Histological study of the elongated esophagus in a rat model

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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.

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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 ± 38.7

µm; that of the non-traction group was 85.5 ± 40.7 µm, and that of the traction group

was 93.4 ± 21.4 µm. 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.

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

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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).

REFERENCES

1.   Foker, J. E., Linden, B. C., Boyle, E. M., Jr., and Marquardt, C. Development of a true primary repair for the full spectrum of esophageal atresia. *Ann Surg* **226**: 533-541; discussion 541-533, 1997.

2.   Al-Qahtani, A. R., Yazbeck, S., Rosen, N. G., Youssef, S., and Mayer, S. K. Lengthening technique for long gap esophageal atresia and early anastomosis. *J Pediatr Surg* **38**: 737-739, 2003.

3.   Gaglione, G., Tramontano, A., Capobianco, A., and Mazzei, S. Foker's technique in oesophageal atresia with double fistula: a case report. *Eur J Pediatr Surg* **13**: 50-53, 2003.

4.   Skarsgard, E. D. Dynamic esophageal lengthening for long gap esophageal atresia: experience with two cases. *J Pediatr Surg* **39**: 1712-1714, 2004.

5.   Lopes, M. F., Reis, A., Coutinho, S., and Pires, A. Very long gap esophageal atresia successfully treated by esophageal lengthening using external traction sutures. *J Pediatr Surg* **39**: 1286-1287, 2004.

6.   Foker, J. E., Kendall, T. C., Catton, K., and Khan, K. M. A flexible approach to achieve a true primary repair for all infants with esophageal atresia. *Semin Pediatr Surg* **14**: 8-15, 2005.

7.   Lopes, M. F., and Botelho, M. F. Midterm follow-up of esophageal anastomosis for

esophageal atresia repair: long-gap versus non-long-gap. *Dis Esophagus* **20**: 428-435, 2007.

8.   Mochizuki, K., Obatake, M., Taura, Y., Inamura, Y., Kinoshita, A., Fukuda, A.,

Kosaka, T., Takatsuki, M., Nagayasu, T., and Eguchi, S. A modified Foker's technique for

long gap esophageal atresia. *Pediatr Surg Int* **28**: 851-854, 2012.

9.   Khan, K. M., Sabati, A. A., Kendall, T., and Foker, J. E. The Effect of Traction on

Esophageal Structure in Children with Long-Gap Esophageal Atresia. *Digestive Diseases

and Sciences* **51**: 1917-1921, 2006.

10.   Lopes, M. F., Catré, D., Cabrita, A., Pires, A., and Patrício, J. Effect of traction

sutures in the distal esophagus of the rat: a model for esophageal elongation by Foker's

method. *Diseases of the Esophagus* **21**: 570-573, 2008.

11.   Livaditis, A., Radberg, L., and Odensjo, G. Esophageal end-to-end anastomosis.

Reduction of anastomotic tension by circular myotomy. *Scand J Thorac Cardiovasc Surg* **6**:

206-214, 1972.

12.   Mahour, G. H., Woolley, M. M., and Gwinn, J. L. Elongation of the upper pouch and

delayed anatomic reconstruction in esophageal atresia. *J Pediatr Surg* **9**: 373-383, 1974.

13.   Hendren, W. H., and Hale, J. R. Electromagnetic bougienage to lengthen

esophageal segments in congenital esophageal atresia. *N Engl J Med* **293**: 428-432, 1975.

14.   Schullinger, J. N., Vinocur, C. D., and Santulli, T. V. The suture fistula technique in

the repair of selected cases of esophageal atresia. *J Pediatr Surg* **17**: 234-236, 1982.

15.   Brown, S., and Nixon, H. H. Spontaneous anastomosis of the esophagus in

esophageal atresia. *J Pediatr Surg* **18**: 293-295, 1983.

16.   Kimura, K., Nishijima, E., Tsugawa, C., Collins, D. L., Lazar, E. L., Stylianos, S.,

Sandler, A., and Soper, R. T. Multistaged extrathoracic esophageal elongation procedure for

long gap esophageal atresia: Experience with 12 patients. *J Pediatr Surg* **36**: 1725-1727,

2001.

17.   Zaritzky, M., Ben, R., Zylberg, G. I., and Yampolsky, B. Magnetic compression

anastomosis as a nonsurgical treatment for esophageal atresia. *Pediatr Radiol* **39**: 945-949,

2009.

18.   Park, J., Puapong, D. P., Wu, B. M., Atkinson, J. B., and Dunn, J. C. Enterogenesis

by mechanical lengthening: morphology and function of the lengthened small intestine. *J

Pediatr Surg* **39**: 1823-1827, 2004.

19.   Stark, R., Zupekan, T., Bondada, S., and Dunn, J. C. Y. Restoration of mechanically

lengthened jejunum into intestinal continuity in rats. *Journal of Pediatric Surgery* **46**:

2321-2326, 2011.

20.   Zhao, J., Liao, D., Yang, J., and Gregersen, H. Phasic and tonic smooth muscle

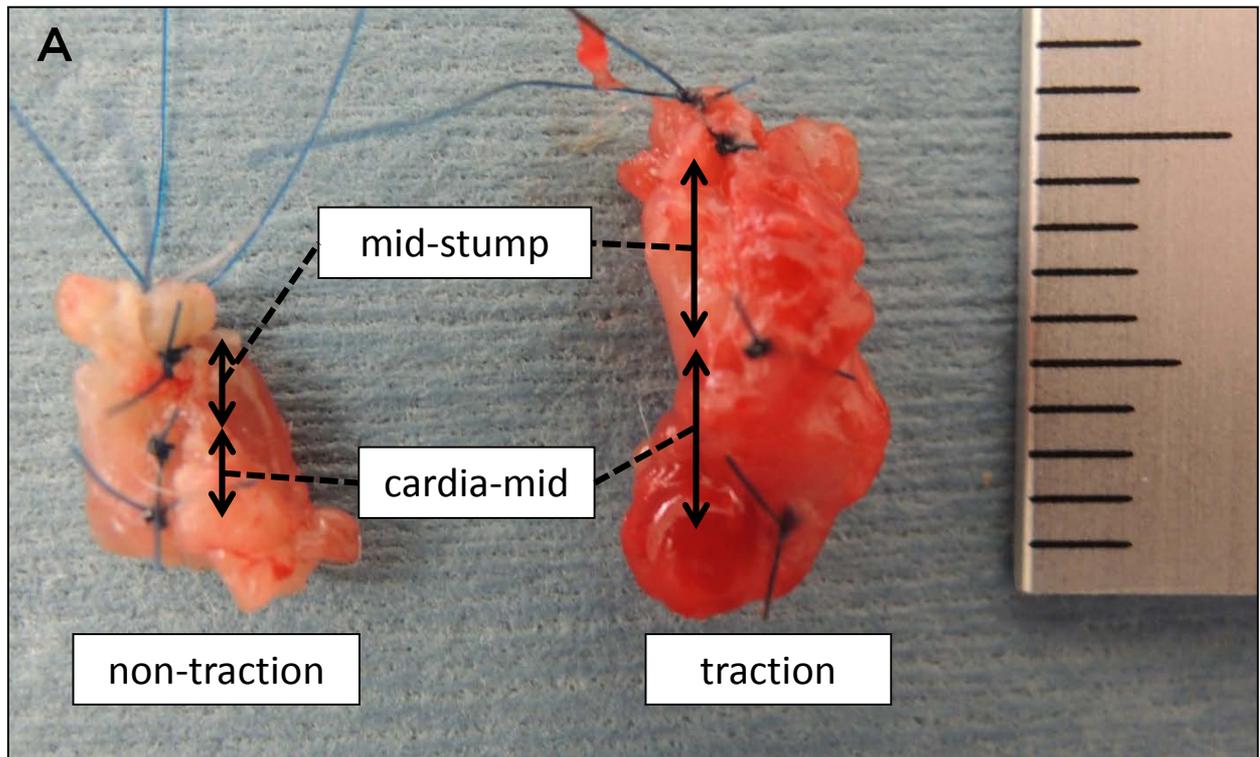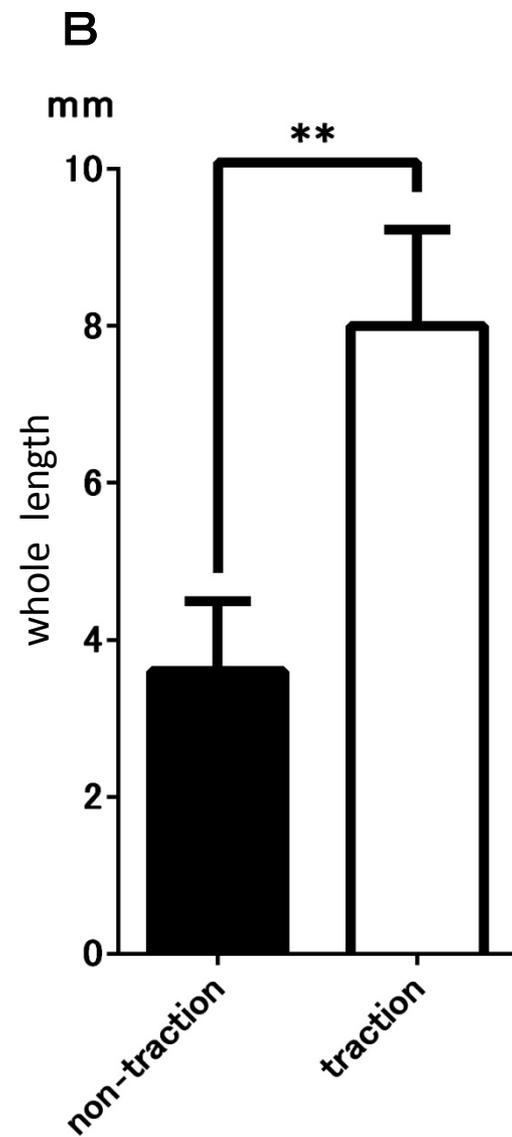function of the partially obstructed guinea pig intestine. *J Biomed Biotechnol* **2011**: 489720,
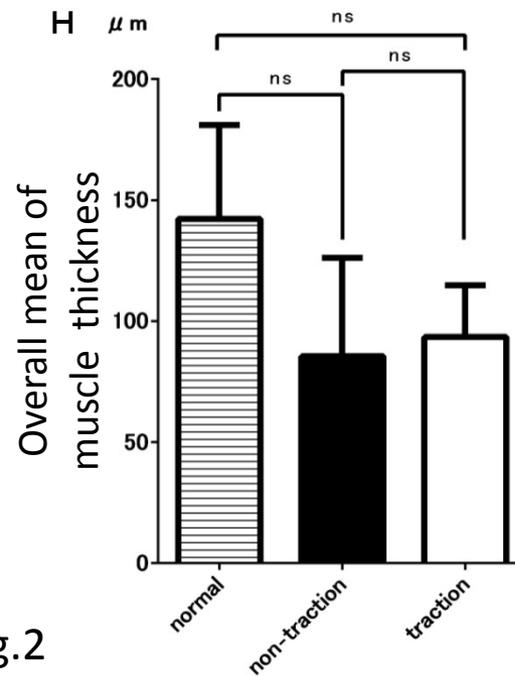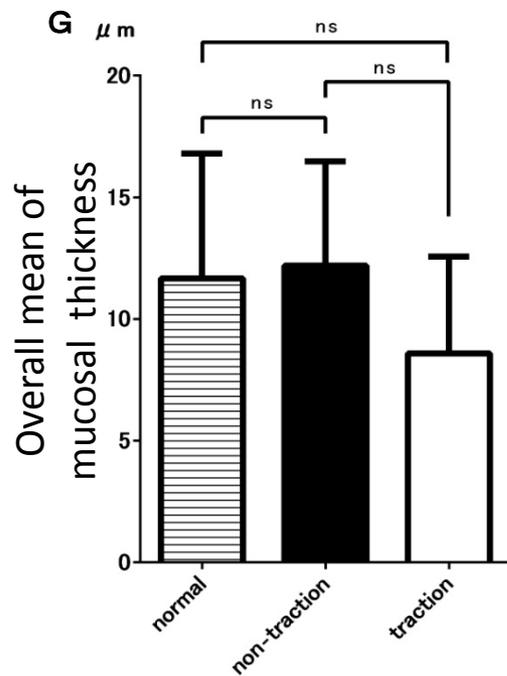
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2011.

Fig. 1

normal　　**non-traction**　　**traction**

A　　B　　C

X100　　X100　　X100

D　　E　　F

X400　　X400　　X400

G μm

Overall mean of mucosal thickness

ns

ns

ns

20

15

10

5

0

normal　non-traction　traction

H μm

Overall mean of muscle thickness

ns

ns

ns

200

150

100

50

0

normal　non-traction　traction

Fig.2

Fig.3

normal | non-traction | traction

nNOS

A   X400 | B   X400 | C   X400

S-100

D   X200 | E   X200 | F   X200
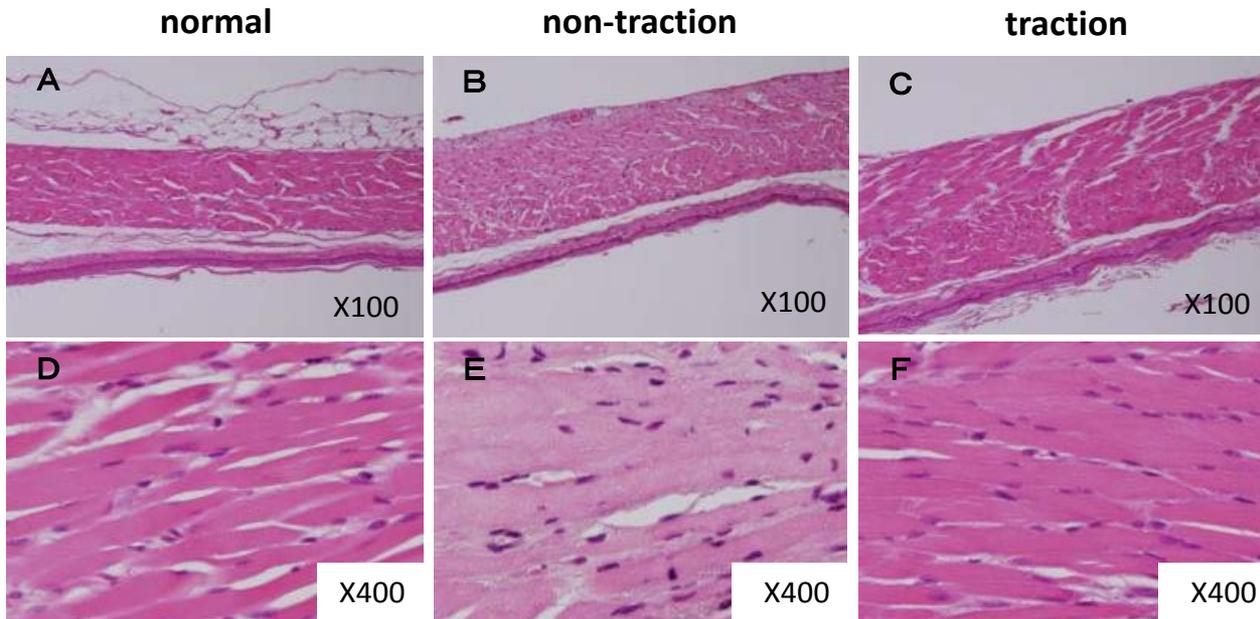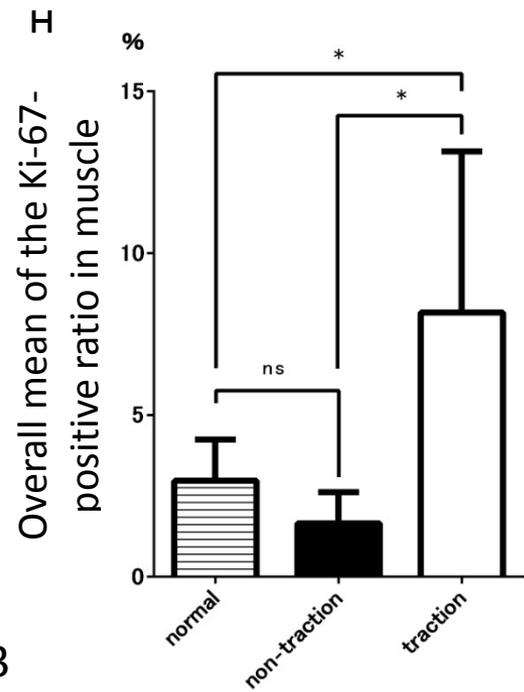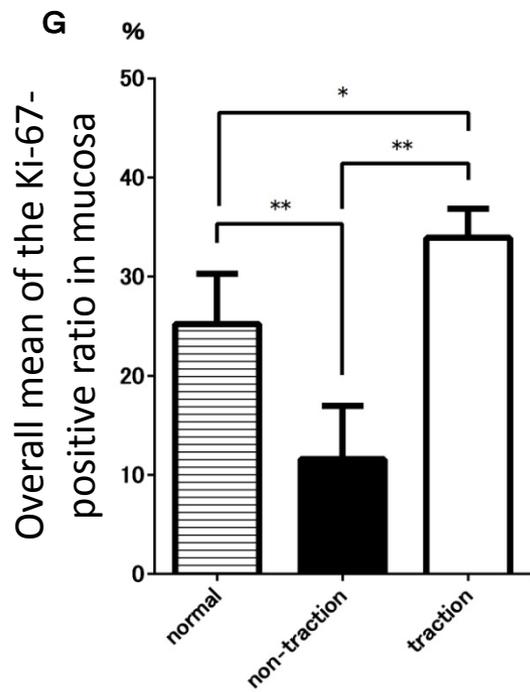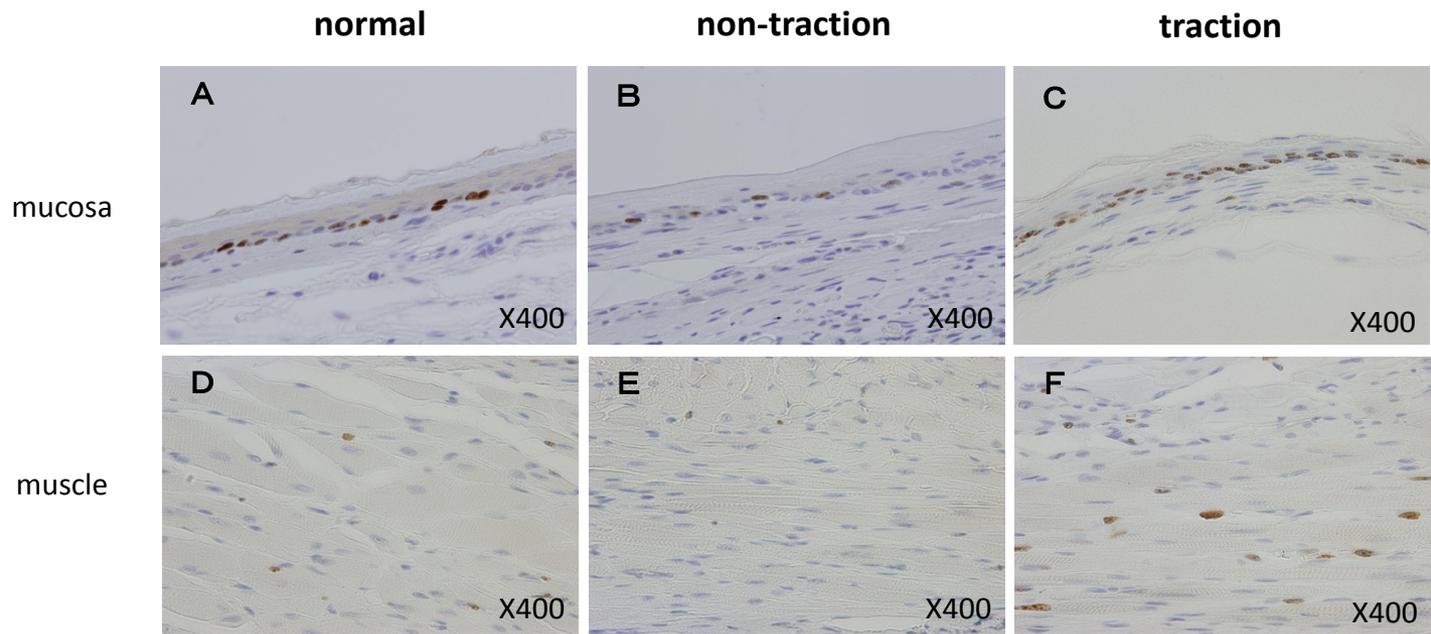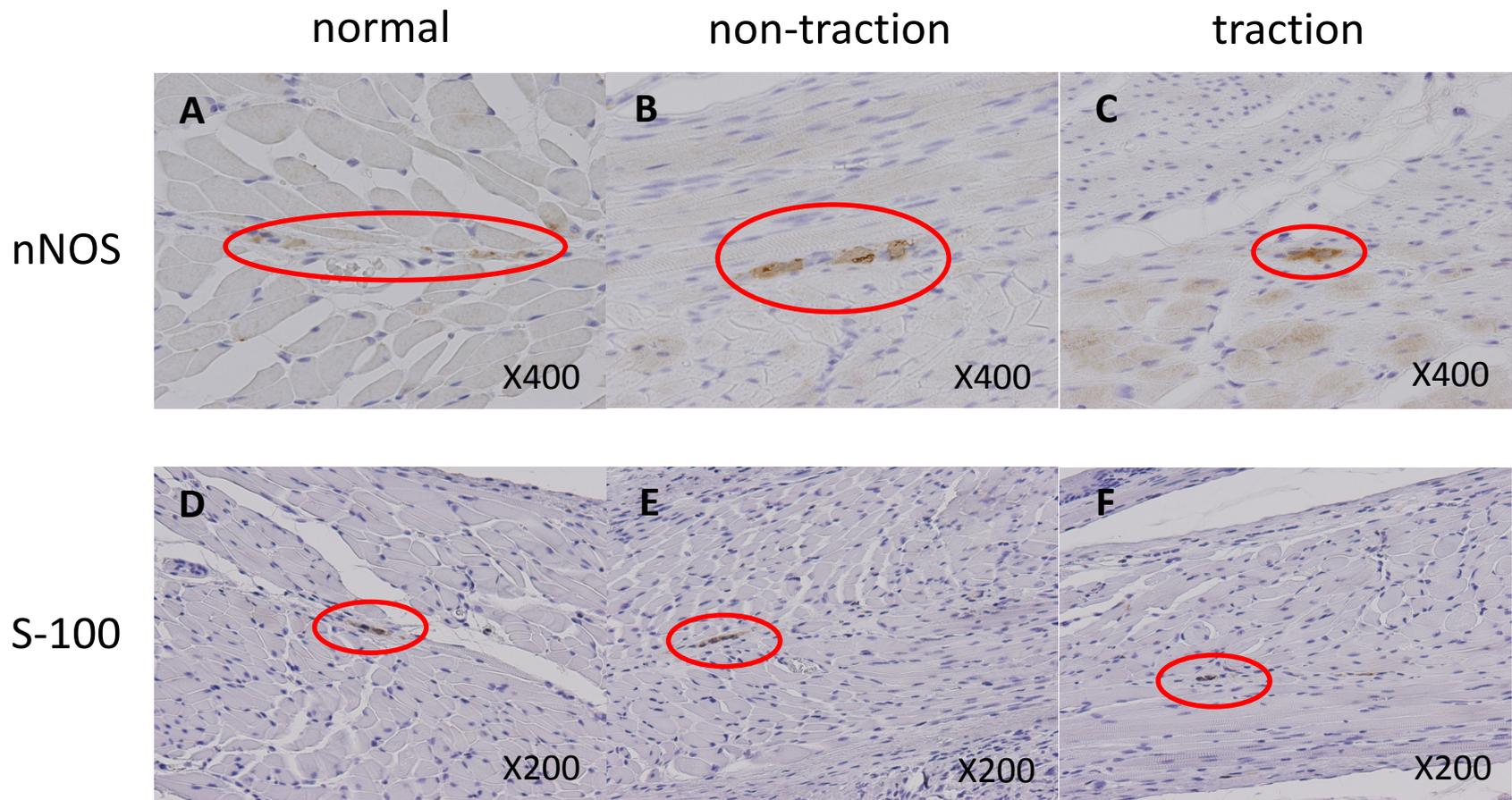
Fig.4

Table1. Comparison of the two segments of the esophagus

	cardia-mid	mid-stump	p-value
Length (mm)			
non-traction	1.8 ± 0.4	1.8 ± 0.4	>0.99
traction	3.6 ± 0.5	4.4 ± 1.1	0.20
Thickness of the mucosa　(μm)			
normal	11.1 ± 5.4	12.3 ± 6.1	0.75
non-traction	12.3 ± 4.8	12.1 ± 4.4	0.96
traction	10.1 ± 4.2	7.1 ± 3.9	0.28
Thickness of the muscle　(μm)			
normal	154.2 ± 41.9	130.5 ± 39.2	0.38
non-traction	90.3 ± 45.1	80.7 ± 38.0	0.72
traction	114.0 ± 28.1	72.8 ± 30.7	0.06
Ki-67-positive ratio in mucosa　(%)			
normal	25.0 ± 2.8	25.5 ± 7.9	0.90
non-traction	11.0 ± 7.2	12.2 ± 5.1	0.77
traction	36.6 ± 5.9	31.3 ± 5.9	0.19
Ki-67-positive ratio in muscle　(%)			
normal	3.1 ± 1.3	2.8 ± 1.6	0.75
non-traction	2.1 ± 1.6	1.2 ± 0.4	0.27
traction	7.3 ± 4.7	9.0 ± 5.5	0.62