Histological study of the elongated esophagus in a rat model

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Author Contributions

S.Inoue, T.Kosaka, and M.Takatsuki contribute to conception and design of study.

S.Inoue, T.Kosaka, and M.Takatsuki contribute to analysis and interpretation of data.

S.Inoue contributes to collection and assembly of data and drafting of the article.

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S.Eguchi contribute to final approval of the article
ABSTRACT [223 words]

**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 histological 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 non-traction group underwent a sham operation, and the normal group was the 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 non-traction 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, 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.

Key Words: esophageal elongation, proliferation, long-gap, esophageal atresia, Foker’s method, esophagus
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 non-traction 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 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, by using a rat model that simulates Foker’s method.
MATERIALS AND METHODS

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 U.S. National Academy of Sciences and published by the U.S. 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): the traction group rats underwent daily stretching of the distal segment of the esophagus; the non-traction 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 Nylone suture (Alfresa Pharma, Osaka, Japan) at the cardia, 2 mm 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, USA) 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 Nylone 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 days 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 non-traction 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 (0.5mg/kg body weight). At the 7th 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.

**Histological analysis**

*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 were measured using the software WinROOF ver. 6.3 (Mitani, Tokyo). The thickness was recorded at ×10 magnification, and three points per microscopic field were measured. Six microscopic fields were checked per slide: 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.

**Immunohistochemistry**

Immunohistochemistry was performed by the diamino benzidine method after ethanol and xylene deparaffinization. Antigen retrieval was performed by the microwave method with Dako REAL Target Retrieval Solution x10 (Dako, Tokyo). After peroxidase blocking for 15 min at room temperature (RT), the following primary antigens were used: Ki67 (Anti-Ki67 antibody, Abcam, Cambridge, UK), 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 [BSA], 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 were counted using the WinROOF ver. 6.3 software by counting the positive cells and total
cells per microscopic field. Six microscopic fields per slide were checked: 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.

**Statistical analyses**

All results are presented as means ± SD. 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 ANOVA for three groups. P-values < 0.05 were considered significant.

**RESULTS**

All animals survived the surgical procedure. No animal became sick, however in contrast to the non-traction group, the traction group showed reduced activity. The animals reduced weight. The weight loss of traction group was -44 ± 5.6 g, and that of non-traction was -27.6 ± 7.6 g. There was a significant difference between them.

**Length of the distal esophagus**

There was a significant difference between the non-traction and traction groups in the
whole length of the esophagus. The whole length of the non-traction group was 3.6 ± 0.9 mm, and that of the traction group was 8.0 ± 1.2 mm (P<0.01, Fig. 1). However, there were no significant differences between the two segments within the non-traction and traction groups. In the non-traction group, the length of the cardia-mid was 1.8 ± 0.4 mm, and that of the mid-stump was 1.8 ± 0.4 mm. In the traction group, the length of cardia-mid was 3.6 ± 0.5 mm, and that of mid-stump was 4.4 ± 1.1 mm (Table 1). The esophagus was elongated uniformly by traction.

**Histology**

*Thickness of esophageal mucosa and muscle*

Our hematoxylin-eosin staining of the esophagus demonstrated an intact epithelial and muscle lining in each group. However, compared to the non-traction group, the traction group revealed mild inflammation and adhesion around the insertion of the traction sutures (Fig. 2A–F).

The overall mean of the mucosal thickness of the normal group was 11.7 ± 5.1 μm; that of the non-traction group was 12.2 ± 4.3 μm, and that of the traction group was 8.6 ± 4.0 μm. There were no significant differences between the normal, non-traction, and traction groups in the thickness of the mucosa (P=0.42, Fig. 2G) In addition, there
were no significant differences between the two segments among the three groups (Table 1).

The overall mean of the muscle thickness of the normal group was $142.3 \pm 38.7 \mu m$; that of the non-traction group was $85.5 \pm 40.7 \mu m$, and that of the traction group was $93.4 \pm 21.4 \mu m$. The thickness of the muscle in the non-traction and traction groups was thinner than that of the normal group and there is a strong trend, but the between-group differences were not significant ($P=0.05$, Fig. 2H). Although the thickness of the muscle of the mid-stump tended to be thinner than that of the cardia-mid in the traction group, there were no significant differences between the two segments in all three groups (Table 1).

**Immunohistochemistry**

*Ki-67-positive ratio in mucosa and muscle*

The overall mean of the Ki-67-positive ratio in the mucosa of the normal group was $25.2 \pm 5.1\%$; that of the non-traction group was $11.6 \pm 5.4\%$, and that of the traction group was $33.9 \pm 2.9\%$. In the traction group, the Ki-67-positive ratio in the mucosa was significantly higher than those of the other two groups. In addition, in the non-traction group, the Ki-67-positive ratio in the mucosa was significantly lower than those of the
other groups (P<0.01, Fig. 3G). There were no significant differences between the two segments among the three groups (Table 1).

The overall mean of the Ki-67-positive ratio in the muscle of the normal group was $3.0 \pm 1.3\%$; that of the non-traction group was $1.7 \pm 1.0\%$, and that of the traction group was $8.2 \pm 5.0\%$. The Ki-67-positive ratio in the muscle of the traction group was significantly higher than those in the other two groups (P=0.01, Fig. 3H). There were no significant differences between the two segments in all three groups (Table 1). The cell proliferation activity was promoted in all parts of the elongated esophagus by traction.

*nNOS and S-100 stain*

nNOS and S-100 stain neural fiber. Auerbach’s plexus was identified between the muscles in all three groups, and could be found at any place in the two segments (Fig. 4).

**DISCUSSION**

We attempted to identify the histological effects of traction on the esophagus, and we observed that (1) the esophagus was elongated uniformly by traction, (2) the cell proliferation activity in both the esophageal mucosa and muscle was promoted by
mechanical traction, and (3) Auerbach’s plexus was identified at all sites of the elongated esophagus.

In our study, the weight loss of traction group rat was significant. We presumed that traction group rat received general anesthesia with isoflurane during adding new clip. We presumed those procedure resulted in the rat’s reduced activity and weight loss.

Similarly to several surgical techniques used for esophageal elongation (1-8, 11-17), in this study the esophagus was elongated by traction as well. To the best of our knowledge, this is the first study to clarify which part of the esophagus was elongated by traction. The esophagus was not elongated near the point of action of the traction; rather, it was elongated uniformly by the traction. Our findings also demonstrated that the cell proliferation activity in the esophageal mucosa and muscle was promoted by traction, and no difference was shown between the two segments. The cell proliferation activity of the elongated esophagus was also promoted uniformly by the traction.

Although the thickness of the esophageal mucosa and muscle tended to be thin after the traction, the differences between the three groups were not significant. Khan et al. (9) reported that the thicknesses of individual mural layers were maintained after the esophageal length was increased with traction in EA patients after suture. Intestinal smooth muscle hypertrophy was seen with mechanical stimulation in an animal model,
and the cell proliferation was increased by mechanical stimulation. (18-20).

We suspect that although the esophagus was first stretched and the thickness of the esophageal mucosa and muscle became thin by traction, the cell proliferation activity was promoted. As a result, the esophagus was elongated and the esophageal mucosa and muscle became thick by the promotion of the cell proliferation activity. It was suggested that the esophageal elongation was caused not only by mechanical stretching but also by the promotion of cell proliferation.

In contrast, the cell proliferation activity in the non-traction group was decreased, and it was lower than that in normal group. We suspect that disuse atrophy was caused in the unstimulated state (non-traction), and that the stimulation of the esophageal lumen by feeding and the force applied to the normally situated esophagus also caused esophageal cell proliferation.

Our rat model originally had a normal esophagus, and we demonstrated that the esophagus was elongated uniformly by traction. Auerbach’s plexus was also identified at all sites of the elongated esophagus, because the esophageal tissue including Auerbach’s plexus was elongated en bloc by the traction. In this study, we could not clarify the functioning of the elongated esophagus. Although the causes of esophageal elongation require further investigation, our findings provide supportive evidence for
the esophageal elongation method, and they may contribute to the treatment of long-gap EA patients.

Conclusions

The esophagus in our animal model was significantly and uniformly elongated by traction, and the cell proliferation activity was promoted in all parts of the elongated esophagus.

Acknowledgments

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Disclosures

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.
Figure legends

Fig. 1. A: The esophagus removed from a non-traction group rat (left) and an elongated esophagus removed from a traction-group rat (right). There are three marking strings on each sample. B: Overall mean in whole-length esophagus. The whole length of the traction group was significantly longer than that of the non-traction group (P<0.01).

**P<0.01.

Fig. 2. A–F: Hematoxylin-eosin staining of the esophagus demonstrated an intact epithelial and muscle lining in each group. G: Overall mean of mucosal thickness. There was no significant difference between the three groups. H: Overall mean of muscle thickness. There was no significant difference between the three groups.

Fig. 3. Ki-67 staining. A: Mucosa of a normal-group rat. B: Mucosa of a non-traction rat. C: Mucosa of a traction rat. D: Muscle of a normal rat. E: Muscle of a non-traction rat. F: Muscle of a traction rat. G: Overall mean of the Ki-67-positive ratio in the mucosa of the traction group was significantly higher than those of the normal group (P<0.05) and the non-traction group (P<0.01). H: Overall mean of the Ki-67-positive
ratio in the muscle of the traction group was significantly higher than those of the
normal group (P<0.05) and the non-traction group (P<0.05). *P<0.05, **P<0.01.

**Fig. 4.** Auerbach’s plexus. A–C: nNOS staining. D–F: S-100 staining. A,D: Normal
group. B,E: non-traction group. C,F: Traction group. Auerbach’s plexus was stained
(circled).
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Fig. 1

(A) Sample images showing mid-stump and cardia-mid regions with non-traction and traction conditions.

(B) Bar graph comparing whole length measurements under non-traction and traction conditions. The graph indicates a statistically significant difference (**).
Fig. 2

Overall mean of mucosal thickness

Overall mean of muscle thickness

A B C
D E F

G H

\( \mu \text{m} \)

ns  ns
ns  ns
ns  ns

Fig. 2
Overall mean of the Ki-67-positive ratio in mucosa

Overall mean of the Ki-67-positive ratio in muscle

Fig. 3
Fig. 4
### Table 1. Comparison of the two segments of the esophagus

<table>
<thead>
<tr>
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<th>cardia-mid (mm)</th>
<th>mid-stump (mm)</th>
<th>p-value</th>
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<tr>
<td><strong>Length</strong></td>
<td></td>
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<tr>
<td>non-traction</td>
<td>1.8 ± 0.4</td>
<td>1.8 ± 0.4</td>
<td>&gt;0.99</td>
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<tr>
<td>traction</td>
<td>3.6 ± 0.5</td>
<td>4.4 ± 1.1</td>
<td>0.20</td>
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<td><strong>Thickness of the mucosa (µm)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>normal</td>
<td>11.1 ± 5.4</td>
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<tr>
<td>non-traction</td>
<td>12.3 ± 4.8</td>
<td>12.1 ± 4.4</td>
<td>0.96</td>
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<tr>
<td>traction</td>
<td>10.1 ± 4.2</td>
<td>7.1 ± 3.9</td>
<td>0.28</td>
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<tr>
<td><strong>Thickness of the muscle (µm)</strong></td>
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<tr>
<td>normal</td>
<td>154.2 ± 41.9</td>
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<td>114.0 ± 28.1</td>
<td>72.8 ± 30.7</td>
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<tr>
<td><strong>Ki-67-positive ratio in mucosa (%)</strong></td>
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<td></td>
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<tr>
<td>normal</td>
<td>25.0 ± 2.8</td>
<td>25.5 ± 7.9</td>
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<tr>
<td><strong>Ki-67-positive ratio in muscle (%)</strong></td>
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<tr>
<td>normal</td>
<td>3.1 ± 1.3</td>
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<td>0.75</td>
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