Özet
Amaç: İnterstisyel Cajal hücrelerinin (İCH) azalmış olması intestinal motilite bozuklukları ile ilişkilendirilmektedir. Bu çalışmada deneysel özefagus atresişi (ÖA) rat modelinde, rat fetüs özefaguslarında ICC 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 gruptuları oluşturuldu. Adriyamisin gruba 6 ve 9. gebelik günlerinde 4 doz, 2 mg/kg'dan intraperitoneal adriyamisin e juris edildi. Rat fetüslerinin özefagusunda, immünohistokimyasal yöntemle (c-kit, CD117) İCH varlığı değerlendirildi. Mikroskobik değerlendirme ortalamalı 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 fetüsler sayısının, kontrol grubunda 5 (%72), ÖA eşlik etmediği grupda 3 (%42), ÖA eşlik ettiği grupda 1 (%14) olarak belirlendi. ÖA'nın eşlik ettiği adriyamisin gruba İCH dağılımında anlamli bir azalma olduğu belirlendi (p < 0.05). ÖA'nın eşlik ettiği adriyamisin gruba İCH dağılımında anlamli bir fark olmadı (p > 0.05). Tartışma: ÖA'lı rat fetüslerin İCH yoğunluğunun ÖA olmayan rat fetüsler ile karşılaştırıldığında daha düşük olduğu görüldü. Bu bulgular, ÖA ve adriyamisinın(Media: 905067021777 F.: +90 3882121411 E-Mail: caner.isbir@gmail.com) interstisyel Cajal hücrelerinin dağılımı ile ilişkilendirebilir.

Keywords
Esophageal Atresia; Interstitial Cells of Cajal; 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 (0 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, and 1 (14%) fetus in the adriamycin group with EA. It was found that there was a marked reduction of ICCs distribution in the adriamycin group with EA compared to control group. There was no significant change in 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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Supporting Information
S1: Supporting Figure 1. S2: Supporting Figure 2. S3: Supporting Figure 3. S4: Supporting Figure 4. S5: Supporting Figure 5. S6: Supporting Figure 6.

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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, İstanbul, 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 ethylene-diamine-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

<table>
<thead>
<tr>
<th>Visual scoring system</th>
<th>Score 1= if cell amount is between 0-2</th>
<th>Score 2= if cell amount is between 3-4</th>
<th>Score 3= if cell amount is more than 4</th>
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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 was 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).

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
interstitial cells of Cajal (ICCs) are 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 pyloricstenosis [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.

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


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