the antigen. The samples were treated with polyclonal primer antibody (A4502: CD117, c-kit, DAKO, California, USA) at 1:100 dilution at room temperature for 45 min. Then, the chromogenic material with peroxidase and the labeled secondary antibody (ACT500: DAB Chromogen/Substrate Kit, Scy-Tek, Utah, USA) was applied to the samples. Upon waiting 20 min at room temperature, the emergence of the brown color was considered as positive staining. The counterstaining of the samples was performed with Hematoxylin-eosin for the morphological evaluation. ICCs were evaluated morphologically as a result of microscopic observations by staining the cells with Hematoxylin-eosin. The c-kit (CD117) positive cells were detected in all groups according to the immunohistochemical findings.

Under x40 magnification light microscope, ICCs were counted in 10 different microscopic sites which were selected randomly by the pathologist and expressed as count per unit area (100 µm2). The pathologist was blinded with respect to experimental groups when preparing and evaluating the specimens. Morphological differences were used to distinguish the ICCs from the mast cells. The counting results of the ICCs were scored from 1-3, according to the average number of ICCs. The scoring system was as follows: score 1= if cell count is between 0 and 2, score 2= if cell count is 3 or 4, Score 3= if cell count is more than 4 (Table1).

Table 1. The visual scoring system for the evaluation of the ICC distributions in fetal rat esophagus

Visual scoring system
Score 1= if cell amount is between 0-2
Score 2= if cell amount is between 3-4
Score 3= if cell amount is more than 4

Statistical analysis

The statistical differences between the groups were evaluated by using chi-square and Kruskal-Wallis test. The p-value less

than 0.05 were considered as statistically significant.

Results

Macroscopic observations

In this study, 28 fetuses was obtained from 5 pregnant rats in which there were one pregnant rat in the control group and four pregnant rats in the adriamycin group. Seven fetuses were obtained in the control group. In the adriamycin group, EA and distal TEF (Type C) were determined in 10 (47%) out of 21 fetuses. Seven fetuses from each group were included to the study. In the adriamycin group, three out of 10 fetuses with EA, and four out of 11 fetuses without EA in which did not obtained convenient esophageal tissue were excluded from the study. Biopsy specimens could only be obtained from the distal esophagus, since the proximal esophagus was embedded in the neck and it was not technically possible to obtain a proper tissue for biopsy. Therefore, tissue samples were taken from the 1/3 distal esophagus of the control and experimental group fetuses without EA and the distal esophagus of the fetuses with EA (Figure 1).

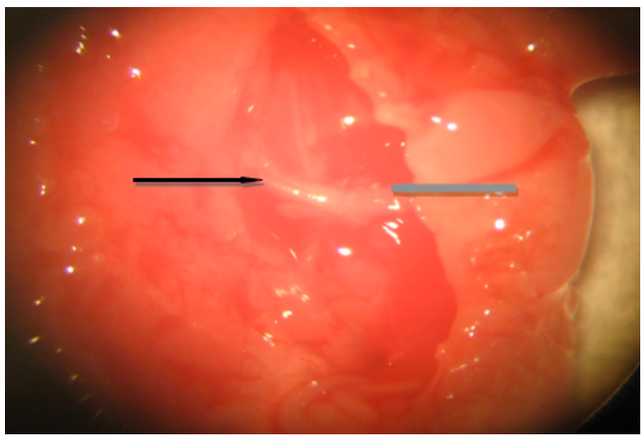


Figure 1. Type-C esophageal atresia in a rat fetus (x10 magnification), tracheoesophageal fistula (black arrow), gastroesophageal junction (gray arrow)

Changes in the number and density of ICCs

The ICCs score distributions of fetuses were as follows: the score was 2 (28%) in 2 fetus and score 3 was (72%) in 5 fetus of the control group whereas the score was 1 (14%) in 1 fetus, score 2 was (43%) in 3 fetus and score 3 was (43%) in 3 fetus of the adriamycin group without EA. There was no c-kit (-) sections in the both control and adriamycin group without EA. In the adriamycin group with EA, the score was 1 (72%) in 5 fetus, score 2 was (14%) in 1 fetus and score 3 was (14%) in 1 fetus (In the adriamycin group with EA, there were 3 sections which were stained c-kit (-) (Figures 2-4).

When the ICCs scores of each group were evaluated, we have found that there was a marked reduction curve of ICCs distribution of the group with EA ($p < 0.05$). When we compared the control group and the group without EA, there was no statistically significant difference between them regarding the ICCs scores distributions ($p > 0.05$) (Table 2).

Discussion

ICCs had been first described in the gastrointestinal tract that was thought to be originating from the precursors of the smooth muscle cells serving as a pacemaker to initiate peristalsis [10]. ICCs in the gastrointestinal system are located in the myenteric plexus, inside the circular muscle layer and between the muscle fibers on the gut wall [10]. Intrinsic pacemaker cells included ICCs maintain an electrical activity for the peristaltic waves [10]. ICCs are important cells for organs with peristaltic

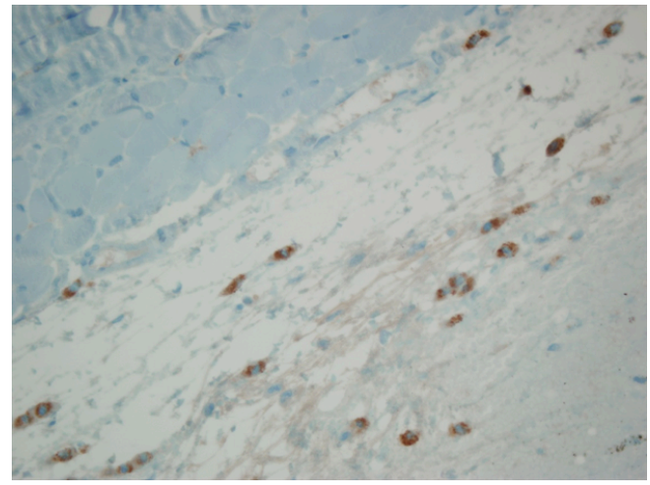


Figure 2. ICCs were not observed between muscles of the esophageal tissues in the rat fetuses with EA, while there are many mast cells staining c-kit positive (c-kit, x40)

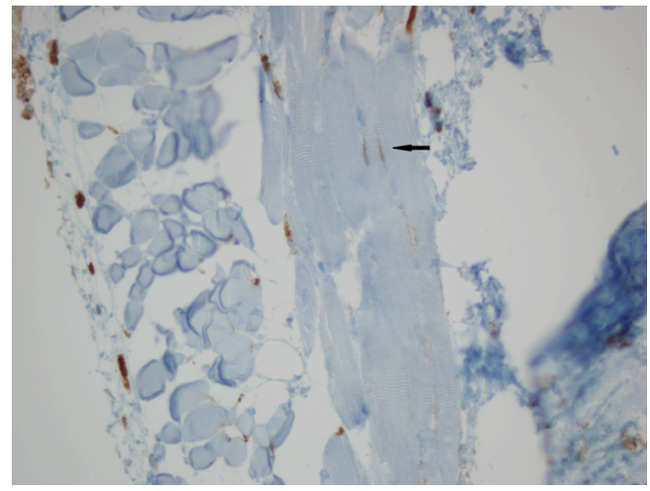


Figure 3. C-kit (CD117) positive ICCs (arrow) in intermuscular area of the fetal rat esophagus in the control group (c-kit, original magnification x40)

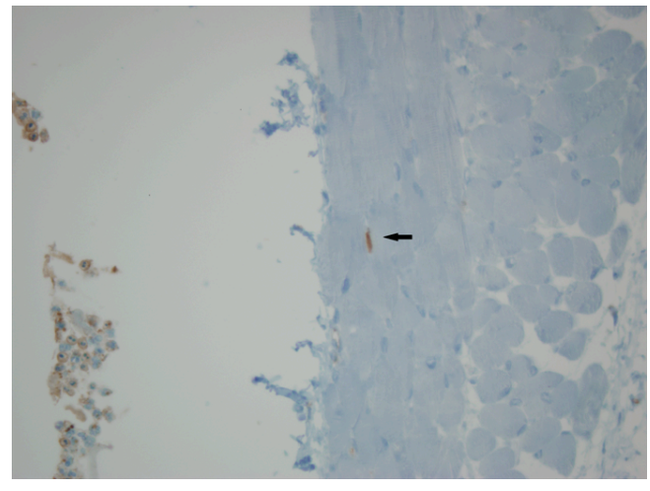


Figure 4. C-kit (+) ICCs (arrow) in the rat fetus esophagi of the adriamycin group without EA in the intermuscular area (c-kit, original magnification x40)

Table 2. The visual scores distribution of the interstitial cells of Cajal in the fetal rat fetuses

Groups	Score 1 (n)	Score 2 (n)	Score 3 (n)
Control	0	2	5
EA atresia (-)†	1	3	3
EA atresia (+)*	5	1	1

Esophageal atresia (EA)
† p = 0.427 when the atresia (-) group compared with control group
* p = 0.018 when the atresia (+) group compared with control group

function. ICCs produce signals for rhythmic contractions and their absence is associated with peristaltic dysfunction. ICCs show c-kit positivity by means of tyrosine kinase surface receptors which can be stained with c-kit antibodies [18]. Peristaltic dysfunction is closely related with the aplasia, hypoplasia or dysplasia of these cells. ICCs have been shown to be absent or decreased in some gastrointestinal system diseases related to peristalsis and motility disorders such as Hirschsprung's disease, neuronal intestinal dysplasia, infantile hypertrophic pyloric stenosis [11-15,19]. It is demonstrated that a remarkable decrease of ICC in small bowel wall of patients with intestinal atresia [14,15].

It is reported in some studies that esophageal motility failure in EA is associated with the neural damage due to operation, anatomical branching defects of the vagus nerve and Auerbach plexus hypoplasia [5-9,20,21]. The motility defects seen in the esophagus of EA-TEF cases, in which surgical dissection is limited at the lower esophagus, strongly suggests that the motility problems may result from the innervation deficiency of the esophagus [22]. Additionally, the detection of the irregular peristaltic pattern and accompanying GERD before the corrective operation in isolated TEF cases is also showed that innervation defects of the esophagus may be congenital [23].

It has been recently stated that ICCs are closely contact with smooth muscle cells and nerve endings [10,18]. Thus, it is possible that ICCs numbers and the distributions may also be affected in the EA in which smooth muscle structure is disrupted congenitally.

After the animal models of EA were described, several studies have been conducted regarding the pathophysiology of the EA [8,9]. Romeo et al [20] have showed that a decline in the relaxation response in the lower esophageal segments of the babies who had not surgery for EA with manometer in the newborn period. In an experimental EA rat model, Turgay et al [24] have showed a decline in the relaxation response in the lower esophageal segments of the rat fetuses with adriamycin induced EA. However, there is no data about the relation between the ICCs and the insufficient esophageal relaxation response in EA. Midrio et al [25] have performed a post-mortem study investigating the ICCs numbers and distributions in the esophagus of the babies with EA. They have found that the density of the ICCs was significantly decreased in the biopsy samples obtained from both distal and proximal segments of the atretic esophagus [25].

In the current study, we have found that ICCs numbers and densities significantly decreased in the rat fetuses with EA compared to the fetuses without EA. These findings support the idea that ICCs density may be congenitally abnormal in EA. We conclude that a decline in the ICCs density may negatively affect the esophageal peristaltic activity in the EA by decreasing the relaxation response in the smooth muscle tissue. This may be related to dismotility seen in the operated esophagus due to EA and TEF.

In conclusion, the scarcity of the ICCs in EA may result in the esophageal motility problems with different clinical findings observed after the operation. Nevertheless, further histological and physiological studies should be done to determine the relation between the absence of ICCs and esophageal peristalsis disorders. Additionally teratogenic effects of adriamycin on the fetal esophagus should also evaluated.

Competing interests

The authors declare that they have no competing interests.

References

1. Lija HE, Wester T. Outcome in neonates with esophageal atresia treated over the last 20 years. Pediatr Surg Int 2008;24(5):531-6.
2. Spitz L. Esophageal atresia. Lessons I have learned in a 40- year experience. J Pediatr Surg 2006;41(10):1635-40.
3. Tomaselli V, Volpi ML, Dell'Agnola CA, Bini M, Rossi A, Indriolo A. Long-term evaluation of esophageal function in patients treated at birth for esophageal atresia. Pediatr Surg Int 2003;19(1-2):40-3.
4. Shono T, Suita S, Arima T, Handa N, Ishii K, Hirose R, et al. Motility function of the esophagus before primary anastomosis in esophageal atresia. J Pediatr Surg 1993;28(5):673-6.
5. Kirkpatrick JA, Cresson SL, Pilling GP 4th. The motor activity of the esophagus in association with esophageal atresia and tracheoesophageal fistula. Am J Roentgenol Radium Ther Nucl Med 1961;86:884-7.
6. Davies MR. Anatomy of the extrinsic motor nerve supply to mobilized segments of the oesophagus disrupted by dissection during repair of oesophageal atresia with distal fistula. Br J Surg 1996;83(9):1268-70.
7. Cheng W, Bishop AE, Spitz L, Polak JM. Abnormal enteric nerve morphology atretic esophagus of fetal rats with adriamycin-induced esophageal atresia. Pediatr Surg Int 1999;15(1):8-10.
8. Qi BQ, Uemura S, Farmer P, Myers NA, Hutson JM. Intrinsic innervation of the oesophagus in fetal rats with oesophageal atresia. Pediatr Surg Int 1999;15(1):2-7.
9. Qi BQ, Merei J, Farmer P, Hasthorpe S, Myers NA, Beasley SW, et al. The vagus and recurrent laryngeal nerves in the rodent experimental model of esophageal atresia. J Pediatr Surg 1997;32(11):1580-6.
10. Sanders KM, Ward SM. Interstitial cells of Cajal-a new perspective on smooth muscle function. J Physiol 2006;576(3):721-6.
11. Taniguchi K, Matsuura K, Matsuoka T, Nakatani H, Nakano T, Furuya Y, et al. A morphological study of the pacemarker cells of the aganglionic intestine in hirschsprung's disease utilizing Is/Is model mice. Med Mol Morphol 2005;38(2):123-9.
12. Jeng YM, Mao TL, Hsu WM, Huang SF, Hsu HC. Congenital interstitial cells of cajal hyperplasia with neuronal intestinal dysplasia. Am J Surg Pathol 2000;24(11):1568-72.
13. Vanderwinden JM, Liu H, Menu R, Conreur JL, De Laet MH, Vanderhaeghen JJ. The pathology of infantile hypertrophic pyloric stenosis after healing. J Pediatr Surg 1996;31(11):1530-4.
14. Schoenberg RA, Kluth D. Experimental small bowel obstruction in chick embryos: Effects on the developing enteric nervous system. J Pediatr Surg 2002;37(5):735-40.
15. Tander B, Bicakci U, Sullu Y, Rizalar R, Ariturk E, Bernay F, et al. Alterations of Cajal cells in patients with small bowel atresia. J Pediatr Surg 2010;45(4):724-8.
16. Diez-Pardo JA, Baoquan Q, Navarro C, Tovar JA. A new rodent experimental model of esophageal atresia and tracheoesophageal fistula: preliminary report. J Pediatr Surg 1996;31(4):498-502.
17. Merei J, Hasthorpe S, Farmer P, Hutson JM. Visceral anomalies in prenatally adriamycin-exposed rat fetuses: a model for the VATER association. Pediatr Surg Int 1999;15(1):11-6.
18. Sanders KM, Ordög T, Koh SD, Torihashi S, Ward SM. Development and plasticity of interstitial cells of Cajal. Neurogastroenterol Motil 1999;11(5):311-38.
19. Harberson J, Thomas RM, Harbison SP, Parkman HP. Gastric neuromuscular pathology in gastroparesis: analysis of full-thickness antral biopsies. Digestive Diseases and Sciences 2010;55(2):359-70.
20. Romeo G, Zuccarello B, Proietto F, Romeo C. Disorders of the esophageal motor activity in atresia of the esophagus. J Pediatr Surg 1987;22(2):120-4.
21. Nakazato Y, Landing BH, Wells TR. Abnormal Auerbach plexus in the esophagus and stomach of patients with esophageal atresia and tracheoesophageal fistula. J Pediatr Surg 1986;21(10):831-7.
22. Burgess JN, Carlson HC, Ellis FH Jr. Esophageal function after successful repair of esophageal atresia and tracheoesophageal fistula. A manometric and cinefluorographic study. J Thorac Cardiovasc Surg 1968;56(5):667-73.

23. Gundry SR, Orringer MB. Esophageal motor dysfunction in an adult with a congenital tracheoesophageal fistula. Arch Surg 1985;120(9):1082-3.
24. Turgay M, Yildiz F, Utkan T, Ulak G, Gacar N, Erden F. Impaired esophageal reactivity in adriamycin-induced rat esophageal atresia: an in vitro study. J Pediatr Surg 2001;36(10):1569-73.
25. Midrio P, Alaggio R, Strojna A, Gamba P, Giacomelli L, Pizzi S, et al. Reduction of interstitial cells of Cajal in esophageal atresia. J Pediatr Gastroenterol Nutr 2010;51(5):610-7.

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