Experiment on endoscopic balloon dilation for esophageal stenosis after endoscopic submucosal dissection in pigs.

Sayoko Kinowaki[1], Yuichi Shimizu[2], Masayoshi Ono[1], Yang ZiJian[1], Ikko Tanaka[1], Yoshihiko Shimoda[1], Masaki Inoue[1], Marin Ishikawa[1,3], Keiko Yamamoto[2], Shoko Ono[2], Shunsuke Ohnishi[1], Naoya Sakamoto[1]

[1] Department of Gastroenterology, Hokkaido University Graduate School of Medicine, Kita 15 jo Nishi 7 chome, Kitaku, Sapporo 060-8638, Japan

[2] Division of Endoscopy, Hokkaido University Hospital, Kita 14 jo Nishi 5 chome, Kitaku, Sapporo 060-8648, Japan

[3]Department of Cancer Pathology, Hokkaido University Graduate School of Medicine, Kita 15 jo Nishi 7 chome, Kitaku, Sapporo 060-8638, Japan

Short Title : Experiment on esophageal　EBD

Corresponding author (Reprint Requests): Yuichi Shimizu, MD.　Division of Endoscopy, Hokkaido University Hospital, Kita 14 jo Nishi 5 chome, Kitaku, Sapporo 060-8648, Japan.

TEL: 81-11-716-1161 (5920)　FAX : 81-11-706-7867

e-mail address : yshimizu@med.hokudai.ac.jp

Author contributions:

Kinowaki S and Shimizu Y contributed equally to this work; Shimizu Y designed the research study; Kinowaki S, Ono M, Zijian Y and Ohnishi S performed experiments in pigs; Shimizu Y, Yamamoto K, Ono S, Ohnishi S and Sakamoto N supervised the research; Tanaka I, Shimoda Y and Inoue M contributed to collection and analysis of the data. Ishikawa M pathologically supervised the research; Kinowaki S and Shimizu Y wrote the manuscript; All authors have read and approved the final manuscript.

KEY WORDS: balloon dilation, esophageal stenosis, ESD.

Abbreviations:

ESD, endoscopic submucosal dissection

EBD, endoscopic balloon dilation

H&E, hematoxylin and eosin

SD, standard deviation

Introduction:

Owing to recent developments of endoscopic technology, endoscopic submucosal dissection (ESD) has been widely accepted as a first-line treatment for early-stage esophageal cancer［1］.In addition, due to improvements of devices and techniques, ESD has become possible for large lesions, even for total circumference lesions［2-6］.

While ESD for esophageal cancer is a minimally invasive, highly curative and useful treatment, several intraoperative and postoperative complications can occar［7-10］.Especially, the most significant adverse event is considered to be postoperative esophageal stenosis［11.12］.

It has been reported that the risk of stenosis is extremely high up to 66-100% when a circumferential mucosal defect involving over three fourths of the circumference of the esophagus［13.14］.Postoperative esophageal stricture not only impairs patients'quality of life but also causes serious complications such as aspiration pneumonia and undernutrition due to dysphagia. Although local injection or oral administration of steroids has been reported to be effective for preventing esophageal stenosis after ESD［15-20］,there are some cases in which esophageal stenosis occurs even if these methods are used.In fact, it has been reported that esophageal stenosis that require endoscopic balloon dilation occurred in 10-

30.8% of patients who received local steroid injections or oral steroid admistration after ESD ［18.21-23］.

In such cases, restenosis frequently developed and a repeated balloon dilation procedure was therefore often required. The most significant complication of endoscopic balloon dilation is considered to be esophageal perforation. The prevalence of esophageal perforation has been reported to be 1-9%［24-26］ and the risk of perforation would increase with repeated balloon dilations. Esophageal perforation is a complication that must be avoided because it can lead to fatal conditions such as mediastinitis ; however, there has been no study in which the mechanism and risk of perforation during balloon dilation and restenosis were investigated. In addition, perforation during balloon dilation is considered to be related not only to excessive dilation but also to muscle layer injury during the ESD procedure ; however, this point also has not been examined at all.

In this study, we conducted endoscopic balloon dilation experiments on a living porcine esophageal stenosis model and analyzed historical findings of dilated esophagus, esophageal perforation and esophageal restenosis to determine an efficient and safe balloon dilation method.

Methods:

Animal model

The experimental protocol was approved by the Animal Care and Use Committee of Hokkaido University. Female domestic pigs (20-25 kg; Sankyo Labo Service, Tokyo, Japan) were used in this study.The pigs were kept at a room temperature of 24℃ under a constant feeding condition with a dark-light cycle of a 12-hour light period to a 12-hour dark period, and one pig was bred per cage. The experimental protocols are summarized in Figure 1.

ESD

The pigs (n = 12) were anesthetized by intramuscular injection of midazolam (20 mg; Astellas, Tokyo, Japan) and buprenorphine hydrochloride (0.2 mg; Otsuka Pharmaceutical, Tokyo, Japan) followed by inhalation of 5% sevoflurane (Maruishi Pharmaceutical, Osaka, Japan). The pigs were then intubated using a 6 mm intubation tube (Smiths Medical, Tokyo, Japan) and connected to a mechanical ventilator under 3% sevoflurane in oxygen. ESD was performed with continuous monitoring of heart rate and oxygen saturation (Nihon Kohden, Tokyo, Japan) using a single-channel GI endoscope (GIF-Q240/ GIF-240Z; Olympus,Tokyo, Japan) with a transparent attachment hood fitted to the tip (Top, Tokyo, Japan).

Markings for the incision line were placed with a ball-tipped flush knife (flush knife BT) (Fujifilm, Tokyo,Japan) 36 cm and 40 cm from the mouth with total circumference. A 25-gauge needle (Top Corporation) was used to inject a mixed solution into the submucosal layer before

mucosal and submucosal cutting. The mixed solution was 2:1 mixture of 10% glycerine solution (Glyceol; Chugai Pharmaceutical, Tokyo,Japan) and 80 mg/mL hyaluronic acid (Mucoup; Seikagaku Corporation, Tokyo, Japan) with a small amount of indigo carmine. The mixed solution was injected into the submucosal layer of the esophagus in the vicinity of the marking on the anal side to make a bulge in the submucosal layer, and a mucosal incision was performed circumferentially with the flush knife BT. Subsequently, the mixed solution was locally injected into the submucosal layer from the marking on the oral side, and the submucosal layer was exfoliated with the flush knife BT to complete the ESD.

An electrosurgical generator (ESG-100; Olympus) was set to the pulse cut slow mode (40 W) or forced coagulation mode (40 W) for incision of the mucosa and submucosa.

Hemorrhage was controlled using the flush knife BT or Coagrasper (Olympus) in the soft coagulation mode (40 W). For postoperative care, all of the pigs were given liquid starting from the day after ESD and then solids on the following days.

Endoscopic balloon dilation

Balloon dilation was performed three weeks after ESD. The pigs were anesthetized again. After completion of the stenosis had been confirmed by endoscopy, balloon expansion was performed by using the CRE balloon system (CRE™ Fixed Wire Balloon Dilators［Esophageal］ (Boston Scientific Japan)) with a diameter of 12 mm or 15 mm.

The balloon was gradually inflated and kept at sufficient pressure for 1 minute. The pigs underwent dilation with maximum diameter of 12 mm to 15 mm. Immediately after dilation, sacrifice was performed. Two cases of muscle layer non-injury and two cases of muscle layer injury were created for histological evaluation of the esophagus during dilation and dilation-induced perforation (study 1). The presence of muscle layer injury was diagnosed by endoscopic findings during the dilation procedure. Additionally, two control models of stenosis (day 22, without dilation) and two control models of perforation (day 1, perforation during ESD) were created.

Next, pigs with restenosis after balloon dilation were created. After dilation on day 22, the pigs were managed for three weeks and sacrificed on day 43. We performed indian ink marking in the laceration site immediately after dilation for histological evaluation of a part of the submucosal laceration after restenosis. Two cases of muscle layer non-injury and two cases of muscle layer injury during dilation were created for histological evaluation of restenosis after dilation (study 2). Figure 2 shows images of endoscopic balloon dilation(A-D), schema of the dilation model(E), image of measurement of the area of muscle fiber bundle necrosis(F), and schema of the restenosis model(G).

Measurement

Study 1: Histological evaluation of the esophagus during dilation and dilation- induced

muscle layer injury.

Examination 1-1: We evaluated the muscle layer extension effect of balloon dilation by using dilation models (day 22 after ESD). As an index of the muscle layer extension effect, the thickness of the outer longitudinal layer of smooth muscle was evaluated. According to the histological evaluation of a surgically resected esophagus for scleroderma and achalasia, the dilated esophageal wall became markedly thinner than the esophageal wall with contracture［27］. Since we have no method to measure muscle layer extension directly, we evaluated the thickness of the muscle layer as an indirect index of the muscle layer extension effect. Previously, Honda et al. conducted histological evaluation in a dog model in which endoscopic mucosal resection of the esophagus was performed. They reported that the inner circular layer of smooth muscle became atrophic in the process of ulcer healing. We therefore evaluated the thickness of the outer longitudinal layer of smooth muscle as an indirect index of the muscle layer extension effect［28］.

For both groups, surrounding parts distant from the laceration induced by dilation were randomly measured at 10 locations. In addition to the 10 locations, 2 locations beneath the submucosal laceration were measured in the non-injury muscle layer cases, and both sides of the muscle layer laceration site were measured in the muscle layer injury cases. If there were multiple lacerations, the deepest laceration was evaluated. Ten random locations were also measured in the control models of stenosis.

Examination 1-2: We evaluated remaining muscle layer thermal damage caused by the ESD procedure by using dilation models (day 22 after ESD). As an index of muscle layer damage, the area of muscle fiber bundle necrosis measured per field of view in the inner circular muscle layer was evaluated. For both groups, surround parts distant from the laceration were randomly measured at 10 locations. In addition to the 10 locations, 2 locations beneath the submucosal laceration were measured in the non-injury muscle layer cases, and both sides of the muscle layer laceration site were measured in the muscle layer injury cases. Both sides of the muscle layer laceration site were also measured in the control models of perforation (day 1, perforation during ESD).

Study 2: Histological evaluation of the esophagus when restenosis after balloon dilation was formed.

Examination 2-1: We evaluated the histological change of restenosis, mainly the parts of laceration, by using restenosis models (day 43 after ESD). As an index of the degree of restenosis, the thickness of the fibrous plexus was measured. For both groups, the laceration scar site (ink-marked site) was measured and parts distant from the laceration scar were randomly measured at 10 locations.

Examination 2-2: We evaluated the histological change of recovery of the injured muscle layer by using restenosis models. As an index of recovery from muscle layer laceration, the

thickness of the entire muscular layer was measured. For the muscle layer injury cases, the muscle layer laceration scar site was measured and parts distant from the laceration scar were randomly measured at 10 locations.

Examination 2-3: We evaluated the remaining muscle layer thermal damage caused by the ESD procedure. In the same way as examination 1-2, the area of muscle fiber bundle necrosis measured per field of view in the inner circular muscle layer was counted. For both groups, two locations beneath the laceration scar site were measured and parts distant from the laceration were randomly measured at 10 locations.

Histological and immunohistochemical examinations

All of the pigs were sacrificed by intravenous injection of 20 mL of 15 % potassium chloride (Terumo) after general anesthesia. The anterior neck and abdomen were incised, and transhiatal esophagectomy was performed. The resected esophagus was immediately placed on a rubber plate and fixed with pins. The esophagus was fixed in 40 g/L formaldehyde saline solution, embedded in paraffin, and cut into 5-mm sections. Sections were made along the minor axis of the esophagus. Tissue sections were stained with hematoxylin and eosin (H&E), and Masson’s trichrome staining was performed to emphasize collagen fibers. For all cases, two suitable section slides (for example, deepest part of a laceration) for each case were evaluated. The values of thickness and quantity were measured using NDP.view2 software [Hamamatsu Photonics K.K., Hamamatsu, Japan]).

Statistical analysis

Since our study was the first study in which the effect of dilation on esophageal stenosis in pigs was assessed, we had no data from which to calculate sample size in advance. Therefore, we first aimed to perform an experiment with a total of twelve pigs and post hoc power analysis to verify the sample size. Subsequently, we estimated that a total of twelve pigs was a sufficient number as a result of analysis.

Data are expressed as means (SD). Parameters in the two groups were compared by an unpaired t-test. Differences were considered statistically significant at $P < 0.05$. All analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) ［29］.

Results:

All of 12 porcine models (2 perforation control models, 2 stenosis control models, 2 dilation models with muscle layer injury, 2 dilation models with muscle layer non-injury, 2 restenosis models with muscle layer injury and 2 restenosis models with muscle layer non-injury) were successfully created. There was no finding of ulceration at the stenosis site (scar formation having been completed) in any of the stenosis control models and dilation models.

Endoscopic findings at the sites of stenosis in all of the stenosis control models, dilation models (before dilation) and restenosis models were severe stenosis (like a pin-hole). As for the factor that could affect the muscle layer injury, maximum diameters of the balloons for dilation were 12.8±0.5 mm in 4 pigs with muscle layer injury and 12.0±0.0 mm in 4 pigs with muscle layer non-injury (p=0.45). Procedure times for ESD were 112.5±18.0 min in 4 pigs with muscle layer injury and 92.2±12.2 min in 4 pigs with muscle layer non-injury (p=0.3). There were no significant differences in these factors between the two groups. We endoscopically confirmed muscle layer injury during dilation in all of the 4 pigs. Although cases with minor perforation might have been included in the 4 pigs, no clinical finding suggesting mediastinitis was found in any cases after dilation and all of the pigs ate solid foods from the day after dilation.

Study 1: Histological evaluation of the esophagus during dilation and dilation- induced muscle layer injury.

Examination 1-1: Thickness of the outer longitudinal layer of smooth muscle (an index of the muscle layer extension effect of dilation models).In control models of stenosis, it was 1307±313 ㎛. In cases of muscle layer non-injury, it was 1243±322 ㎛ in surrounding random locations and it was 803±145 ㎛ beneath the submucosal laceration. In cases of muscular layer injury, it was 1248±305 ㎛ in surrounding random locations and it was 884±312 ㎛ on

both sides of the muscle layer laceration site. The results are summarized in Figure 3. There was no difference between stenosis control models and surrounding random locations of either the muscle layer non-injury group or injury group ($p= 0.444$ and 0.513, respectively). The thickness in the laceration site was significantly smaller than that in surrounding random locations in both the muscle layer non-injury and injury groups ($p= 0.005$, $p<0.001$, respectively).

Examination 1-2: Area of muscle fiber bundle necrosis per field of view in the inner circular muscle layer (an index of muscle layer thermal damage). In control models of stenosis, it was $1.6\pm2.1\times10^3$

than in surrounding locations($p<0.001$). It was most significant in perforation control models ($p=0.028$ compared with the laceration site in the muscle layer injury group).

Study 2: Histological evaluation of the esophagus when restenosis after balloon dilation was formed.

As for the effect of excessive dilation, lengths of the short axis at the site of maximal constriction in resected specimens were measured. The lengths were 7 mm and 13 mm in the 2 pigs with muscle layer injury and 7 mm and 13 mm in the 2 pigs with muscle layer non-injury. There was no difference between the two groups.

Examination 2-1: Thickness of the fibrous plexus (an index of the degree of restenosis). In control models of stenosis, it was 1406±167 ㎛. In cases of muscle layer non-injury, it was 1359±196 ㎛ in surrounding random locations and it was 1322±136 ㎛ in the laceration scar site. In cases of muscular layer injury, it was 1457±253 ㎛ in surrounding random locations and it was 1383±146 ㎛ in the laceration scar site. The results are summarized in Figure 5A. The thickness of the fibrous plexus in the laceration scar in both cases of muscular layer non-injury and injury was similar to that in surrounding locations and in stenosis control models($p=0.74$ and 0.23, respectively in cases of muscular layer non-injury . $p=0.22$ and 0.063 respectively in cases of muscular layer injury). Figure 5B shows histological findings of the resected esophagus with restenosis (a muscle layer non-injury case).

Examination 2-2: Thickness of the entire muscular layer (an index of recovery from muscle layer laceration). In cases of muscular layer injury, it was 2513±265 ㎛ in surrounding random locations and it was 1106.9±212.8 ㎛ in the laceration scar site. The results are summarized in Figure 5C. The thickness of the entire muscular layer in the laceration scar site was significantly smaller than that in surrounding locations($p<0.001$). Figure 5D shows histological findings of the resected esophagus with restenosis (a muscle layer injury case).

Examination 2-3: Area of muscle fiber bundle necrosis per field of view in the inner circular muscle layer (an index of muscle layer thermal damage). In control models of stenosis, it was 15.8 ±20.6×10^2 ㎡(study 1). In cases of muscle layer non-injury, it was 1.5±3.7×10^2 ㎡ in surrounding random locations and it was 2.7±3.5×10^2 ㎡ in the laceration scar site. In the cases of muscular layer injury, it was 7.4±20.2×10^2 ㎡ in surround random locations and it was 4.5±7.7×10^2 ㎡ in laceration scar site. The results are summarized in Figure 6. There was no significant difference between each measurement in both the muscle layer non-injury and injury groups ($p=0.21$ and 0.68, respectively). It was most significant in stenosis control models created on day 22 ($p<0.001$ compared with surround locations in the muscle layer injury group).

Discussion:

Esophageal stenosis after ESD is forms about three weeks after treatment and is generally treated by balloon dilation; however, the risk of perforation or muscular layer injury caused by dilation is generally difficult to predict in advance. As for adverse events of dilation for esophageal stenosis after ESD, Kishida et al. reported clinical outcomes of 121 patients who underwent endoscopic dilation for esophageal stenosis caused by ESD (median number of dilation procedures:7, median duration of dilation:86 days). They reported that esophageal perforation occurred in 5 patients (4.1%) and that perforation occurred in a relatively early period after ESD (median period of 18 days after ESD; range,8 – 29 days) ［30］.

In this study (study 1), in cases with muscle layer injury caused by balloon dilation, the area of muscle fiber bundle necrosis in the inner circular layer of smooth muscle was significantly larger at the injury site than in surrounding locations, Although the area (at the injury site) was most significant in the models of perforation during ESD, the area was significantly larger in muscle layer injury cases than in muscle layer non-injury cases. It is thought that necrosis remained even three weeks after ESD, when the scar had completely formed, and that the muscular layer would remain fragile in that part. Moreover, in study 2, the muscular layer injury did not recover even after the restenosis was completed three weeks after dilation. We consider that muscle layer injury during balloon dilation would be irreversible and that fragility would persist afterward. Muscle fiber bundle necrosis was observed particularly in muscle

layer injured cases on day 22 but was scarcely observed on day 43 in study 2. The area of muscle fiber bundle necrosis was smaller in the restenosis model even with muscle layer injury than in the stenosis control model. Muscle fiber bundle necrosis was considered to be gradually absorbed more than three weeks after ESD. Thus, in patients who are suspected of having thermal damage to the muscle layer during the ESD procedure, such as patients with muscular layer exposure or patients in whom coagulation using was performed for a penetrating branch vessel, careful dilation using a balloon with a narrow diameter should be performed in the initial stage and dilation using a balloon with a larger diameter should be performed more than six weeks after ESD.

As for the extension effect of balloon dilation, the outer longitudinal layer was extended only beneath the submucosal fissure. An extension effect was scarcely observed in parts distant from the laceration. We therefore assumed that increasing the number of fissures by, for example, mucosal incision prior to dilation may lead to efficient dilation. Regarding mucosal incision during esophageal dilatation, Hordijk et al. conducted a randomized prospective study for 62 patients with postoperative anastomotic stenosis. They reported that clinical outcomes in a group of patients in whom a bougie was used and in a group of patients who underwent mucosal incision (3 or 4 incisions) were equivalent, and they suggested the possible usefulness of the combined use of both treatments［31］.

In addition, Liu reported good results of combined mucosal incision and temporary stent

insertion in 7 patients with refractory benign stenosis with a relatively short stenosis length of 1-3 cm caused mainly by postoperative anastomosis［32］. We speculated that it would be useful to make a mucosal incision prior to balloon dilation in order to increase the number of lacerations for esophageal stenosis. The development of a technique and/or device that can reduce the risk of perforation during incision for a long stenosis such as post-ESD stenosis is needed.

At the time of restenosis after initial balloon dilatation, the fibrotic plexus of the submucosal fissure part returned to a status similar to that before dilation and little difference from the surrounding fibrotic plexus was observed. The main reason for the esophageal benign stenosis becoming refractory is restenosis after dilation. As for prevention of restenosis after balloon dilatation, Hanaoka et al. conducted a randomized controlled trial for 65 patients with anastomotic stenosis after esophagectomy to assess the efficacy of adding a steroid injection to balloon dilation to reduce restenosis and they reported a preventive effect of local steroid injection on restenosis compared with the saline injection group［33］. We consider that some intervention to prevent refibrosis, such as local injection of a steroid into the laceration site, should be performed during balloon dilatation, even for patients with a relatively large length of post-ESD stenosis.

Several limitations of this study should be mentioned. First, in practical endoscopic treatment for esophageal cancer with a large size, local injection of steroids just after ESD

and/or prophylactic endoscopic balloon dilation before the completion of scar formation are often performed for the patients. It would be better to create porcine models with these interventions before dilation for obtaining more practical findings. However, local injection of steroids itself could cause muscle layer injury and prophylactic balloon dilation also could affect histological findings of balloon dilation for stenosis. Since there has been no study in which histological findings of the dilated esophagus, esophageal perforation and esophageal restenosis were analyzed, we focused on histological findings regarding balloon dilation without other interventions that might make the findings of this study confusing. Further studies using porcine models with these interventions prior to balloon dilation are required. Second, because we have no method to measure muscle layer extension directly, we evaluated the thickness of the outer longitudinal layer of smooth muscle as an indirect index of the muscle layer extension effect. A muscle layer with contracture caused by fibrosis should remain thick; however, this indirect index would not be so accurate.

In conclusion, the esophageal dilation experiment conducted in pigs showed that thermal damage to the muscle layer persists until the completion of stenosis and that once a muscle layer is injured, it does not completely recover afterward. Because the extension effect was observed only at the laceration site and it returned to a status similar to that before dilation afterward, additional intervention such as stenosis incision or steroid local injection during balloon dilation would be necessary for prevention of restenosis.

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Figure legends:

Figure 1. Experimental protocol. Two control models of stenosis (day 22, without dilation) and two control models of perforation (day 1, perforation during ESD) were created. For the dilation models, balloon dilation was performed three weeks after ESD. Immediately after dilation, sacrifice was performed. Two cases of muscle layer non-injury and two cases of muscle layer injury were created for histological evaluation of the esophagus during dilation and dilation-induced perforation (study 1). Next, pigs with restenosis after balloon dilation were created. After dilation on day 22, the pigs were managed for three weeks and sacrificed on day 43. Two cases of muscle layer non-injury and two cases of muscle layer injury during dilation were created for histological evaluation of restenosis after dilation (study 2).

Figure 2. Procedure of endoscopic balloon dilation (a muscle layer injury case). A, Endoscopic finding of esophageal stenosis on day 22 after endoscopic submucosal dissection. B, Endoscopic finding during dilation with maximum diameter of 12 mm. C, Endoscopic finding immediately after dilation. D, Excised esophagus.

Measurements in each study. E, Shema of the dilation model (study 1). a: Thickness of the outer longitudinal layer of smooth muscle (index of extension effect). b: Area of muscle fiber

bundle necrosis (index of thermal damage) . F, Image of measurement of the area of muscle fiber bundle necrosis per field of view (x 400) by using image analyzing software. The necrotic muscle fiber bundle became dark red and its microstructure was destroyed.

G, Schema of the restenosis model (study 2). a: Thickness of the fibrous plexus (index of the degree of restenosis). b: Thickness of the entire muscular layer (index of recovery from muscle layer laceration). c: Area of muscle fiber bundle necrosis (index of thermal damage).

Figure 3. Histological examination of the extension effect of balloon dilation (examination 1-1). A, Thickness of the outer longitudinal layer of smooth muscle. Thicknesses were measured in 10 random surrounding locations. Thicknesses at the laceration site were measured beneath the laceration in muscle layer non-injury cases and in both sides of the muscle layer laceration in muscle layer injury cases.

B, Histological finding of resected esophagus immediately after balloon dilation (a muscle layer injury case with a deep laceration and a shallow laceration, Masson’s trichrome staining, scale bar : 5mm).

Figure 4. Histological examination of muscle layer thermal damage on day 22 (examination 1-2). A, Area of muscle fiber bundle necrosis per field of view in the inner circular muscle layer. Areas of necrosis were measured at 10 random surrounding locations. Areas of necrosis at

the laceration site were measured beneath the laceration in muscle layer non-injury cases and in both sides of the muscle layer laceration in muscle layer injury cases.

B, Histological finding of resected esophagus immediately after balloon dilation (a muscle layer injury case, Masson's trichrome staining, scale bar : 2.5 mm). C, High power view in side of laceration (x 400).

Figure 5. Histological examination of the degree of restenosis (examination 2-1). A, Thickness of the fibrous plexus. Thiknesses were measured in 10 random surrounding locations. Thickness was measured at the laceration scar site.

B, Histological finding of resected esophagus with restenosis after balloon dilation (a muscle layer non-injury case, Masson's trichrome staining, scale bar : 1 mm).

Histological examination of recovery from muscle layer laceration for muscle layer-injury cases (an index of recovery from muscle layer laceration). (examination 2-2).C, Thickness of the entire muscular layer. Thicknesses were measured at 10 random surrounding locations. Thickness was measured at the laceration scar site.

D, Histological finding of resected esophagus with restenosis after balloon dilation (a muscle layer injury case, Masson's trichrome staining, scale bar : 1 mm).

Figure 6. Histological examination of the remaining muscle layer thermal damage on day 43

(examination 2-3). A, Area of muscle fiber bundle necrosis per field of view in the inner circular muscle layer. Areas were measured at 10 random surrounding locations. Area was measured beneath the laceration scar site.

B, Histological finding of resected esophagus with restenosis after balloon dilation (a magnified view beneath the laceration scar site of a muscle layer injury case, Masson's trichrome staining, scale bar : 0.25 mm).

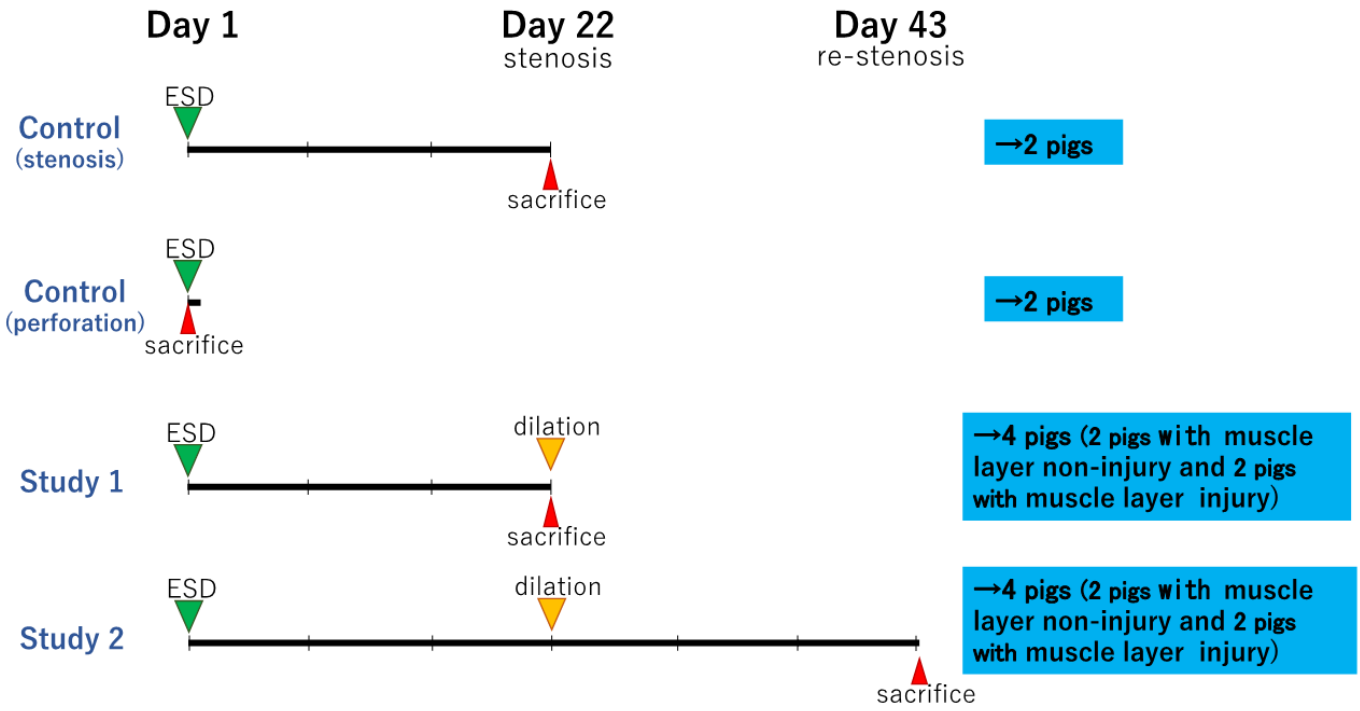

Day 1
Day 22
stenosis
Day 43
re-stenosis
Control
(stenosis)
ESD
sacrifice
→2 pigs
Control
(perforation)
ESD
sacrifice
→2 pigs
Study 1
ESD
dilation
sacrifice
→4 pigs (2 pigs with muscle layer non-injury and 2 pigs with muscle layer injury)
Study 2
ESD
dilation
sacrifice
→4 pigs (2 pigs with muscle layer non-injury and 2 pigs with muscle layer injury)

Figure 1

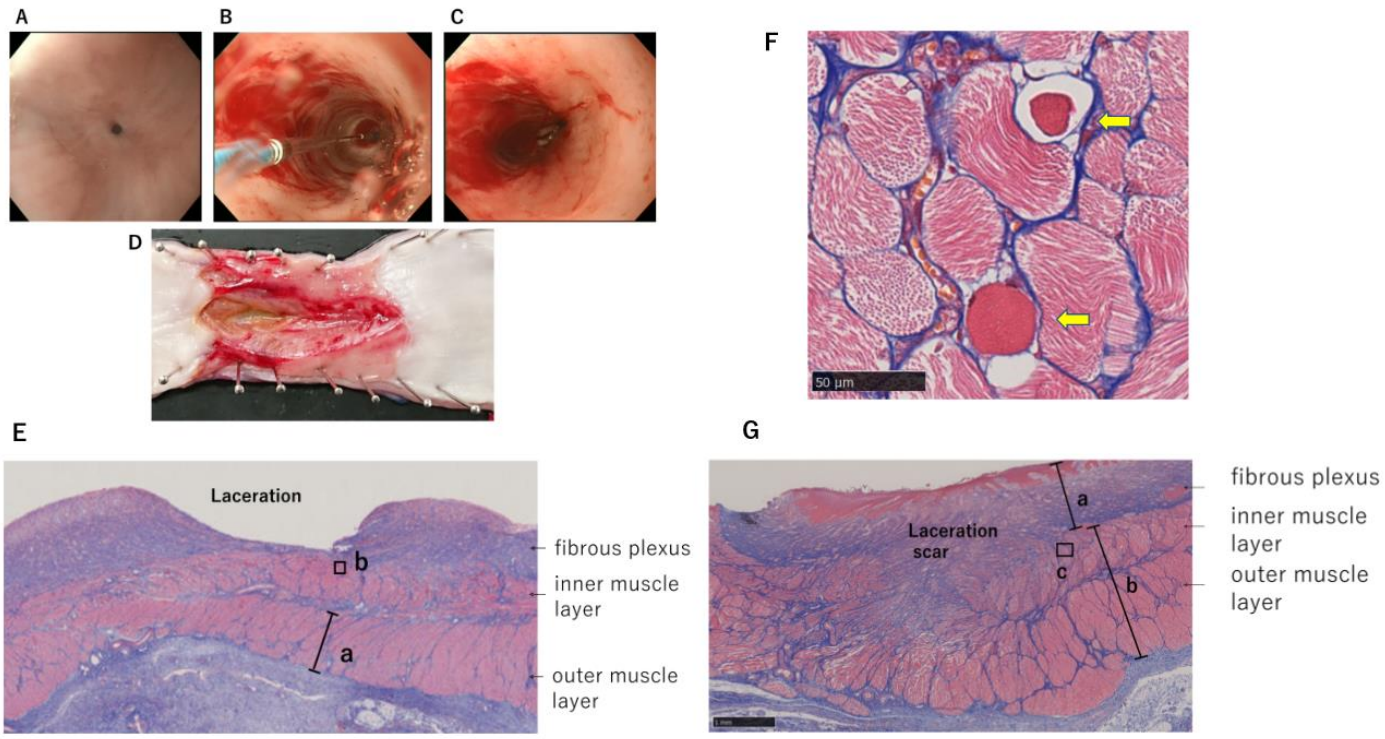

A
B
C
D
F
50 µm
E
Laceration
b
a
fibrous plexus
inner muscle layer
outer muscle layer
G
Laceration scar
a
c
b
fibrous plexus
inner muscle layer
outer muscle layer

Figure 2

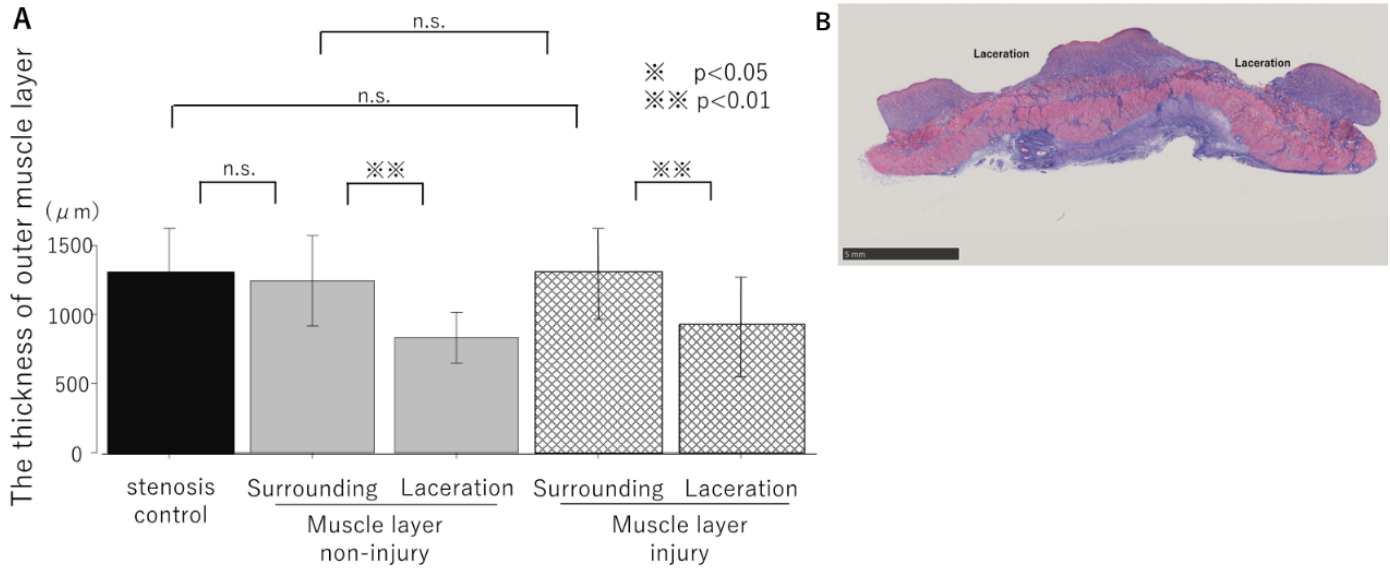

A
B
n.s.
n.s.
※ p<0.05
※※ p<0.01
n.s.
※※
※※
The thickness of outer muscle layer
(μm)
1500
1000
500
0
stenosis control
Surrounding
Laceration
Muscle layer non-injury
Surrounding
Laceration
Muscle layer injury
Laceration
Laceration
5 mm

Figure 3

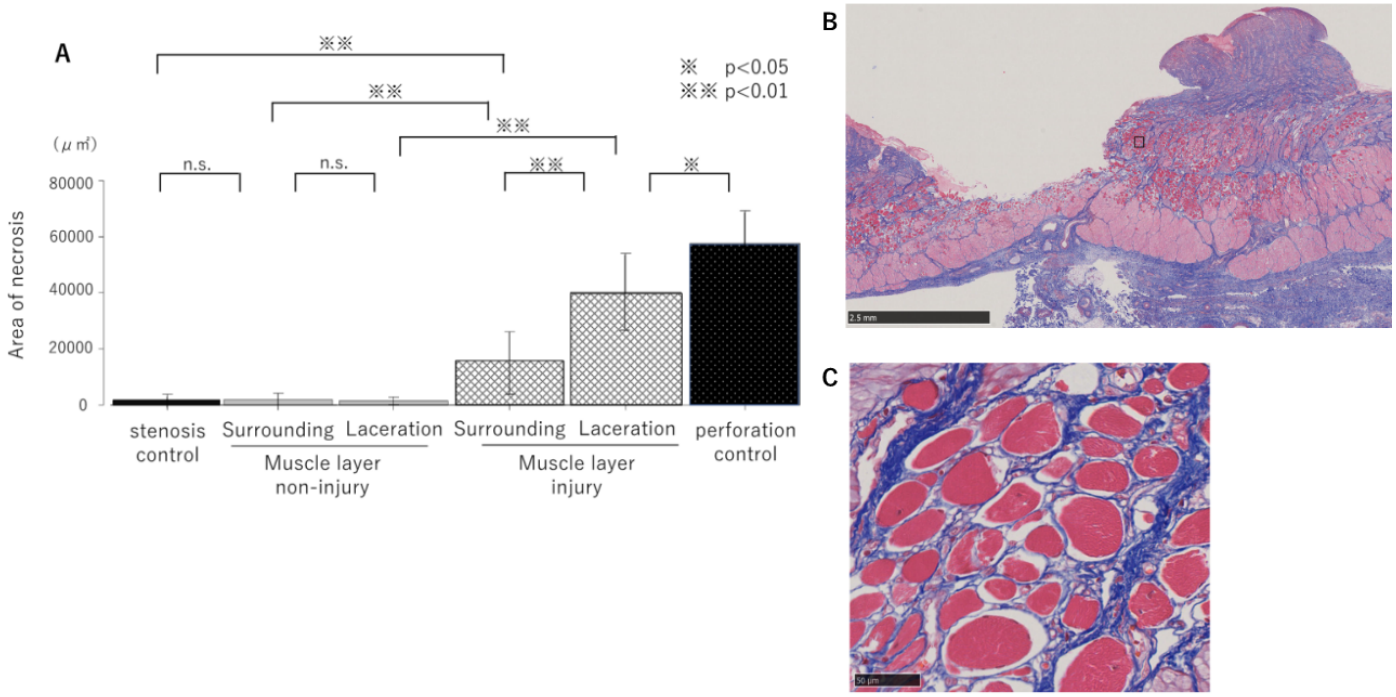
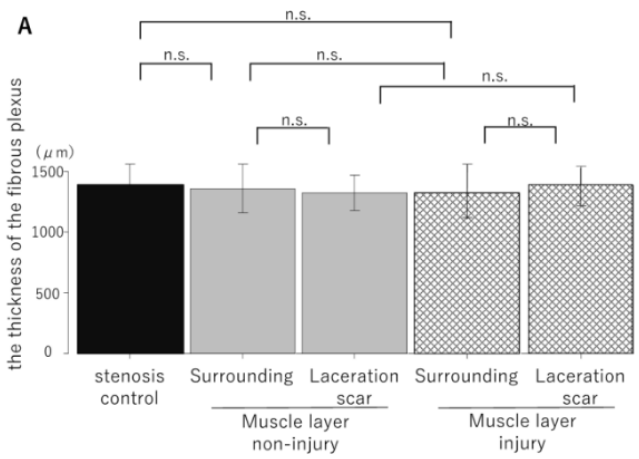
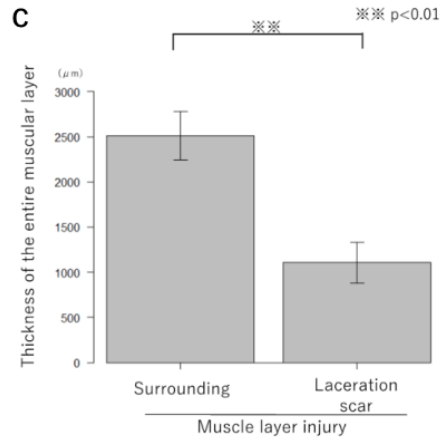
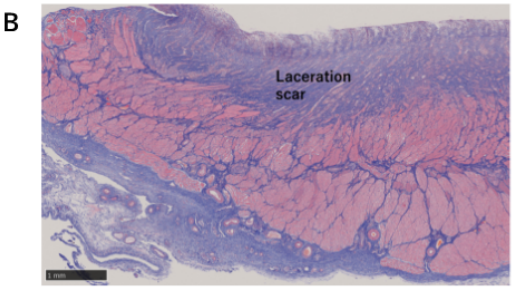

A
※※
※※
※※
※ p<0.05
※※ p<0.01
(μ㎡)
n.s.
n.s.
※※
※
80000
60000
40000
20000
0
Area of necrosis
stenosis control
Surrounding
Laceration
Muscle layer non-injury
Surrounding
Laceration
Muscle layer injury
perforation control
B
2.5 mm
C
50 μm

Figure 4


A
n.s.
n.s.
n.s.
n.s.
n.s.
n.s.
the thickness of the fibrous plexus
(μm)
1500
1000
500
0
stenosis control
Surrounding
Laceration scar
Surrounding
Laceration scar
Muscle layer non-injury
Muscle layer injury


C
※※
※※ p<0.01
Thickness of the entire muscular layer
(μm)
3000
2500
2000
1500
1000
500
0
Surrounding
Laceration scar
Muscle layer injury


B
Laceration scar

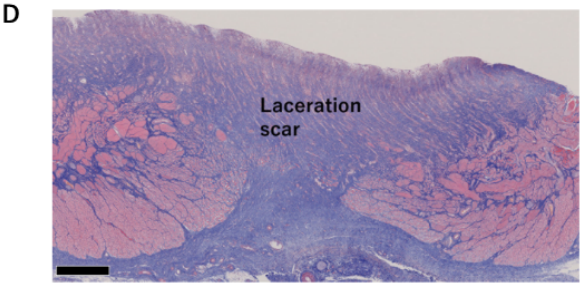

D
Laceration scar

Figure 5

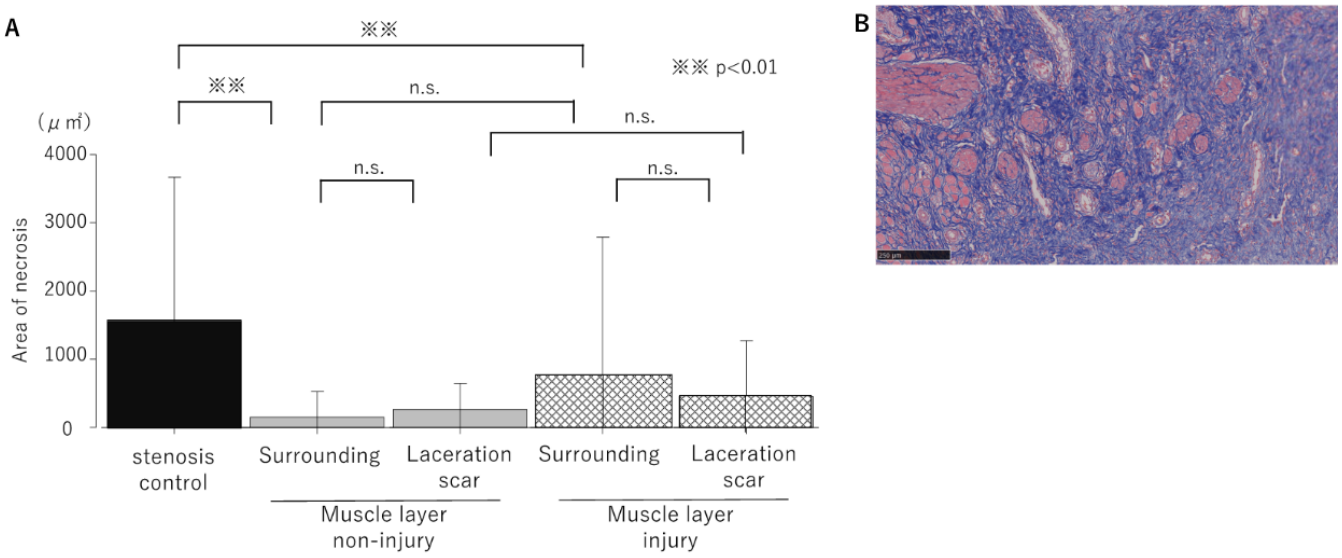

A
※※
※※ p<0.01
※※
n.s.
n.s.
n.s.
n.s.
(μm²)
4000
3000
2000
1000
0
Area of necrosis
stenosis control
Surrounding
Laceration scar
Surrounding
Laceration scar
Muscle layer non-injury
Muscle layer injury
B

Figure 6