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Histological study of the elongated esophagus in a rat model

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ABSTRACT

Background: Esophageal elongation by traction suture is used in pediatric patients to manage long-gap esophageal atresia (EA). There was no histological evidence of the esophageal elongation. Here, we sought to clarify the histologic effects of traction on the esophagus by using a rat EA model simulating Foker's method.

Materials and methods: Rats were randomly assigned into three groups ($n = 5$ each). The traction group underwent daily stretching of the distal segment of the esophagus. The nontraction group underwent a sham operation, and the normal group served as controls. Seven days after the operation, the distal segments of the esophagus were removed. The length and thickness were measured, and samples were stained with Ki-67, nNOS, and S-100.

Results: The whole length of the esophagus in the traction group was significantly longer than that in the nontraction group ($P < 0.01$). The thickness of esophageal mucosa and muscle tended to become thin by traction, but not significantly. The Ki-67-positive ratio of mucosa and muscle was significantly higher in the traction group ($P < 0.05$). There were no significant differences in Ki-67 between two segments (cardia-middle and middle-stump) in any group. Auerbach's plexus was identified at all sites of elongated esophagus by nNOS and S-100 staining.

Conclusions: By traction, the esophagus was elongated uniformly and cell proliferation activity was promoted in all parts of the elongated esophagus in the rat EA model.

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1. Introduction

Esophageal elongation by traction suture has been used for the management of long-gap esophageal atresia (EA) in pediatric patients [1–8]. Foker's method is one of the surgical techniques used to elongate the esophagus. It has been shown to elongate the esophageal length, allowing for direct esophageal anastomosis, and it can be carried out in a clinical setting for EA patients [1]. Khan *et al.* [9], using high-resolution ultrasound, reported that there was no significant difference in the thickness of the individual mural layer between a traction group and a nontraction group of EA patients after the use of traction suture.

However, it is difficult to evaluate the histological findings of the elongated esophagus in humans. Although an animal model of the esophageal elongation has been developed [10], the mechanism of the elongation of the esophagus by traction has remained unclear, and it is not known whether esophageal elongation is due simply to mechanically stretching or is caused by cell proliferation. We made a hypothesis that the

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esophagus was elongated by not only mechanical stretching but also the promotion of cell proliferation. The aim of the present study was to clarify the histological effects of traction on the esophagus, especially regarding cell proliferation, using a rat model that simulates Foker's method.

2. Materials and methods

2.1. Experimental procedures

Our animal model was based on the Lopes model [10]. All rats received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the US National Academy of Sciences and published by the US National Institutes of Health (NIH publication 86-23, revised 1985). The animal protocol was approved by the Animal Experimentation Committee of Nagasaki University.

Six-to-seven-week-old male Sprague–Dawley rats (Charles River Laboratories Japan, Yokohama, Japan), weighing 180–262 g, were randomly assigned into three groups ($n = 5$ each) as follows: the traction group rats underwent daily stretching of the distal segment of the esophagus; the nontraction group rats underwent a sham operation, and the normal group rats served as controls.

After the achievement of general anesthesia with isoflurane, pentobarbital at 40 mg/kg was given intraperitoneally. The rat was placed in the supine position, and its abdomen was prepped in a standard surgical manner. A vertical abdominal incision was made and the peritoneal cavity was entered. After the abdominal esophagus was marked with 6-0 Nylon suture (Alfresa Pharma, Osaka, Japan) at the cardia, 2 and 4 mm from the cardia, the abdominal esophagus was divided at 4 mm from the cardia, and the distal esophageal segment was closed using 7-0 Prolene sutures (Ethicon, Cornelia, GA) and measured again to check the initial length.

To maintain the esophageal tension by traction, the gastric anterior wall close to the cardia was attached to the anterior abdominal wall with sutures. The continuity of the digestive tract was restored by an end-to-side esophagojejunal anastomosis with the continuous Gambee technique using 7-0 Prolene, which joined the proximal esophageal segment and the jejunum at 5 cm from the duodenojejunal ligament. Two horizontal mattress sutures of 6-0 Nylon were deeply placed at the edge of the stump of the distal esophageal segment. These stitches were passed through the subcutaneous tissue, from the site of the abdominal incision to the dorsum. Once in the exterior, the stitches were fixed under maximum esophageal tension (traction group) using a 0.5-mm-width titanium ligation clip (Vesocclude Medical, Raleigh, NC).

After the closing of the abdominal wall incision, the rat was allowed to recover from anesthesia and was kept alive for 7 d after operation. During this period, a new 0.5-mm-wide clip was added daily to each traction group rat to increase the tension uniformly. The nontraction group was operated in the same manner but no tension or stretching was applied to the distal esophagus.

Postoperatively, the rats were allowed free cage activity and took water and feed freely. And all rats received carprofen administered subcutaneously once daily (0.5 mg/kg body weight). At the seventh postoperative day, after general anesthesia, the distal esophagus was removed, and we measured whole length of the distal esophagus and the distance between the strings in the tension-free state. All surviving rats were euthanized by an anesthesia overdose, and blood was removed from the inferior vena cava. As the control, five normal esophagi were removed by the same procedure.

2.2. Histological analysis

2.2.1. Thickness of the esophageal mucosa and muscle

Tissue samples were fixed in 4% paraformaldehyde phosphate buffer solution (Wako Chemical Industries, Miyazaki, Japan) and embedded in paraffin, sectioned (5 μm), and stained with hematoxylin-eosin. Images were obtained on an optical microscope, and the thickness of the esophageal mucosa and muscle was measured using the software WinROOF version 6.3 (Mitani, Tokyo, Japan). The thickness was recorded at ×10 magnification, and three points per microscopic field were measured. Six microscopic fields were checked per slide as follows: three from the cardia to the middle point (2 mm from the cardia), and the others from the middle point to the distal stump (4 mm from the cardia). The means of these values were computed.

2.2.2. Immunohistochemistry

Immunohistochemistry was performed by the diaminobenzidine method after ethanol and xylene deparaffinization. Antigen retrieval was performed by the microwave method with Dako REAL Target Retrieval Solution x10 (Dako, Tokyo, Japan). After peroxidase blocking for 15 min at room temperature (RT), the following primary antigens were used: Ki67 (Anti-Ki67 antibody; Abcam, Cambridge, United Kingdom), nNOS (Anti-nNOS antibody; Abcam), and S-100 (Polyclonal Rabbit Anti-S-100 antibody; Dako). S-100 (ready-to-use) was incubated for 60 min at RT. Ki67 (1:100 dilution) and nNOS (1:1000 dilution) were incubated overnight at 4°C. Anti-rabbit IgG-peroxidase antibody produced in goat (1:300 dilution with 1% bovine serum albumin; Sigma–Aldrich, St. Louis, MO) was used as the secondary antibody and incubated for 60 min at RT.

According to the Ki67 results, images were obtained on an optical microscope, and the positive ratio (positive/total cells) of the esophageal mucosa and muscle was counted using the WinROOF version 6.3 software by counting the positive cells and total cells per microscopic field. Six microscopic fields per slide were checked as follows: three from the cardia to the middle point (cardia-mid), and the others from the middle point to the distal stump (mid-stump). The means of these values were computed.

2.3. Statistical analyses

All results are presented as means ± standard deviation. Overall comparisons between groups were made with the GraphPad Prism 6 software program (GraphPad Software, San Diego, CA). The significance of differences was assessed by Mann–Whitney *U*-test for two groups and by one-way analysis of variance for three groups. P values < 0.05 were considered significant.

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