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Covering post-endoscopic submucosal dissection ulcers in miniature swine with hexanoyl (Hx:C6) group–modified alkaline-treated gelatin porous film (HAG) induces proper healing by decreasing inflammation and fibrosis

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Porous film induces proper ulcer healing

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Acronyms and Abbreviations

HAG, Hexanoyl (Hx:C6) group–modified alkaline-treated gelatin porous film

VEGF, vascular endothelial growth factor

ESD, endoscopic submucosal dissection

αSMA, alpha-smooth muscle actin

Key words

endoscopic submucosal dissection, gastric ulcer, fibrosis

Abstract

Background and aims: Hexanoyl (Hx:C6) group–modified alkaline-treated gelatin porous film (HAG) is a newly developed degradable hydrogel characterized by strong adhesiveness and high affinity for vascular endothelial growth factor (VEGF). The aim of this study was to clarify the effect of HAG sheets on the healing process of post–endoscopic submucosal dissection (ESD) porcine gastric artificial ulcers.

Methods: 1）To evaluate the adhesiveness of HAG sheets over time ,we performed ESD to create one artificial ulcer and covered the lesion with one HAG sheet using one miniature swine. We observed two ulcers by endoscopic and microscopic examinations. 2）To examine the effect of HAG sheets on post-ESD ulcer healing , we performed ESD using five miniature swine. The artificial ulcers were covered with [P]HxAlGltn sheets, or left non-covered after ESD (day 0), followed by macroscopic and microscopic examinations. On day 7 and 14, we observed two ulcers by endoscopic examinations. On day 14, Animals were sacrificed and histological examination was performed on the three stomachs that could be extirpated.

Results: 1) On day 7, adhesion of HAG sheets was observed. 2) Gastric ulcer area on day 7 was significantly larger in the covered ulcers than in the non-covered ulcers ($p = 0.046$). On day 14, although there was no significant difference in ulcer area irrespective of covering (p=0.357), the covered ulcers tended to repair less fold convergence than non-covered ulcers. The covered ulcers ~~sheets~~ significantly decreased inflammatory cell infiltration (p=0.011), but significantly increased the abundance of macrophages (p=0.033), in submucosal layers. Also, the abundance of alpha–smooth muscle actin (αSMA)–positive cells in submucosal layers of the covered ulcers was significantly reduced (p=0.044), leading to a decrease in collagen accumulation. In addition, fibrosis and atrophy of the

muscularis propria were significantly lower for covered ulcers than for non-covered ulcers. Furthermore, microvessels and VEGF-positive cells were significantly more abundant in the submucosal layers of the covered ulcers ($p<0.001$ and $p=0.024$, respectively). ***Conclusions:*** HAG sheets induced post-ESD ulcer healing with less submucosal inflammation and muscularis propria injury and have the potential to decrease excess scarring.

Introduction

Once gastrointestinal mucosal injury has occurred, many inflammatory cells infiltrate the submucosal and muscularis propria, followed by development of fibrosis[1] . These phenomena result from impairment of the mucosal barrier, and exposure to luminal antigens throughout the healing process induces fibrosis and severe inflammation[2] . Therefore, the healing process of large post-endoscopic submucosal dissection (ESD) ulcers often leads to excess scarring or stricture.

In practice, gastric ulcers heal with scarring but rarely with stricture, even when they are deep or large. However, persistent reflux esophagitis often results in esophageal stricture, causing symptoms such as dysphagia [3]. We recently reported that esophageal stricture is also a major complication of ESD [4]. Even in patients who are asymptomatic conditions before ESD, once severe and difficult-to-treat esophageal stricture occurs, repeated endoscopic balloon dilatation is required due to refractory dysphasia. This is a critical issue not only at the esophagus, but also at the pylorus[5,6].

Hexanoyl (Hx:C_6) group–modified alkaline-treated gelatin porous film (HAG) is a newly developed degradable hydrogel (Fig. 1A) [7]. This highly porous material bonds strongly to porcine intestinal surfaces and has a high affinity for vascular endothelial growth factor (VEGF). Indeed, when HAG is implanted in rat subcutaneous tissues, sustained release of VEGF causes the local density of microvessels to increase in the vicinity of the hydrogel. Hence, it is likely that when injured mucosa is shielded with a sheet of HAG, the sheet adheres tightly and remains until it is gradually degraded, resulting in reduced exposure to luminal contents. In addition, the sheet is predicted to facilitate healing of injured mucosa by inducing angiogenesis.

In this study, we performed gastric ESD in miniature swine, covered the lesions

with HAG sheets, and investigated how the HAG sheets affected the post-ESD ulcer healing process in the absence of antacid.

Materials and Methods

Experimental animals

Six miniature swine (6 months of age, 19–21 kg, male, from the Kagoshima Miniature Swine Research Center, Kagoshima Japan.) were used this study. The miniature swine were injected intramuscularly with 15 mg/kg ketamine (Daiichi Sankyo Propharma, Tokyo, Japan) and 2 mg/kg xylazine (Bayer Yakuhin, Osaka, Japan) for premedication, and then administered sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) by inhalation. An endotracheal tube (100/199/065; Smith Medical Japan, Tokyo, Japan) was inserted, and anesthesia was maintained using sevoflurane. Animals were allowed free access to water on the day of ESD, and solid food on the next day. Animal care, housing, and surgery were in accordance with the Rules and Regulation of the Committee for Animal Research of Kagoshima University, Japan. Because this was an animal feasibility study, no IRB approval or written consent was required.

ESD procedure

To make artificial gastric ulcers, an expert endoscopist performed ESD using an upper gastrointestinal endoscope (GIF-Q260J; Olympus, Tokyo, Japan) and a video scope system (EVIS LUCERA CV-260SL; Olympus). A glycerin solution containing a small amount of epinephrine and indigo carmine (Glycerol injection; Hikari Pharmaceutical, Tokyo, Japan) was injected into the submucosa of the planned ulcer site. The mucosa and submucosal layer were incised with an electric knife (Dual Knife, KD-650L; Olympus) and an electrosurgical generator (Pulse Cut Fast mode, 30W, ESG-100; Olympus).

A paper 20 mm in diameter was placed at the planned ulcer site. The

circumference of the paper was marked to create ulcers 30 mm in diameter [8~~7~~]. We confirmed ulcer diameters using measuring forceps (M2-K4; Olympus) (Fig. 1B~~C~~).

HAG sheets

Hexanoyl (Hx:C6) group–modified alkaline-treated gelatin was prepared by reaction between Hx chloride and the primary amino groups of alkaline-treated gelatin derived from porcine skin (Fig. 1A). Porous HxAlGltn films were fabricated by the salt-leaching method, using NaCl particles as a porogen. The solid–liquid ratio (NaCl:HxAlGltn 10% L-lactic acid containing dimethyl sulfoxide solution) was fixed at 4:1. Trisuccinimidyl citrate was used to crosslink HxAlGltn molecules. HAG sheets were manufactured in a disk shape with a long diameter of 30 mm [7].

Delivery of HAG sheets and covering of artificial gastric ulcers

First, because of its strong adhesiveness, the HAG sheet was placed in a storage bag(Fig.1C) [8]. The bag was held with grasping forceps (FG-47L-1; Olympus), followed by insertion of an endoscope through an over tube (FOT large type; Sumitomo Bakelite, Tokyo, Japan) into the stomach. The storage bag was then placed near the ulcer site, and the HAG sheet was grasped and delivered to the ulcer area using forceps (Fig. 1D).

To evaluate the adhesiveness of HAG sheets, we used one miniature swine. On day 0, we performed ESD to create one artificial ulcer and covered the lesion with one HAG sheet. On day 4, we created another artificial ulcer by ESD using in the same swine and covered it with one HAG sheet. On day 7, we observed two ulcers by endoscopy and sacrificed the swine. (Fig.2A).

To determine the effect of HAG sheets on post-ESD ulcer healing, we used five

miniature swine. On day 0, we made two artificial ulcers of the same size (30 mm in diameter) in the antrum of each animal's stomachs. One of the two open ulcers was covered with HAG sheets, and the other was left uncovered as a control.

On days 7 and 14, covered and non-covered ulcers were endoscopically measured using majoring forceps, and the areas of open ulcers (mm^2) were calculated using the following formula: 3.14 × 0.25 × long axis (mm) × minor axis (mm). On day 14, the number of gastric ulcer folds in five swine were endoscopically counted, and three of the five swine were sacrificed by intravenous administration of a lethal dose of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan). (Fig.2B). The other two swine were sacrificed on day 21 and day 28, respectively (not shown data).

Histological examinations

After the specimens were fixed in 10% neutral-buffered formalin (Kenei Pharmaceutical, Osaka, Japan) for 48 h, each lesion was sliced at intervals of 4 mm. Each piece was then embedded in a paraffin block, cut into sections (thickness 2 μm), and stained with hematoxylin–eosin (HE), azan, and Masson trichrome staining. HAG sheets remaining on ulcer areas were examined by Masson trichrome staining. Inflammatory cells infiltrating into the submucosal layers in 12 random fields of the view (×200 magnification) were counted on HE-stained tissues under a microscope. Damage to the muscularis propria was evaluated by Azan staining. The thickness of the muscularis propria was quantified based on the ratio of the thickness of the muscularis propria in the ulcer part (U) to that of the non-ulcer(NU) part (U/NU). The degree of fibrosis and atrophy of the muscularis propria in the ulcer part were graded numerically using a modified version of Honda's and Chang-Il Kwon scoring system [9,10] . Grades were defined as follows: 1, absence of atrophic

or fibrotic changes in the muscularis propria; 2, atrophy or fibrosis reaching up to the upper third of the muscularis propria; 3, atrophy or fibrosis was reaching up to the middle third of the muscularis propria; and 4, atrophy or fibrosis reaching up to the lower third of the muscularis propria.

Immunohistochemistry

Immunohistochemical staining was performed using standard enzyme-labeled antibody methods [11]. The numbers of neutrophils and macrophages were determined using rabbit polyclonal anti-myeloperoxidase (MPO) antibody at 1:50 dilution (Abcam, Cambridge, MA, USA) and goat polyclonal anti-Iba1 antibody at 1:4000 dilution (Abcam), respectively. Myofibroblasts and fibrosis were identified using mouse monoclonal anti-alpha smooth muscle actin (α-SMA) antibody (1:1000 dilution; Progen Biotechnik, Heidelberg, Germany) and anti–collagen I alpha 1 antibody (1:400 dilution; Novus Biologicals, Littleton, CO, USA), respectively. Expression of VEGF and blood vessels was evaluated using a mouse monoclonal anti-VEGF antibody at 1:50 dilution (Novus Biologicals) and rabbit polyclonal anti–von Willebrand factor antibody at 1:5000 dilution (Abcam), respectively. The samples were then incubated with anti-mouse or anti-rabbit secondary antibody (Nichirei, Tokyo, Japan). Immunohistochemical double stainings were performed using mouse monoclonal anti-VEGF antibody at 1:50 dilution and goat polyclonal anti-Iba1 antibody at 1:2000 dilution.

To evaluate fibrosis in the submucosal layer, we randomly selected 12 visual fields and quantified the areas stained with α-SMA and Type I collagen using ImageJ, version 1.50b (National Institutes of Health, Bethesda, MD, USA). Also, MPO-positive cells, Iba1-positive cells, VEGF-positive cells, and blood vessels positive for von

Willebrand factor were counted using ImageJ.

Statistical analysis

Statistical significance of differences between the two groups was calculated using Student's *t*-test or the Mann–Whitney test, depending on the result of the Shapiro–Wilk test and Levine's test for normality and equality of variance. *P* values <0.05 were considered significant. All statistical analyses were performed with the IBM SPSS Statistics Base 23 software program (IBM Corp., Armonk, NY, USA).

Results

HAG sheets adhere tightly to ulcer beds and remain seven days after ESD

ESD was performed safely in all swine, and no complications, including perforation and bleeding, were observed throughout the experimental period. When open ulcers made by ESD were covered with HAG sheets, the sheets adhered within a few minutes, and could not be peeled off (Fig. 1D). Although animals were allowed free access to water on the day of surgery and solid food on the next day, white deposits of remnant sheets were observed on the surface of ulcer 3 days after shielding (Fig.3A). Histologically, HAG sheets were observed on the surface of granulation tissues (Fig. 3B, C). Seven days after shielding, although white deposits disappeared from the surface of ulcer (Fig.3D), a part of the HAG sheet could still be histologically detected in granulation tissues (Fig. 3E, F). These results indicate that, although the animals had free access to solid food, HAG sheets tightly adhered to ulcer beds and shielded the surface-lacking mucosa from harmful gastric contents for at least 7 days.

Ulcers covered with HAG sheets tend to repair with less fold convergence

We endoscopically observed gastric ulcers 7 and 14 days after ESD. On day 7, gastric ulcers covered with HAG sheets remained significantly larger than non-covered ulcers (non-covered: 125 ± 6 mm^2; covered: 269 ± 51 mm^2; p=0.046). However, the covered ulcers were reduced in size, although there was no significant difference in ulcer areas on day 14, irrespective of covering (non-covered and covered ulcers: 28 ± 23 mm^2 and 40 ± 16 mm^2, respectively, p=0.357) (Fig. 4A, B). On day 14, the number of ulcer folds were smaller in covered than non-covered ulcers, but the difference was not statistically

significant (non-covered and covered ulcers: 4.4 ± 2.6% and 1.8 ± 1.6%, respectively, p=0.096) (Fig. 4C).

HAG sheets decrease inflammation and facilitate angiogenesis in submucosal layers

We next evaluated infiltrating inflammatory cells, neutrophils and macrophages. The number of inflammatory cells in the submucosal layers was significantly smaller in covered than non-covered ulcers (non-covered and covered ulcers: 360 ± 27 and 278 ± 17, respectively, p=0.011) (Fig.5A, B, C). In addition, MPO-positive cells infiltrating the submucosal layers were significantly less abundant in covered ulcers (non-covered and covered ulcers: 14.1 ± 2.4 and 7.0 ± 2.9, respectively, p=0.032) (Fig.5D, E, F), whereas the number of submucosal macrophages was significantly higher in covered ulcers (non-covered and covered ulcers: 54.0 ± 11.8 and 92.9 ± 17.6, respectively, p=0.033) (Fig.5G, H, I).

Next, we examined submucosal areas positive for α-SMA and type I collagen. When compared to non-covered ulcers, α-SMA–positive area significantly decreased in HAG sheet–covered ulcers (non-covered and covered ulcers: 14.7 ± 1.3% and 10.6 ± 2.1%, respectively, p=0.044) (Fig.6A, B, C). The submucosal areas positive for type I collagen were smaller in covered than non-covered ulcers, but the difference was not statistically significant (non-covered and covered ulcers: 21.8 ± 1.6% and 18.1 ± 3.3%, respectively, p=0.163) (Fig.6D, E, F).

Because HAG sheets have high affinity for VEGF, leading to its sustained release, we examined angiogenesis in submucosal layers immunohistochemically. The number of submucosal microvessels positive for von Willebrand factor was significantly higher in covered ulcers (non-covered and covered ulcers: 20 ± 1 and 34 ± 2, respectively, p<0.001) (Fig.7A, B, C). Interestingly, VEGF-positive cells were significantly more

abundant in covered ulcers (non-covered and covered ulcers: 13.2 ± 3.5 and 21.9 ± 2.5, respectively, p=0.024) (Fig. 7D, E, F). Some of the VEGF-positive cells were also positive for Iba1 (Fig. 7G, H), indicating that VEGF was partly produced by macrophages infiltrating in the submucosal layers. These results suggest that covering ulcers with HAG sheets facilitates angiogenesis through VEGF, which is released from the HAG sheets and produced partly by infiltrating macrophages.

HAG sheets decrease damage to the muscularis propria

Next, we evaluated thickness and damage, such as fibrosis and atrophy, of the muscularis propria, which is closely associated with excess scarring [9]. We calculated the ratio of the thickness of the muscularis propria in ulcer areas vs. non-ulcer areas (U/NU ratio). The U/NU ratio was significantly lower in covered ulcers (non-covered and covered ulcers: 1.68 ± 0.14 and 1.27 ± 0.19, respectively, p=0.031) (Fig.8A, B). Additionally, the degree of fibrosis and atrophy of the muscularis propria was significantly lower in covered ulcers (non-covered: 3.3 ± 0.6; covered: 1.3 ± 0.6; p=0.043) (Fig.8C, D).

Discussion

The structural integrity of the gastric mucosa is constantly challenged by harmful agents, including acid pepsin, bile acid, alcohol, and drugs[12] . However, once the ulcerative gastric mucosal defect occurs, persistent exposure to gastric contents could induce severe inflammation and fibrosis, leading to excess scarring[13,14] . Suppressing inflammation improve quality of ulcer healing [15, 16]. In this study, we covered artificial gastric ulcers with sheets of HAG, a bio-degradable hydrogel, immediately after ESD. Over the course of the healing process, the covered ulcers exhibited a decrease in not only submucosal inflammatory infiltration, but also fibrosis and atrophy of the muscularis propria.

The porous structure of the HAG sheets enhanced wet-tissue bonding when the Hx group was included, and facilitated absorption of moisture from wet-tissue surfaces in comparison with a flat structure[7] . Additionally, the Hx group penetrated the cell membranes of the tissue surface, and easily interacted with the hydrophobic region of the extracellular matrix. In this study, HAG sheets had not peeled off even after food intake, and HAG remnants were still detected in the granulation tissues until 7 days after ESD (Fig.3). These results indicate that HAG sheets could avoid exposure of post-ESD ulcers to harmful gastric contents during the healing process.

Recently, Kwon et al. reported that when bio-sheet grafts were applied to artificial ulcers, ulcer healing was unexpectedly delayed due to physical hindrance[10] . In this study, size reduction in HAG-covered ulcers was significantly delayed on day 7. However, the size of covered ulcers gradually decreased over the subsequent 7 days, and there was no significant difference in size between covered and non-covered ulcers on day 14 (Fig.4). HAG is a biodegradable hydrogel, and only a few HAG remnants were detected in the granulation tissues of covered ulcers on day 7 (Fig.3E, F). Although HAG

sheets could directly inhibit wound contraction, resulting in a temporary delay in ulcer healing, the sizes of covered ulcers were reduced in parallel with gradual degradation of HAG remnants via proteolysis with protease. Therefore, in addition to its strong adhesiveness, another advantage of HAG is its biodegradability.

Inflammatory response initiated in the wounded tissue is associated with an early recruitment of neutrophils[17] . Neutrophils secrete chemokines and cytokines, which control the subsequent recruitment of monocytes that differentiate into macrophages. Many different cell types, including macrophages, fibroblasts, and contractile myofibroblasts, participate in wound repair[18] . Myofibroblasts derived from fibroblasts in the wound bed express smooth muscle actin (SMA) and generate strong contractile forces that facilitate wound contraction and repair. In this study, HAG reduced infiltrating neutrophils and α-SMA–positive areas (Fig.5, 6). Although expression of type I collagen was not significantly reduced, a decrease in α-SMA–positive areas may have contributed to the reduction in wound contraction. Furthermore, HAG binds to VEGF and induces angiogenesis [7], an important component of gastric erosion and ulcer healing [19]. In this study, the density of microvessels increased in the submucosal layers of HAG-covered ulcers. Therefore, HAG probably binds to endogenous VEGF produced in the wound microenvironment, leading to persistent release of VEGF. Interestingly, HAG increased the abundance of macrophages, some of which were positive for VEGF, infiltrating the submucosal layers (Fig.5, 7). Ulcerative mucosal defects require remodeling in addition to epithelial migration, proliferation, maturation, and dedifferentiation[19, 20]. Recent studies reported that macrophages infiltrating injured tissues transform from inflammatory M1 to anti-inflammatory M2 macrophages during the healing process, and that expression of VEGF is characteristic of M2 or wound-healing macrophages, which play a

critical role in the remodeling of injured tissues[21, 22] . Therefore, HAG sheets could effectively inhibit exposure of harmful gastric contents to mucosal defect, and ameliorate submucosal inflammation, leading to early induction of remodeling phase in wound healing process.

Damage to the muscularis propria is closely associated with wound contraction and stricture[9, 23] . Honda et al observed fibrosis and atrophy in the muscularis propria in animals exhibiting esophageal stricture following esophageal mucosal resection[9] . Additionally, severe inflammation is also induced by stimulation from the passage of food and digestive juices[23], and resection of a large amount of the esophageal mucosa often causes esophageal ulcer and postoperative stricture. We recently reported that mucosal defects occupying more than three-quarters of the esophageal circumference are a risk factor for stricture[4] . This is a critical issue not only at the esophagus, but also at the pylorus [5,6]. Efforts to prevent stricture have resulted in the development of tissue-engineered cell sheets[24,25,26] , polyglycolic acid (PGA) sheets with fibrin glue[8, 27, 28] , biodegradable stents[29] , amniotic membrane grafts[30] , high-density collagen patches[31] , and adipose tissue–derived stromal cells[9] . However, these materials provide only limited success. In this study, covering ulcers with HAG sheets markedly decreased fibrosis and atrophy of the muscularis propria (Fig.8). These results suggest that HAG sheets adhered tightly, effectively reducing exposure to gastric juice, bile acid, and food, leading to a decrease in the number of inflammatory cells infiltrating deeply in the muscularis propria. Damage to the muscularis propria was reported to persist even after epithelialization in a canine model of artificial esophageal ulcer[21]. Although further investigations are needed to determine how damage to the muscularis propria affects scarring following epithelialization, our results imply that shielding of post-ESD ulcers with

HAG sheets has the potential to reduce excess scarring (Fig.9).

We consider it necessary to evaluate a post-ESD ulcer model in the esophagus and duodenum. However, because of its strong adhesiveness, we could not deliver HAG sheets to ulcers in a small space such as the esophagus or duodenum. For studies using a post-ESD ulcer model in the esophagus and duodenum, we will need to change the shape of the material and improve the delivery system.

This study may have some limitations. First, the evaluations were performed on a small number of animals. Second, we did not evaluate post-ESD gastric ulcers over the long term. Hence, further studies with larger numbers of cases and clearer definitions of optimized configurations are needed. Third, in clinical practice, antacids are used after gastric ESD [5]. In this study, we investigated how the HAG sheets affected the post-ESD ulcer healing process in the absence of antacid. For practical use, further studies using both HAG sheets and antacid should be performed. Fourth, several submucosal injection fluids (saline/ hypertonic saline-epinephrine /hyaluronic acid, etc.) are used for ESD [5]. Future studies should examine the effects of those submucosal injection fluids on HAG sheets.

In conclusion, HAG sheets, which tightly adhere to ulcer beds, effectively prevent exposure to harmful gastric contents and induce ulcer healing with less submucosal inflammation and damage to the muscularis propria. Furthermore, the sheets facilitated angiogenesis. Due to their bonding and biodegradability, HAG sheets are advantageous as a material for covering post-ESD ulcer, and could lead to proper ulcer healing with less scarring.

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Statement of Ethics

All experiments were approved by the Animal Care and Use Committee of Kagoshima University (approval no. MD 15063). This animal experiments conform to institutional standards of the Committee for Animal Research of Kagoshima University.

Disclosure Statement

The authors have no conflicts of interest to declare.

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None.

Author Contributions

HM and FS were responsible for the experimental design. HM, FS, MK and YN performed the animal experiments. HM and YM performed the stomach histochemical examinations. HI, YK, SA, ST and SH were responsible for statistical analyses. AN and TT created and provided HAG sheets. AI made substantial contributions to the conception and design of the study and the drafting of the manuscript. HM and FS wrote the manuscript. All authors were involved in manuscript revisions.

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Figure legends

Figure 1. Covering an artificial gastric ulcer with HAG sheets. Electron micrograph of HAG sheets with interconnected pores (**A**). Artificial ulcer (30 mm in diameter) in the stomach antrum, made by ESD (**B**). HAG sheets were placed in a storage bag (**C**). HAG sheets adhered within a few minutes, and could not be peeled off (**D**).

Figure 2. Study design. Evaluation the adhesiveness of HAG sheets (A). Evaluation the effect of HAG sheets itself on post-ESD ulcer healing (B).

Figure 3. HAG sheets remain on the ulcer surface for up to 7 days. On day 3, HAG remained as white deposits on the ulcer surface (**A**), and was histologically observed on the surface of the granulation tissues (arrowhead, **B**, **C**). Although HAG became endoscopically invisible (**D**), a small amount of HAG was still detected on day 7 (arrowhead, **E**, **F**). Masson trichrome staining (**B**, **C**, **E**, **F**), original magnifications × 12.5 (**B**, **E**) and × 100 (**C, F**).

Figure 4. Changes in endoscopic findings of gastric ulcers covered with or without HAG sheets. Representative endoscopic findings on days 0, 7, and 14. HAG-covered ulcers, yellow arrow; non-covered ulcers, white arrow (**A**). Changes in area of gastric ulcers. Although the areas of covered ulcers (HAG) were significantly larger than those of non-covered ulcers (Control) on day 7 (p=0.046), there was no significant difference in areas between covered and non-covered ulcers (p=0.357) on day 14 (**B**). The number of ulcer folds were smaller in covered than non-covered ulcers, but the difference was not statistically significant (p=0.096) (**C**).

Figure 5. Covering with HAG sheets decreases inflammatory cell infiltration but increases the abundance of macrophages in the submucosal layers. Inflammatory cells in submucosal layers of non-covered and covered ulcers (A, B, respectively). The number of inflammatory cells was significantly lower in HAG-covered ulcers (HAG) than in non-covered ulcers (Control) (p=0.011) (C). Immunohistochemistry of MPO-positive cells of non-covered and covered ulcers (D, E, respectively). MPO-positive cells were significantly less abundant in covered ulcers than in non-covered ulcers (p=0.032) (F). Macrophages positive for Iba1 in the submucosal layers of non-covered and covered ulcers (G, H, respectively). The number of cells positive for Iba1 was significantly higher in covered ulcers than in non-covered ulcers (p=0.033) (I). H&E staining (A, B), immunohistochemistry for MPO (D, E), and Iba1 (G, H); original magnification × 200.

Figure 6. HAG sheets decrease α-SMA–positive area in submucosal layers. Areas positive for α-SMA in submucosal layers of non-covered and covered ulcers (**A**, **B**, respectively). α-SMA–positive areas in submucosal layers were significantly lower in HAG-covered ulcers (HAG) than in non-covered ulcers (Control) (p=0.044) (**C**). Areas positive for type I collagen in submucosal layers of non-covered and covered ulcers (**D**, **E**, respectively). Type I collagen–positive areas were reduced in covered ulcers, but the difference was not statistically significant (p=0.163) (**F**). Immunohistochemistry for α-SMA (**A**, **B**) and type I collagen (**D**, **E**); original magnification × 200.

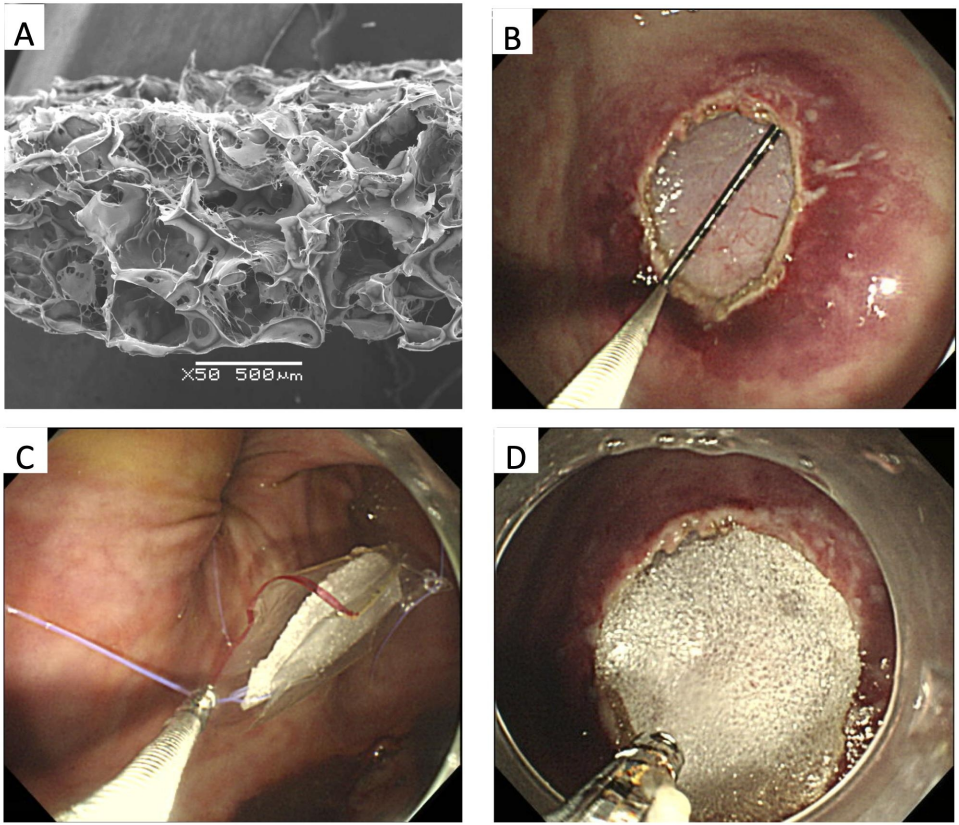
Figure 7. HAG-covering increases the density of microvessels and the abundance of VEGF-positive cells. Microvessels positive for von Willebrand factor were evaluated in submucosal layers of non-covered and covered ulcers (**A, B**, respectively). Submucosal

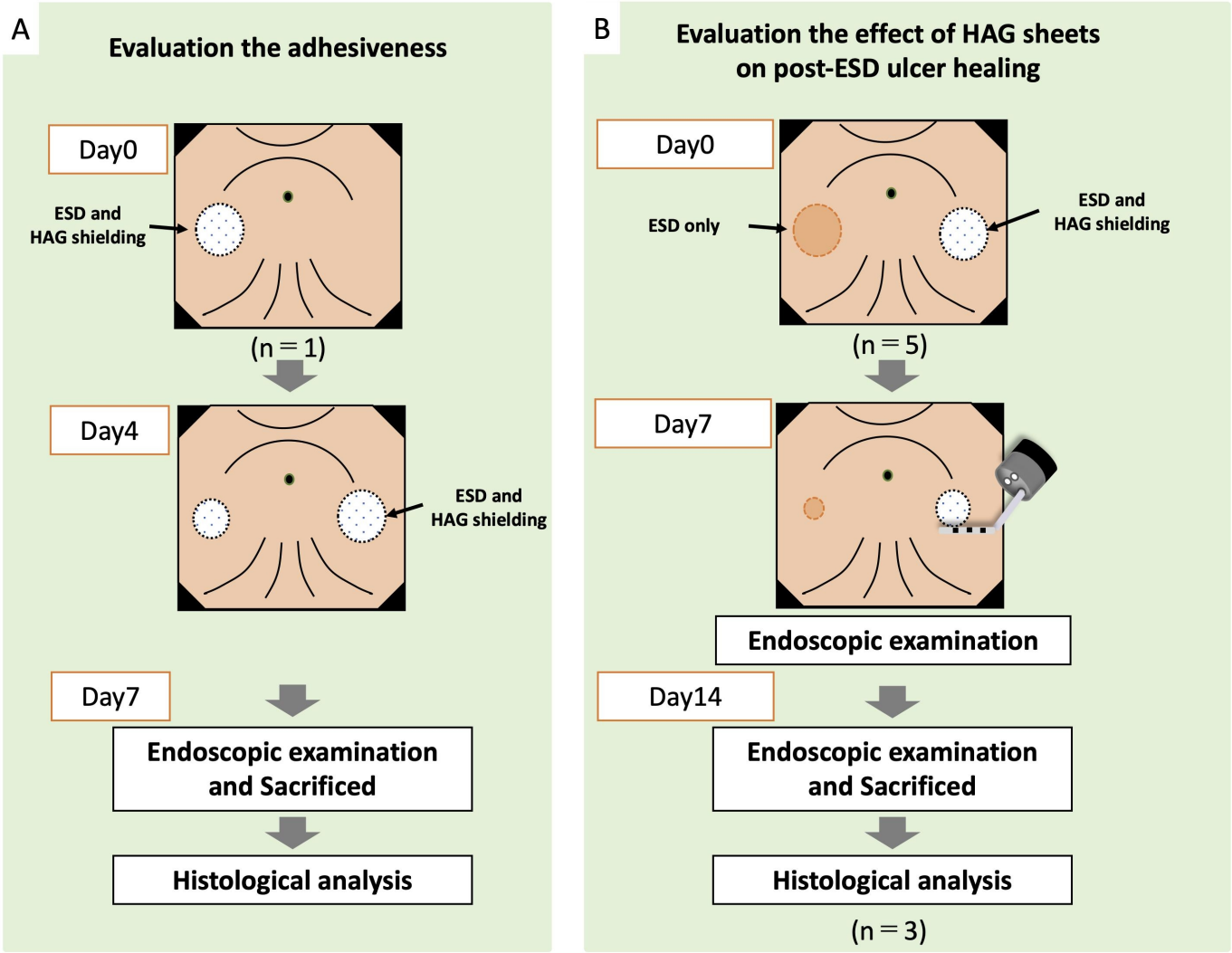
microvessels were more abundant in HAG-covered ulcers (HAG) than in non-covered ulcers (Control) ($p<0.001$) (**C**). VEGF positive-cells in submucosal layers of non-covered and covered ulcers were evaluated (**D**, **E**, respectively). The number of cells positive for VEGF was higher in covered ulcers than in non-covered ulcers ($p=0.024$) (**F**). Some VEGF-positive cells (brown) were also positive for Iba1 (blue) (**G**, **H**). Immunohistochemistry for von Willebrand factor (**A**, **B**), VEGF (**D**, **E**), double staining for VEGF and Iba1 (**G**, **H**); original magnifications × 200 (**A**, **B**, **D**, **E**, **G**) and × 400 (**H**).

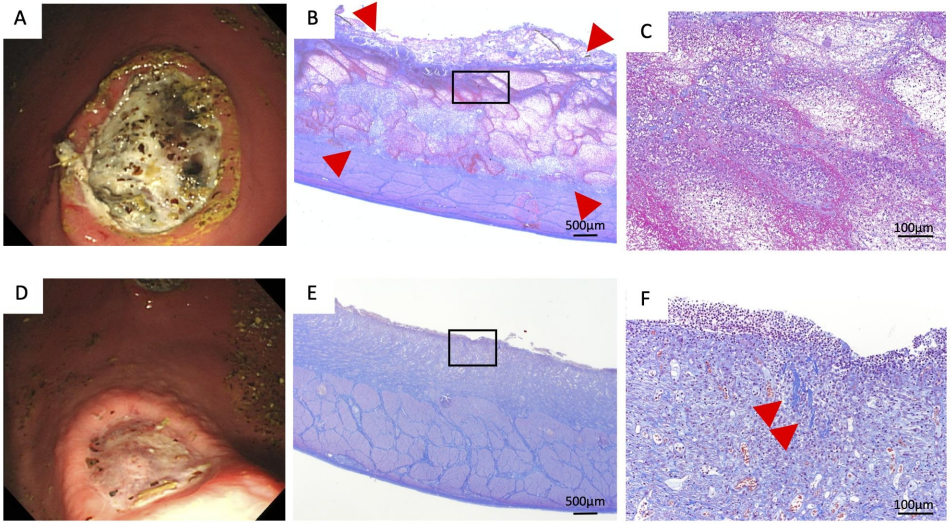
Figure 8. Covering ulcers with HAG sheets decreases damage to the muscularis propria. The thickness of the muscularis propria was quantified as the ratio of the thickness of the ulcer part (U) vs. that of the non-ulcer (NU) part (U/NU ratio), as described in Materials and Methods (**A**). The U/NU ratio was significantly lower in HAG-covered ulcers (HAG) than in non-covered ulcers (Control) ($p=0.031$) (**B**). The degree of fibrosis and atrophy of the muscularis propria in the ulcer part was evaluated as described in Materials and Methods (**C**). Fibrosis and atrophy of muscularis propria were significantly less severe in covered ulcers than in non-covered ulcers ($p=0.043$) (**D**). Azan staining (**A** and **C**); original magnification ×20.

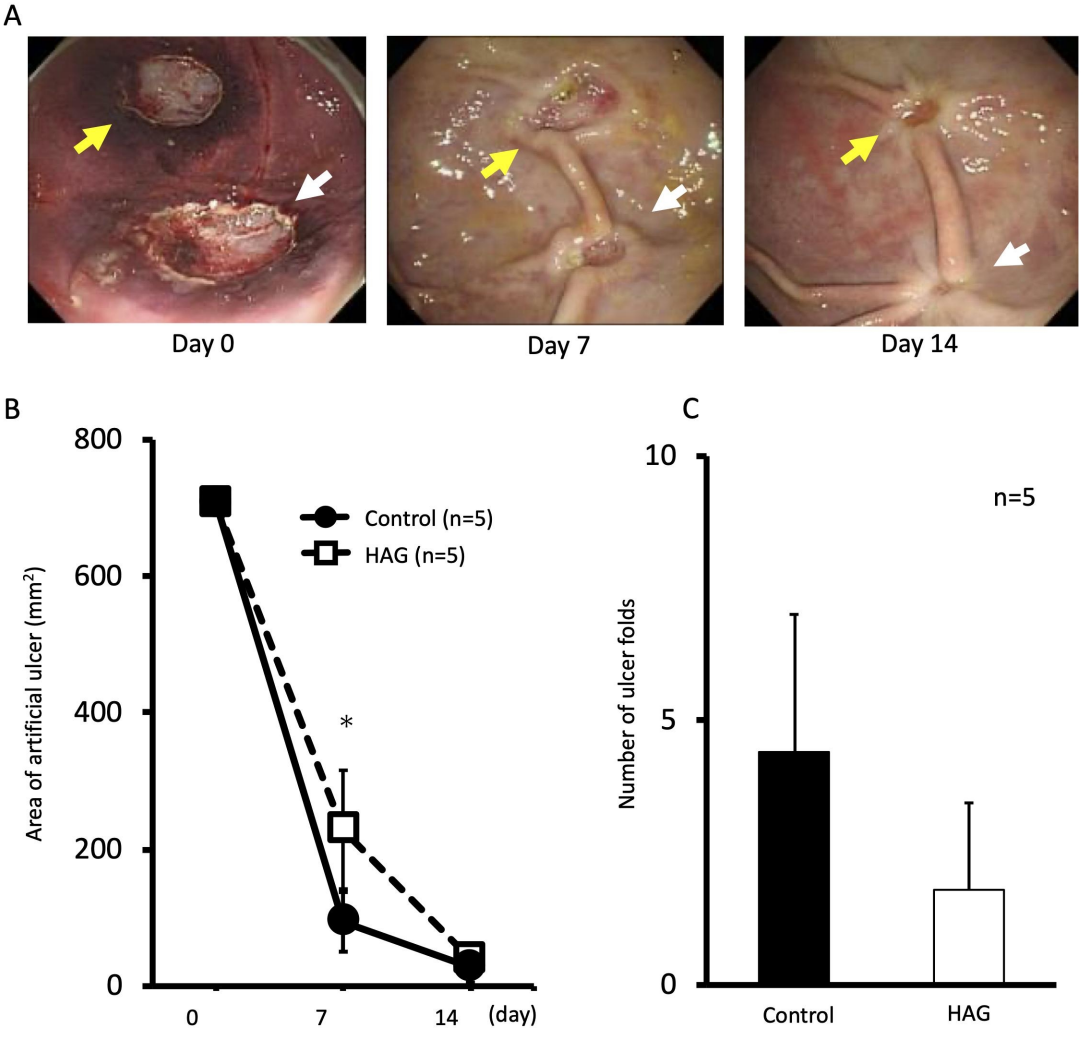
Figure 9. Schematic illustration of the effect of HAG sheets on post-ESD ulcer healing. The sheet tightly adheres to ulcer areas. As a consequence, it protects the open ulcer against exposure to luminal contents, including gastric and bile acids as well as food. Therefore, it induces ulcer healing with less submucosal inflammation and damage to the muscularis propria. In addition, the sheet interferes with tissue contractility during the healing process. Furthermore, the sheets had high affinity for endogenous VEGF and thus

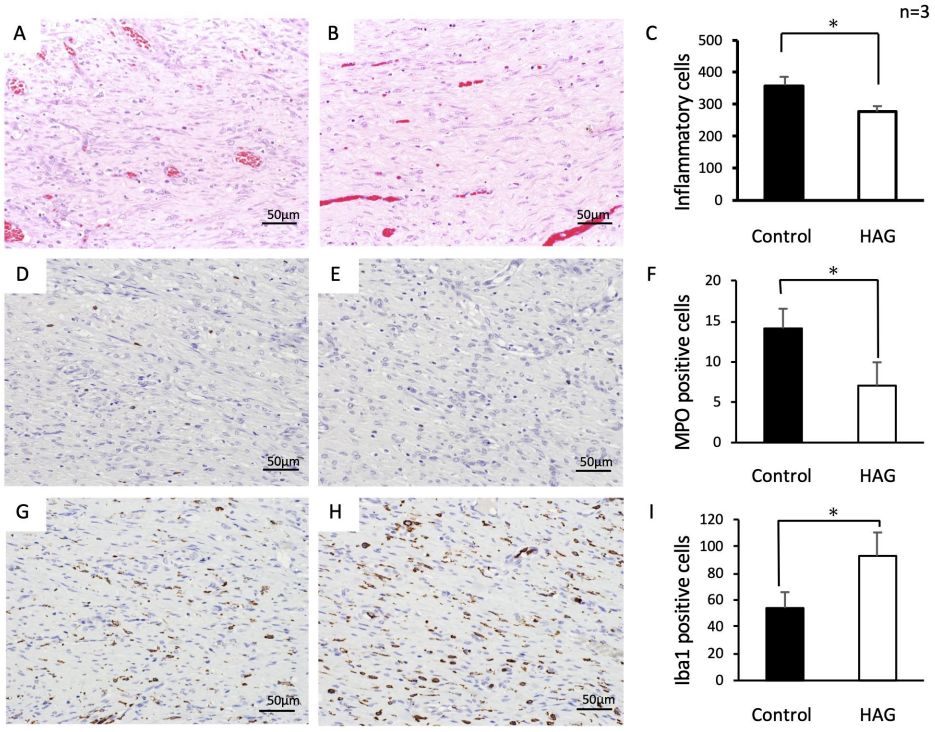
facilitated angiogenesis. Therefore, HAG sheets may suppress excess scarring.

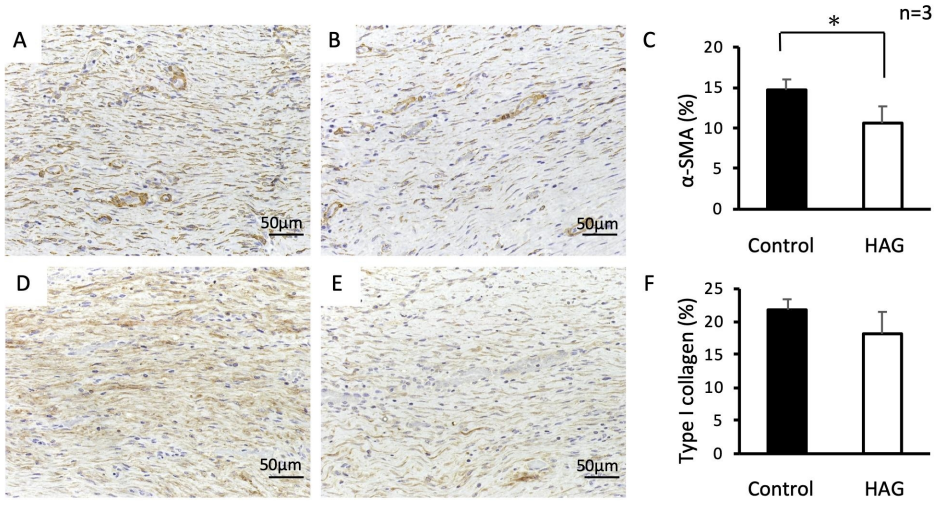

A
X50 500μm
B
C
D

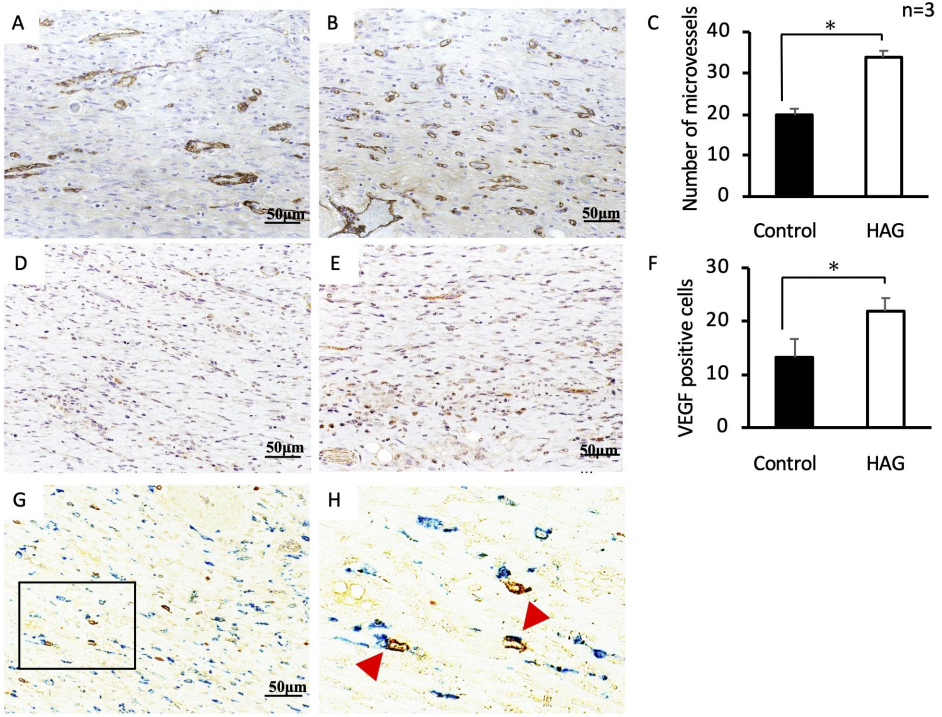

A
Evaluation the adhesiveness
Day0
ESD and
HAG shielding
(n = 1)
Day4
ESD and
HAG shielding
Day7
Endoscopic examination
and Sacrificed
Histological analysis
B
Evaluation the effect of HAG sheets
on post-ESD ulcer healing
Day0
ESD only
ESD and
HAG shielding
(n = 5)
Day7
Endoscopic examination
Day14
Endoscopic examination
and Sacrificed
Histological analysis
(n = 3)

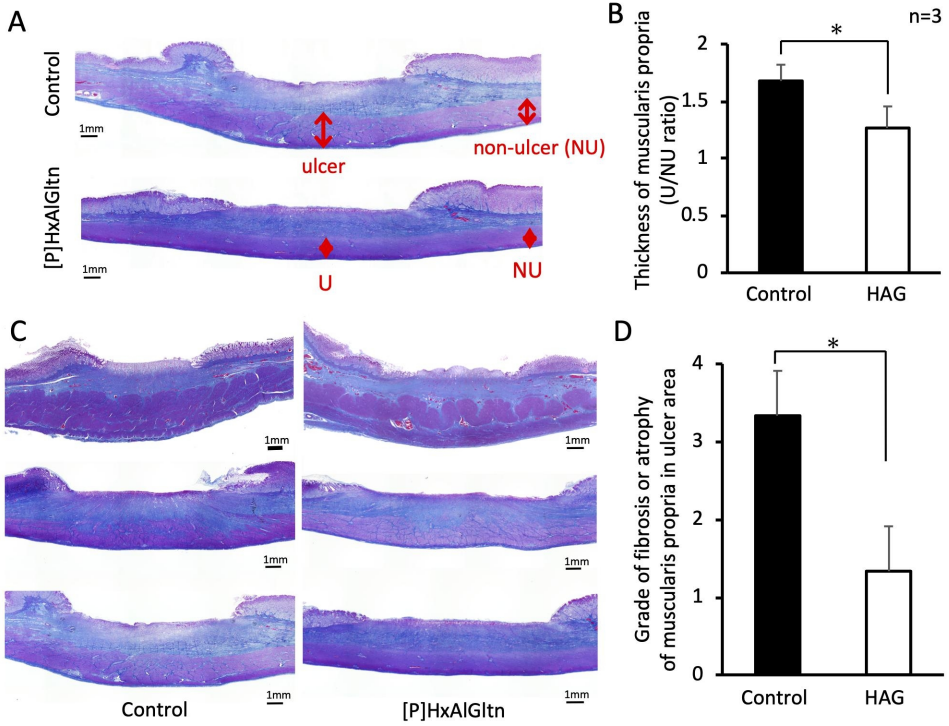

A
B
C
500μm
100μm
D
E
F
500μm
100μm

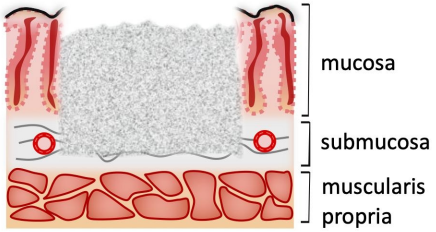

A
Day 0
Day 7
Day 14
B
800
600
400
200
0
Area of artificial ulcer (mm²)
Control (n=5)
HAG (n=5)
*
0
7
14
(day)
C
10
5
0
Number of ulcer folds
n=5
Control
HAG


A
B
C
n=3
*
500
400
300
200
100
0
Inflammatory cells
Control
HAG
50μm
D
E
F
20
15
10
5
0
MPO positive cells
G
H
I
120
100
80
60
40
20
Iba1 positive cells


A
B
C
D
E
F
50μm
50μm
50μm
50μm
n=3
*
α-SMA (%)
20
15
10
5
0
Control
HAG
Type I collagen (%)
25
20
15
10
5
0
Control
HAG

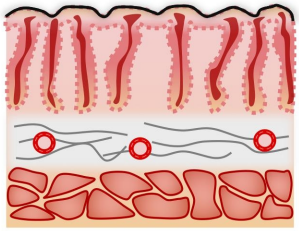

A
B
C
n=3
Number of microvessels
40
30
20
10
0
*
Control
HAG
50μm
D
E
F
VEGF positive cells
30
20
10
0
*
Control
HAG
G
H
50μm


A
Control
1mm
ulcer
non-ulcer (NU)
[P]HxAlGltn
1mm
U
NU
B
n=3
*
Thickness of muscularis propria (U/NU ratio)
2
1.5
1
0.5
0
Control
HAG
C
1mm
Control
[P]HxAlGltn
D
*
Grade of fibrosis or atrophy of muscularis propria in ulcer area
4
3
2
1
0
Control
HAG



Covering post-endoscopic submucosal dissection ulcers with a HAG sheet

Ulcer healing



HAG sheets possibly suppress excess scarring