



HOKKAIDO UNIVERSITY

Title	Preventive effect of mesenchymal stem cell culture supernatant on luminal stricture after endoscopic submucosal dissection in the rectum of pigs
Author(s)	Tsuda, Momoko; Ohnishi, Shunsuke; Mizushima, Takeshi et al.
Citation	Endoscopy, 50(10), 1001-1016 https://doi.org/10.1055/a-0584-7262
Issue Date	2018-10
Doc URL	https://hdl.handle.net/2115/73707
Rights	This is an Accepted Manuscript of an article published by Thieme Publishing Group in Endoscopy on October 2018, available online at https://www.thieme-connect.de/products/ejournals/abstract/10.1055/a-0584-7262
Type	journal article
File Information	Endoscopy50_1001.pdf



**Preventive effect of mesenchymal stem cell-culture
supernatant on luminal stricture after endoscopic submucosal
dissection in the rectum of pigs**

Momoko Tsuda,¹ Shunsuke Ohnishi,^{1,*} Takeshi Mizushima,¹ Hidetaka Hosono,¹

Kenichi Yamahara,² Marin Ishikawa,³ Satoshi Abiko,¹ Takehiko Katsurada,¹ Yuichi

Shimizu³ and Naoya Sakamoto¹

¹Department of Gastroenterology and Hepatology, Hokkaido University Graduate
School of Medicine, Sapporo, Japan.

²Department of Transfusion Medicine and Cell Therapy, Hyogo College of Medicine,
Nishinomiya, Japan.

³Division of Endoscopy, Hokkaido University Hospital, Sapporo, Japan.

Correspondence to: Dr. Shunsuke Ohnishi,

Department of Gastroenterology and Hepatology, Hokkaido University Graduate School

of Medicine, N15, W7, Kita-ku, Sapporo 060-8638, Japan.

Phone: +81-11-716-1161; Fax: +81-11-706-7867;

E-mail: sonishi@pop.med.hokudai.ac.jp

Abstract

Background and study aims: Mesenchymal stem cells (MSCs) are valuable in regenerative medicine, and MSC culture supernatant (MSC-CS) reportedly inhibits inflammation and fibrosis. We investigated whether colorectal luminal stricture develops after circumferential endoscopic submucosal dissection (ESD) in the colorectum and whether the development of luminal stricture could be prevented using MSC-CS enema.

Methods: In the first experiment, we performed circumferential ESD in the rectums or distal colons of pigs. We sacrificed the pigs on day 22 and measured the degree of luminal stricture. In the second experiment, we performed circumferential ESD in the rectums of pigs and administered MSC-CS gel or control gel enema after ESD for 4 days. We sacrificed the pigs on day 8 or 22 to measure the degree of luminal stricture and performed histological analysis.

Results: Severe luminal stricture was observed in the rectum but not in the distal colon. Moreover, fiber accumulation in submucosa and hypertrophy of the muscularis propria were observed in the rectum but not in the distal colon. The degree of luminal stricture in the rectum was significantly lower in the MSC-CS group than in the control group. Furthermore, MSC-CS attenuated myofibroblast activation and hypertrophy of the muscularis propria on day 22 and reduced inflammatory cell infiltration on day 8.

Conclusions: Luminal stricture after ESD developed only in the rectum due to the difference in myofibroblast activation and fiber accumulation. In addition, MSC-CS enema prevented luminal stricture after ESD, possibly by inhibiting the inflammatory reaction and fibrosis.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths worldwide [1]. Treatment options for CRC include endoscopic resection, chemotherapy, surgery, and radiotherapy. Endoscopic resection, such as endoscopic mucosal resection and endoscopic submucosal dissection (ESD), is preferred for early stage CRC and large colorectal polyps [2].

ESD for gastrointestinal neoplasms has been widely adopted in the last decade because it enables *en bloc* resection of any tumor size and location and excellent long-term outcomes [3-5]. However, ESD often causes stricture when widely dissected [6,7]. Esophageal stricture occurs when over three-fourths of the circumference is dissected and sometimes requires multiple balloon dilation sessions, reducing the quality of life for patients [7]. However, whether ESD causes postoperative stricture in the colorectum remains controversial [6,8,9].

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into a variety of lineages, including bone, cartilage, or fat, and are present in adult tissues [10]. At present, MSCs have been investigated in regenerative medicine due to their differentiation ability and potential to improve damaged tissues through the secretion of a variety of growth factors and anti-inflammatory molecules [11,12].

The fetal membrane consists of amnion and chorion, which envelop the developing

1
2
3
4
5
6 fetus. Although human fetal membrane is usually discarded as medical waste after
7
8
9 delivery, fetal tissues have been found to be rich sources of MSCs [13,14]. We
10
11 previously demonstrated that systemic administration of amnion-derived MSCs
12
13 improved the condition of rats with chemically induced severe colitis [15,16], radiation
14
15 proctitis [17], acute and chronic pancreatitis [18], and liver fibrosis [19], possibly
16
17 through secretory factors from the transplanted MSCs. In addition, we recently reported
18
19 that oral administration of MSC culture supernatant (MSC-CS) prevented luminal
20
21 stricture after semi-circumferential ESD in the esophagus of pigs [20].
22
23
24
25
26
27

28 Thus, the aim of this study was to investigate whether luminal stricture develops after
29
30 ESD in the colorectum of large animals and to examine the effects of MSC-CS enema
31
32 on the prevention of luminal stricture after circumferential ESD.
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Material and methods

Animals

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.

Isolation and expansion of human amnion-derived MSCs

The Medical Ethics Committee of Hokkaido University Graduate School of Medicine, Sapporo, Japan approved this study, and all pregnant women gave written informed consent. The human fetal membrane was obtained during cesarean deliveries, and the amnion was separated from the chorion by peeling. MSCs were isolated and expanded as described previously [20].

We previously confirmed that cultured MSCs are multipotent and express surface markers, such as CD44, CD73, CD90, and CD105 but not hematopoietic markers [20], which are characteristic of MSCs [21].

Preparation of MSC-CS gel

MSC-CS was prepared as described previously [20], and mixed with 2% carboxymethyl

cellulose (Wako Pure Chemical Industries, Osaka, Japan) on the day before ESD and stored at 4°C overnight. Serum-free minimal essential medium (MEM) α (DS Pharma Biomedical, Osaka, Japan) mixed with 2% carboxymethyl cellulose was used as a control (standard medium (SM)) gel.

ESD

The pigs were anesthetized as described previously [20]. On the day before ESD, the pigs were pre-treated with intragastric administration of 300 mL polyethylene glycol electrolyte lavage solution (Niflec; EA Pharma, Tokyo, Japan) using an endoscope (GIF-Q240; Olympus, Tokyo, Japan). The next day, ESD was performed under anesthesia, continuous monitoring of the heart rate, three-lead electrocardiography and oxygen saturation (Nihon Kohden, Tokyo, Japan). A single-channel gastrointestinal endoscope (GIF-Q240) with a transparent attachment hood fitted to the tip (Top Corporation, Tokyo, Japan) was used. A 25G needle (Top Corporation) was used to inject a 2:1 mixture of 10% glycerine solution (Glyceol; Chugai Pharmaceutical, Tokyo, Japan) and 80 mg/mL hyaluronic acid (Mucoup; Seikagaku Corporation, Tokyo, Japan) into the submucosal layer before mucosal and submucosal cutting. After injection, a circumferential incision was made using a flush knife BT (DK2620J-B15S-; Fujifilm,

Tokyo, Japan) and an insulation tipped nano knife (KD-612Q; IT nano knife) (Olympus).

Submucosal dissection was then performed using an IT nano knife, as described previously [20]. All ESD procedures were performed by one endoscopist (M.T.) who had experience with >100 human ESD cases. For postoperative care, all pigs were given liquid starting from the day after ESD and then solids on the following days.

Experiment 1: Degree of post-ESD luminal stricture at the rectum or distal colon

ESD was performed in the rectum (2–7 cm from the dentate line, n = 4) or distal colon (10–15 cm from the dentate line, n = 4) of the pigs (Fig. 1A, upper). Enema was not administered in both groups. Colonoscopy was performed using a gastrointestinal endoscope (GIF-Q240) on days 8, 15, and 22, and the pigs were sacrificed on day 22, immediately after endoscopic observation (Fig. 1A, lower).

Experiment 2: Effect of MSC-CS gel enema on the prevention of post-ESD luminal stricture in the rectum

To evaluate the effect of the MSC-CS gel on the prevention of luminal stricture formation after ESD in the rectum, we created two groups: an SM group and an MSC-CS group (n = 3 in each group, Fig. 1B, upper). All pigs were sacrificed on day 22.

Next, we created another SM group and MSC-CS group to assess the effects of the MSC-CS gel on acute reactions after ESD (n = 3 in each group) and sacrificed the pigs on day 8 (Fig. 1B, lower). We assigned the first three pigs to the SM group and the latter three to the MSC-CS group in these experiments. For the MSC-CS group, 50 mL of MSC-CS gel was applied through an 18F tube (Terumo, Tokyo, Japan) onto the wound bed of the rectum immediately after ESD (day 1). The same amount of MSC-CS gel was injected as an enema through an 18F tube once a day from days 2–4 under anesthesia with an intramuscular injection of midazolam (20 mg; Astellas, Tokyo, Japan) and buprenorphine hydrochloride (0.2 mg; Otsuka Pharmaceutical, Tokyo, Japan). A 50 mL aliquot of SM gel enema was used in the SM group.

Assessment of the degree of luminal stricture after ESD

All pigs were sacrificed on day 8 or day 22 by intravenous injection of 20 mL of 15% potassium chloride (Terumo) after general anesthesia. The abdomen was incised, and abdominoperianal resection of the rectum was performed. The resected rectum was immediately placed on a corkboard and fixed with pins. The degree of luminal stricture at the lesion site was calculated according to the following formula as described previously with a minor modification (Fig. 1C) [22]:

Degree of luminal stricture (%) = [1 – (length of short axis at site of maximal

constriction

/ length of short axis at a normal mucosal site on proximal side)] × 100.

We defined severe luminal stricture when endoscope (GIF-Q240) could not pass through the ESD site. Substituting this to the luminal stricture, more than 40% of the luminal stricture could not pass the endoscope (GIF-Q240).

Histological and immunohistochemical examination

The rectum was fixed in 40 g/L of formaldehyde saline, embedded in paraffin and cut into 5-μm sections. Tissue sections were stained with hematoxylin and eosin (H&E), and Masson's trichrome staining was performed to examine the accumulation of collagen fibers. Three fields that vertically crossed the short axis of the ESD site from each pig were randomly selected and photographed. A line was drawn between both ends of the muscularis mucosae and a vertical line was drawn from the middle of this line (Fig. 1D). The thickness of each layer from the post-ESD pigs as well as the normal pigs (n = 3) was measured (NDP View2 software; Hamamatsu, Japan) in a blinded manner.

The tissue sections were stained with anti-α-smooth muscle actin (α-SMA) antibody

(Sigma-Aldrich, St. Louis, Missouri, USA), a marker for myofibroblasts, anti-CD107a antibody (AbD Serotec, Kidlington, UK), a marker for macrophages, anti-CD31 antibody (Santa Cruz Biotechnology, Dallas, Texas, USA), a marker for endothelial cells, and with anti-myeloperoxidase (MPO) antibody (Thermo Scientific, Waltham, Massachusetts, USA), a marker for neutrophils. Nine random submucosa fields from each pig were photographed (high-powered fields (HPF), $\times 400$) and the stained cells were counted in a blinded manner.

Statistical analysis

Since our research is the first to assess colorectal stricture in pigs, we had no data to calculate sample size in advance. Therefore, we first aimed to perform each experiment with $n = 3$ in each group and performed post hoc power analysis to verify the sufficient sample size. As for Experiment 1, we estimated that $n = 4$ in each group was sufficient as a result of the analysis. Data were expressed as the mean (standard deviation).

Parameters in the two groups were compared by performing an unpaired t-test.

Differences were considered statistically significant at $P < 0.05$. All analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, California, USA).

Results

The rectum, but not the distal colon, was susceptible to luminal stricture after ESD

First, we performed ESD in the rectum or distal colon without the administration of enemas, and observed the changes in the lumen using an endoscope every week for 3 weeks. Luminal narrowing was first observed in the rectum group on day 8, and the endoscope could not pass through the rectum on days 8, 15, and 22; however, this did not occur in the distal colon group (Fig. 2A). We sacrificed the pigs on day 22, excised the colorectum and then opened it longitudinally. Although we performed ESD with a 5-cm long axis, the scar area was markedly contracted to <3 mm of the long axis in both groups (Fig. 2B). However, severe luminal stricture was observed only in the rectum group but not the distal colon group (degree of luminal stricture: 60.7% (16.8) vs. 16.1% (12.6), $P < 0.01$, $n = 4$ in each group, Fig. 2C).

Histological analysis of the colorectum after ESD

We next performed histological analysis of the colorectum removed on day 22. H&E staining demonstrated that the colorectal wall was markedly thicker in the rectum group, with excessive granulation and hypertrophy of the muscularis propria (Fig. 3A). On the contrary, in the distal colon group, granule formation was very small, and hypertrophy of the muscularis propria was barely observed, although the scar area was markedly

1
2
3
4
5
6 contracted. Masson's trichrome staining demonstrated that ESD had caused severe
7
8 fibrotic change in the rectum group, and the fiber accumulation extended throughout the
9
10 muscularis propria (Fig. 3B). In contrast, fiber accumulation was limited to the
11
12 submucosal layer in the distal colon group. We then measured the thickness of each
13
14 layer of the colorectum after ESD and compared the results with those in normal tissue.
15
16
17 The increases in the thickness of the submucosa, circular muscle, and longitudinal
18
19 muscle in the rectum were significantly greater than those in the distal colon (Table 1).
20
21
22 Immunohistochemical examination demonstrated that the numbers of activated
23
24 myofibroblasts (Fig. 3C) and infiltrated neutrophils (Fig. 3D) but not macrophages (Fig.
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
3E) were significantly higher in the rectum than in the distal colon (Table 1).

Effect of MSC-CS gel enema on the prevention of luminal stricture after circumferential ESD in the rectum

42 Because severe luminal stricture was observed only in the rectum, we next investigated
43
44 the effect of MSC-CS gel enema on the prevention of luminal stricture after
45
46
47 circumferential ESD in the rectum. MSC-CS gel was administered in the rectum once a
48
49
50 day from days 1–4. We performed endoscopy and sacrificed the pigs on day 22. The
51
52
53
54
55
56
57
58
59
60
endoscope easily passed through the post-ESD scar in the MSC-CS group but not in the

SM group (Fig. 4A). Accordingly, the degree of luminal stricture was significantly lower in the MSC-CS group than in the SM group (14.7% (0.6) vs. 63.9% (27.4), $P < 0.05$, $n = 3$ in each group, Figs. 4B and 4C).

Histological analysis of the rectum after administration of MSC-CS gel enema

We next performed a histological analysis of the rectum on day 22. H&E staining demonstrated that the colorectal wall was markedly thicker in the SM group with hypertrophy of the muscularis propria (Fig. 5A), consistent with the previous experimental result (Fig. 3A). However, hypertrophy of the muscularis propria was barely observed in the MSC-CS group, although the scar area was markedly contracted. Masson's trichrome staining demonstrated that fiber accumulation extended throughout the muscularis propria in the SM group but was reduced and limited to the submucosa in the MSC-CS group (Fig. 5B). We next measured the thickness of the rectal wall and each layer of the rectum. The increases in the thickness of the rectal wall, submucosa, circular muscle, and longitudinal muscle were significantly suppressed by applying the MSC-CS gel (Table 2). However, the fiber thickness in the muscularis propria was not significantly decreased by the MSC-CS gel. Immunohistochemical analysis demonstrated that the numbers of activated myofibroblasts (Fig. 5C) and infiltrated

neutrophils (Fig. 5D) were significantly decreased by the MSC-CS gel (Table 2).

However, the number of infiltrated macrophages (Fig. 5E) and capillary density (Fig. 5F) showed no changes after the application of the MSC-CS gel (Table 2).

Effect of MSC-CS gel enema on the acute reaction after circumferential ESD in the rectum

Because the administration of MSC-CS gel enema from days 1–4 was effective for preventing luminal stricture after rectal ESD, we hypothesized that the MSC-CS gel affects wound healing after ESD in the acute phase. Therefore, we further observed the acute wound healing phase. We administered MSC-CS gel once a day from days 1–4 after ESD, performed endoscopy, and sacrificed the pigs on day 8. There was a lesser degree of luminal stricture in the MSC-CS group than in the SM group (Fig. 6A). The scar area was markedly contracted but the scar length remained approximately 1 cm on day 8 in both groups (Fig. 6B). The degree of luminal stricture was significantly lower in the MSC-CS group than in the SM group (31.2% (5.5) vs. 56.5% (3.4), $n = 3$ in each group, $P < 0.01$, Fig. 6C).

Histological analysis of the rectum after administration of MSC-CS gel enema in the acute phase

We next performed a histological analysis of the rectum on day 8. H&E staining demonstrated that the circular muscle disappeared after the infiltration of inflammatory cells, and the colorectal wall was markedly thickened with hypertrophy of the muscularis propria in the SM group (Fig. 7A). However, the circular muscle remained, and hypertrophy of the muscularis propria was barely observed in the MSC-CS group. Masson's trichrome staining revealed that fiber accumulation extended throughout the muscularis propria in the SM group. However, fiber accumulation was reduced and limited to the submucosal layer by the application of the MSC-CS gel on day 8 (Fig. 7B). The thickness of the rectal wall and longitudinal muscle was significantly reduced on day 8 after the application of the MSC-CS gel (Table 3). Immunohistochemical analysis demonstrated that the numbers of activated myofibroblasts (Fig. 7C) and infiltrated neutrophils (Fig. 7D) and macrophages (Fig. 7E) were significantly decreased on day 8 after the application of the MSC-CS gel (Table 3). However, there was no significant change in the capillary density (Fig. 7F and Table 3).

Discussion

In this study, we found that luminal stricture developed in the rectum, but not in the distal colon after ESD, and was associated with fiber accumulation in submucosa and hypertrophy of the muscularis propria. Next, we demonstrated that an MSC-CS gel enema prevented luminal stricture development after ESD in the rectum and inhibited inflammatory cell infiltration, myofibroblast activation, fiber accumulation, and hypertrophy of the muscularis propria.

Colorectal stricture may develop after any condition that causes scarring to the wall of the colorectum, such as inflammatory bowel disease, tuberculosis, and colorectal anastomoses [23]. Submucosal fibrosis is considered the main cause of stricture in ulcerative colitis [24,25], while hyperplasia of the submucosa and hypertrophy of the muscularis propria are the most significant histopathological features in the stricturing intestine in Crohn's disease [26]. It has been reported that intestinal fibrosis is a major complication of Crohn's disease and caused by contribution of epithelial to mesenchymal transition (EMT) [27]. EMT is a complex and dynamic phenomenon accompanied by epithelial cells acquiring mesenchymal characteristic proteins including α -SMA [28]. However, these strictures in inflammatory bowel disease are considered to be the result of chronic inflammation [29,30]. In the present study,

1
2
3
4
5
6 hypertrophy of the muscularis propria, especially the circular muscle, was observed in
7
8 the rectum group but not in the distal colon group. Moreover, since we have detected
9
10 increased number of α -SMA positive myofibroblasts in the cases with stricture, it is
11
12 possible that EMT is associated with the occurrence of the post-ESD stricture. In
13
14 addition, excessive infiltration of neutrophils, myofibroblast activation, and fibrosis was
15
16 observed in the rectum but not in the distal colon after ESD. Although the underlying
17
18 mechanisms remain to be elucidated, it appears that the inflammatory reaction is
19
20 stronger in the rectum than in the distal colon and contributes to stricture formation.
21
22
23
24
25
26
27

28 There have been several reports on stricture after ESD of large colorectal tumors
29
30 [6,8,9,31,32]. Most of the strictures occurred in the rectum, and only 1 case occurred in
31
32 the colon [31]. Stricture occurs in cases with more than 90% of circumferential mucosal
33
34 defects in ESD. Most of the strictures were diagnosed within 3 months after ESD during
35
36 surveillance endoscopy without any symptoms. Only two patients complained about
37
38 symptoms related to stricture: one patient complained of constipation, and the other of
39
40 abdominal discomfort and severe constipation [6,31]. Strictures were treated with
41
42 endoscopic balloon dilation and/or steroid medication, and no cases required surgery to
43
44 control the stricture. In the present study, luminal stricture occurred only in the rectum,
45
46 not in the distal colon, which supported the results of previous clinical studies. However,
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 it has been suggested that even after the resection of large tumors, the larger lumen and
7
8 possible self-dilation by stool may reduce the risk of post-ESD stricture in the
9
10 colorectum [32]. In the present study, because all pigs received the same food and
11
12 defecated loose stool, it appears that self-dilation by stool occurs to the same extent or
13
14 not at all in all pigs.
15
16
17
18

19
20 The present study demonstrated that an MSC-CS gel enema can prevent luminal
21
22 stricture after circumferential ESD in the rectum. It has been reported that steroid
23
24 treatment prevents esophageal stricture by modifying the appearance of the
25
26 myofibroblasts [33]. However, the appearance of the myofibroblasts did not change in
27
28 our study. MSC-CS gel reduced the infiltration of neutrophils and macrophages and the
29
30 activation of myofibroblasts in the acute phase as well as the infiltration of neutrophils
31
32 and myofibroblast activation in the following weeks. MSC-CS gel also reduced fiber
33
34 accumulation and hypertrophy of the muscularis propria as the key mechanisms for
35
36 preventing luminal stricture. We recently demonstrated that human amnion-derived
37
38 MSCs decrease the inflammatory response in rats with severe colitis, possibly through
39
40 the suppression of macrophage activity [15]. Further studies are required to clarify the
41
42 underlying mechanisms.
43
44
45
46
47
48
49
50
51
52

53 We recently reported that oral administration of non-gel MSC-CS (i.e., without
54
55
56
57
58
59
60

1
2
3
4
5
6 carboxymethyl cellulose) was ineffective for the prevention of esophageal stricture after
7
8
9 ESD [20]. The viscosity of the MSC-CS gel appeared to be suitable for the retention of
10
11 MSC-CS on the wound surface, although the vast majority of the MSC-CS passed
12
13
14 through the wound area due to esophageal peristalsis and only a small amount of
15
16
17 MSC-CS gel remained on the wound surface. In the present study, the gel was colored
18
19
20 in pink and was easy to recognize once they were evacuated. For the first 24 hours after
21
22
23 ESD until administration of second gel, all pigs did not evacuate. Even after the pigs
24
25
26 gradually started to evacuate, we did not recognize the pink colored gel in feces. This
27
28
29 suggests that the gel constantly contacted to the rectum during 4 days of administration
30
31
32 period.

33
34 There were several limitations to the present study. This is an animal study with a
35
36
37 small number of pigs. Because of the difference in species, the wound healing process
38
39
40 observed in this study may not be extrapolated to humans. In addition, we did not
41
42
43 investigate luminal stricture after ESD in the proximal colon; however, it has been
44
45
46 reported that pig anatomy is unsuitable for ESD beyond 20 cm from the anus because
47
48
49 the muscularis propria becomes extremely thin [34].

50
51 In conclusion, luminal stricture after ESD occurs only in the rectum but not in the
52
53
54 distal colon in pigs, which may be explained by the differences in myofibroblast
55
56
57
58
59
60

1
2
3
4
5
6 activation and fiber accumulation, followed by fibrosis in submucosa and hypertrophy
7
8
9 in the muscularis propria. In addition, MSC-CS gel enema prevented luminal stricture
10
11
12 after circumferential ESD in the rectum, possibly through inhibition of the acute
13
14
15 inflammatory reaction after ESD. Because these preliminary results may not be
16
17
18 translated to humans, further studies including clinical trials will be required.
19
20
21

22 **Acknowledgments**

23
24
25 This study was funded by a Grant-in-Aid (C) from the Japan Society for the Promotion
26
27
28 of Science (JSPS, 16K09300) and by the Translational Research Network Programme
29
30
31 of the Japan Agency for Medical Research and Development (AMED).
32
33
34
35
36

37 **Competing interests**

38
39 The authors declare no competing interests.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

References

1. Arnold M, Sierra MS, Laversanne M et al. Global patterns and trends in colorectal cancer incidence and mortality. *Gut* 2017; 66: 683-691
2. Saunders BP, Tsiamoulos ZP. Endoscopic mucosal resection and endoscopic submucosal dissection of large colonic polyps. *Nat Rev Gastroenterol Hepatol* 2016; 13: 486-496
3. Toyonaga T, Man-i M, East JE et al. 1,635 Endoscopic submucosal dissection cases in the esophagus, stomach, and colorectum: complication rates and long-term outcomes. *Surg Endosc* 2013; 27: 1000-1008
4. Mizushima T, Kato M, Iwanaga I et al. Technical difficulty according to location, and risk factors for perforation, in endoscopic submucosal dissection of colorectal tumors. *Surg Endosc* 2015; 29: 133-139
5. Kosaka T, Endo M, Toya Y et al. Long-term outcomes of endoscopic submucosal dissection for early gastric cancer: a single-center retrospective study. *Dig Endosc* 2014; 26: 183-191
6. Ohara Y, Toyonaga T, Tanaka S et al. Risk of stricture after endoscopic submucosal dissection for large rectal neoplasms. *Endoscopy* 2016; 48: 62-70
7. Ono S, Fujishiro M, Niimi K et al. Predictors of postoperative stricture after

- 1
2
3
4
5
6 esophageal endoscopic submucosal dissection for superficial squamous cell
7
8 neoplasms. Endoscopy 2009; 41: 661-665
9
10
11 8. Jung DH, Youn YH, Kim JH et al. Endoscopic submucosal dissection for
12
13 colorectal lateral spreading tumors larger than 10 cm: is it feasible? Gastrointest
14
15 Endosc 2015; 81: 614-620
16
17
18
19
20 9. Abe S, Sakamoto T, Takamaru H et al. Stenosis rates after endoscopic
21
22 submucosal dissection of large rectal tumors involving greater than three
23
24 quarters of the luminal circumference. Surg Endosc 2016; 30: 5459-5464
25
26
27
28 10. Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human
29
30 mesenchymal stem cells. Science 1999; 284: 143-147
31
32
33
34 11. Meirelles Lda S, Fontes AM, Covas DT et al. Mechanisms involved in the
35
36 therapeutic properties of mesenchymal stem cells. Cytokine Growth Factor Rev
37
38 2009; 20: 419-427
39
40
41
42 12. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease.
43
44 Nat Rev Immunol 2008; 8: 726-736
45
46
47
48 13. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C et al. Isolation of
49
50 mesenchymal stem cells of fetal or maternal origin from human placenta. Stem
51
52 Cells 2004; 22: 1338-1345
53
54
55
56
57
58
59
60

14. Alviano F, Fossati V, Marchionni C et al. Term amniotic membrane is a high throughput source for multipotent mesenchymal stem cells with the ability to differentiate into endothelial cells in vitro. *BMC Dev Biol* 2007; 7: 11
15. Onishi R, Ohnishi S, Higashi R et al. Human amnion-derived mesenchymal stem cell transplantation ameliorates dextran sulfate sodium-induced severe colitis in rats. *Cell Transplant* 2015; 24: 2601-2614
16. Miyamoto S, Ohnishi S, Onishi R et al. Therapeutic effects of human amnion-derived mesenchymal stem cell transplantation and conditioned medium enema in rats with trinitrobenzene sulfonic acid-induced colitis. *American Journal of Translational Research* 2017; 9: 940-952
17. Ono M, Ohnishi S, Honda M et al. Effects of human amnion-derived mesenchymal stromal cell transplantation in rats with radiation proctitis. *Cytotherapy* 2015; 17: 1545-1559
18. Kawakubo K, Ohnishi S, Fujita H et al. Effect of fetal membrane-derived mesenchymal stem cell transplantation in rats with acute and chronic pancreatitis. *Pancreas* 2016; 45: 707-713
19. Kubo K, Ohnishi S, Hosono H et al. Human amnion-derived mesenchymal stem cell transplantation ameliorates liver fibrosis in rats. *Transplant Direct* 2015; 1:

e16

20. Mizushima T, Ohnishi S, Hosono H et al. Oral administration of conditioned medium obtained from mesenchymal stem cell culture prevents subsequent stricture formation after esophageal submucosal dissection in pigs. *Gastrointest Endosc* 2017; 86: 542-552 e541
21. Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; 8: 315-317
22. Honda M, Hori Y, Nakada A et al. Use of adipose tissue-derived stromal cells for prevention of esophageal stricture after circumferential EMR in a canine model. *Gastrointest Endosc* 2011; 73: 777-784
23. Garcea G, Sutton CD, Lloyd TD et al. Management of benign rectal strictures: a review of present therapeutic procedures. *Dis Colon Rectum* 2003; 46: 1451-1460
24. De Dombal FT, Watts JM, Watkinson G et al. Local complications of ulcerative colitis: stricture, pseudopolyposis, and carcinoma of colon and rectum. *Br Med J* 1966; 1: 1442-1447
25. Rieder F, Brenmoehl J, Leeb S et al. Wound healing and fibrosis in intestinal

- disease. Gut 2007; 56: 130-139
26. Chen W, Lu C, Hirota C et al. Smooth muscle hyperplasia/hypertrophy is the most prominent histological change in crohn's fibrostenosing bowel strictures: A semiquantitative analysis by using a novel histological grading scheme. Journal of Crohn's & Colitis 2017; 11: 92-104
27. Scharl M, Huber N, Lang S et al. Hallmarks of epithelial to mesenchymal transition are detectable in Crohn's disease associated intestinal fibrosis. Clinical and translational medicine 2015; 4: 1
28. Lee JM, Dedhar S, Kalluri R et al. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 2006; 172: 973-981
29. Gordon IO, Agrawal N, Goldblum JR et al. Fibrosis in ulcerative colitis: mechanisms, features, and consequences of a neglected problem. Inflamm Bowel Dis 2014; 20: 2198-2206
30. Burke JP, Mulsow JJ, O'Keane C et al. Fibrogenesis in Crohn's disease. Am J Gastroenterol 2007; 102: 439-448
31. Hayashi T, Kudo SE, Miyachi H et al. Management and risk factor of stenosis after endoscopic submucosal dissection for colorectal neoplasms. Gastrointest Endosc 2017; 86: 358-369

- 1
2
3
4
5
6 32. Takahashi H, Hayashi Y, Sunada K et al. Complete resection of a 225-mm
7
8 circumferential rectosigmoid intramucosal carcinoma by endoscopic submucosal
9
10
11 dissection. Endoscopy 2016; 48 Suppl 1: E161-162
12
13
- 14 33. Nonaka K, Miyazawa M, Ban S et al. Different healing process of esophageal
15
16 large mucosal defects by endoscopic mucosal dissection between with and
17
18 without steroid injection in an animal model. BMC Gastroenterol 2013; 13: 72
19
20
21
22
- 23 34. Gostout CJ, Knipschild MA. Submucosal endoscopy with mucosal resection: a
24
25 hybrid endoscopic submucosal dissection in the porcine rectum and distal colon.
26
27
28 Gastrointest Endosc 2012; 76: 829-834
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure legends

Figure 1. Experimental protocol.

(A) Experiment 1: (Upper) Excised colorectum of the pig. Endoscopic submucosal dissection (ESD) was performed in the rectum (2–7 cm from the dentate line, $n = 4$) or in the distal colon (10–15 cm from the dentate line, $n = 4$). Scale bar, 10 mm. (Lower)

Experimental schedule for evaluating the degree of luminal stricture after ESD at the rectum or distal colon. ESD was performed in the distal colon or rectum of the pigs on day 1, and colonoscopy (CS) was performed on days 8, 15, and 22 to observe the luminal narrowing. All pigs were sacrificed on day 22.

(B) Experiment 2: Experimental schedule for evaluating the effect of mesenchymal stem cell-culture supernatant (MSC-CS) gel enema. ESD was performed in the rectum of the pigs on day 1, and 50 mL of MSC-CS gel enema was applied from days 1–4 ($n = 3$). Standard medium (SM) gel enema was applied as a control ($n = 3$). All pigs were sacrificed on day 22 (Upper) or on day 8 (Lower).

(C) Formula to calculate the degree of luminal stricture. Scale bar, 10 mm.

(D) Measurement of the thickness of each layer. A line was drawn between the ends of both muscularis mucosae and a vertical line was drawn from the middle of this line. The thickness of each layer was measured along this line. Scale bar, 1 mm.

Figure 2. Luminal stricture after endoscopic submucosal dissection (ESD) in the colorectum.

(A) Endoscopic findings from the colorectum after ESD.

(B) Excised colorectum on day 22. Scale bars, 10 mm.

(C) Degree of luminal stricture on day 22.

Values are the mean (standard deviation) of 4 animals per group. $**P < 0.01$ vs. rectum group.

Figure 3. Histological examination of the colorectum before and after endoscopic submucosal dissection (ESD) on day 22.

(A) Hematoxylin and eosin staining. Scale bars, 1 mm.

(B) Masson's trichrome staining. Scale bars, 1 mm.

(C) Expression of α -smooth muscle actin (α -SMA). Scale bars, 50 μ m.

(D) Expression of myeloperoxidase (MPO). Scale bars, 50 μ m.

(E) Expression of CD107a. Scale bars, 50 μ m.

HPF; high-powered fields ($\times 400$). Values are the mean (standard deviation) of 4 animals per group. $*P < 0.05$ vs. post-ESD rectum, respectively.

Figure 4. Effect of mesenchymal stem cell-culture supernatant (MSC-CS) gel enema on the prevention of luminal stricture in the rectum after circumferential endoscopic submucosal dissection (ESD) on day 22.

(A) Endoscopic findings from the rectum after ESD. (Left) Immediately after ESD, (Middle) Immediately after administration of gel enema, (Right) Immediately before sacrifice on day 22.

(B) Excised rectum on day 22. Scale bars, 10 mm.

(C) Degree of luminal stricture on day 22.

SM, standard medium. Values are the mean (standard deviation) of 3 animals per group.

* $P < 0.05$ vs. the SM group.

Figure 5. Histological analysis of the rectum after circumferential endoscopic submucosal dissection (ESD) and mesenchymal stem cell-culture supernatant (MSC-CS) gel enema on day 22.

(A) Hematoxylin and eosin staining. Scale bars, 1 mm.

(B) Masson's trichrome staining. Scale bars, 1 mm.

(C) Expression of α -smooth muscle actin (α -SMA). Scale bars, 50 μ m.

(D) Expression of myeloperoxidase (MPO). Scale bars, 50 μ m.

(E) Expression of CD107a. Scale bars, 50 μ m.

(F) Expression of CD31. Scale bars, 50 μ m.

SM, standard medium; HPF, high-powered fields ($\times 400$). Values are the mean (standard deviation) of 3 animals per group. $*P < 0.05$, $**P < 0.01$ vs. SM group, respectively.

Figure 6. Effect of mesenchymal stem cell culture supernatant (MSC-CS) gel enema after circumferential endoscopic submucosal dissection (ESD) on day 8.

(A) Endoscopic findings from the rectum.

(B) Excised rectum on day 8. Scale bars, 10 mm.

(C) Degree of luminal stricture on day 8.

SM, standard medium. Values are the mean (standard deviation) of 3 animals per group.

$**P < 0.01$ vs. SM group.

Figure 7. Histological analysis of the rectum after circumferential endoscopic submucosal dissection (ESD) and mesenchymal stem cell-culture supernatant (MSC-CS) gel enema on day 8.

(A) Hematoxylin and eosin staining. Scale bars, 1 mm.

(B) Masson's trichrome staining. Scale bars, 1 mm.

(C) Expression of α -smooth muscle actin (α -SMA). Scale bars, 50 μ m.

(D) Expression of myeloperoxidase (MPO). Scale bars, 50 μ m.

(E) Expression of CD107a. Scale bars, 50 μ m.

(F) Expression of CD31. Scale bars, 50 μ m.

SM, standard medium; HPF, high-powered fields ($\times 400$). Values are the mean (standard deviation) of 3 animals per group. $*P < 0.05$, $**P < 0.01$ vs. SM group, respectively.

Table 1. Histological analysis of the colorectum on day 22 after ESD

	Rectum (n = 4)	Distal colon (n = 4)	Analysis of variance <i>P</i> value
Relative change in thickness of submucosa, fold, mean (SD)	4.9 (1.9)	1.4 (0.3)	0.01
Relative change in thickness of circular muscle, fold, mean (SD)	5.9 (3.3)	1.3 (0.5)	0.03
Relative change in thickness of the fibers between circular and longitudinal muscles, fold, mean (SD)	5.9 (2.3)	2.8 (1.0)	0.05
Relative change in thickness of longitudinal muscle, fold, mean (SD)	4.0 (1.6)	1.4 (0.8)	0.03
α -SMA-positive activated myofibroblasts, cells/HPF, mean (SD)	182.9 (46.5)	100.6 (11.9)	0.01
MPO-positive neutrophils, cells/HPF, mean (SD)	68.5 (11.5)	38.1 (18.6)	0.03
CD107a-positive macrophages, cells/HPF, mean (SD)	8.2 (3.5)	6.0 (1.8)	0.32

SD, standard deviation; HPF, high-powered fields; α -SMA, α -smooth muscle actin;

MPO, myeloperoxidase

Table 2. Histological analysis of the rectum on day 22 after administration of MSC-CS

enema

	SM (n = 3)	MSC-CS (n = 3)	Analysis of variance <i>P</i> value
Wall thickness, mm, mean (SD)	4.5 (0.6)	3.1 (0.3)	0.02
Thickness of submucosa, mm, mean (SD)	0.8 (0.2)	0.4 (0.02)	0.02
Thickness of circular muscle, mm, mean (SD)	0.7 (0.1)	0.4 (0.1)	< 0.01
Thickness of the fibers between circular and longitudinal muscles, mm, mean (SD)	0.7 (0.4)	0.6 (0.1)	0.75
Thickness of longitudinal muscle, mm, mean (SD)	1.3 (0.1)	0.6 (0.1)	< 0.01
α -SMA-positive activated myofibroblasts, cells/HPF, mean (SD)	183.4 (20.7)	66.2 (27.3)	< 0.01
MPO-positive neutrophils, cells/HPF, mean (SD)	50.6 (23.2)	8.2 (0.6)	0.03
CD107a-positive macrophages, cells/HPF, mean (SD)	8.2 (4.1)	6.9 (3.4)	0.69
Capillary density, cells/HPF, mean (SD)	22.3 (5.4)	16.6 (1.7)	0.16

SD, standard deviation; HPF, high-powered fields; SM, standard medium; MSC-CS,

mesenchymal stem cell-culture supernatant, α -SMA, α -smooth muscle actin; MPO,

myeloperoxidase

Table 3. Histological analysis of the rectum on day 8 after administration of MSC-CS enema

	SM (n = 3)	MSC-CS (n = 3)	Analysis of variance <i>P</i> value
Wall thickness, mm, mean (SD)	5.2 (1.0)	3.4 (0.2)	0.04
Thickness of longitudinal muscle, mm, mean (SD)	1.5 (0.1)	0.7 (0.04)	< 0.01
α -SMA-positive activated myofibroblasts, cells/HPF, mean (SD)	96.3 (18.6)	46.1 (14.0)	0.02
MPO-positive neutrophils, cells/HPF, mean (SD)	144.3 (48.7)	55.5 (25.3)	0.04
CD107a-positive macrophages, cells/HPF, mean (SD)	35.4 (6.6)	12.2 (0.7)	< 0.01
Capillary density, cells/HPF, mean (SD)	24.1 (5.7)	14.9 (2.9)	0.07

SD, standard deviation; HPF, high-powered fields; SM, standard medium; MSC-CS, mesenchymal stem cell-culture supernatant, α -SMA, α -smooth muscle actin; MPO, myeloperoxidase

Figure 1

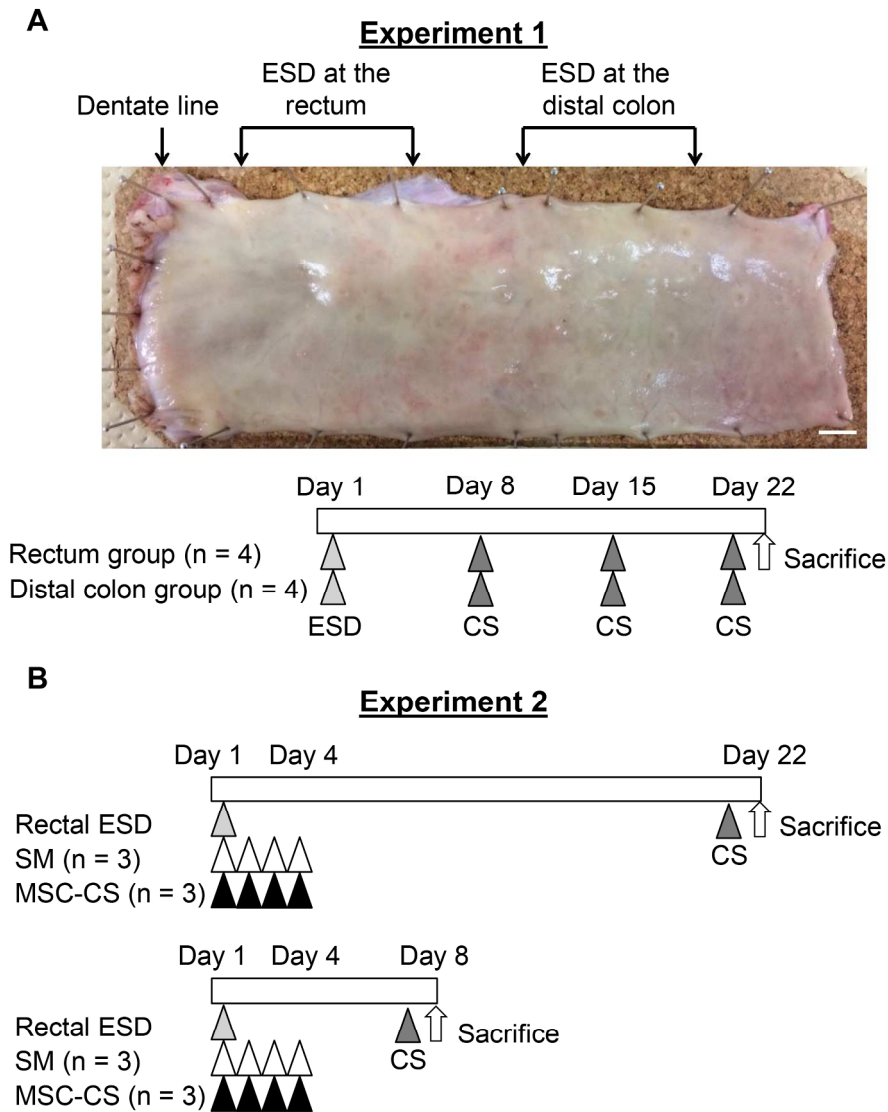
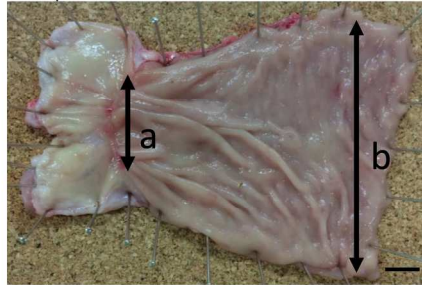


Figure 1. Experimental protocol. (A) Experiment 1: (Upper) Excised colorectum of the pig. Endoscopic submucosal dissection (ESD) was performed in the rectum (2–7 cm from the dentate line, n = 4) or in the distal colon (10–15 cm from the dentate line, n = 4). Scale bar, 10 mm. (Lower) Experimental schedule for evaluating the degree of luminal stricture after ESD at the rectum or distal colon. ESD was performed in the distal colon or rectum of the pigs on day 1, and colonoscopy (CS) was performed on days 8, 15, and 22 to observe the luminal narrowing. All pigs were sacrificed on day 22. (B) Experiment 2: Experimental schedule for evaluating the effect of mesenchymal stem cell-culture supernatant (MSC-CS) gel enema. ESD was performed in the rectum of the pigs on day 1, and 50 mL of MSC-CS gel enema was applied from days 1–4 (n = 3). Standard medium (SM) gel enema was applied as a control (n = 3). All pigs were sacrificed on day 22 (Upper) or on day 8 (Lower).

Figure 1

C

Dentate line



a: short axis at site of maximal constriction

b: short axis at a normal mucosal site on proximal side

$$\text{Degree of luminal stricture (\%)} = (1 - a / b) \times 100$$

D

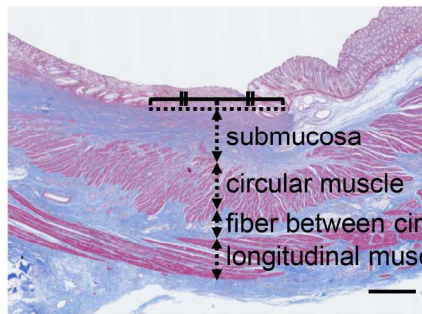


Figure 1. Experimental protocol.

(C) Formula to calculate the degree of luminal stricture. Scale bar, 10 mm.

(D) Measurement of the thickness of each layer. A line was drawn between the ends of both muscularis mucosae and a vertical line was drawn from the middle of this line. The thickness of each layer was measured along this line. Scale bar, 1 mm.

190x254mm (300 x 300 DPI)

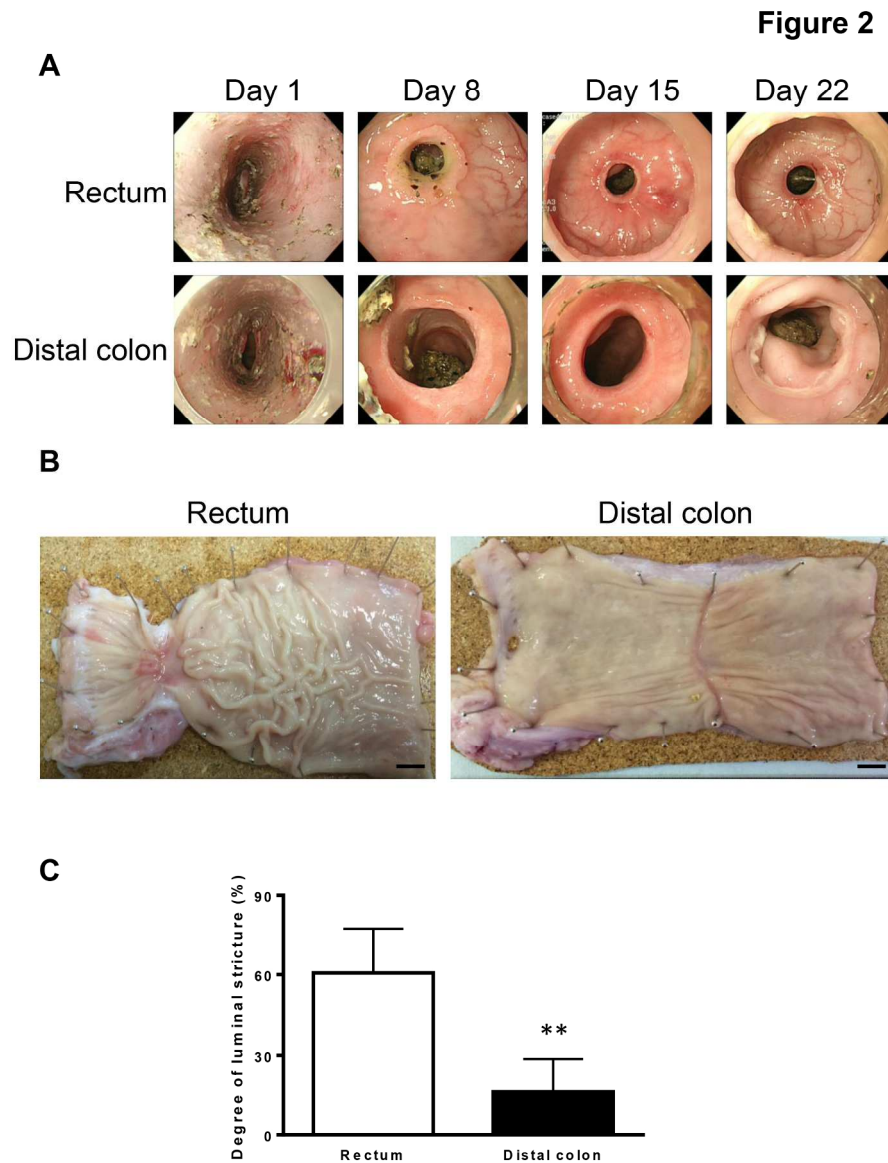


Figure 2. Luminal stricture after endoscopic submucosal dissection (ESD) in the colorectum.
(A) Endoscopic findings from the colorectum after ESD.
(B) Excised colorectum on day 22. Scale bars, 10 mm.
(C) Degree of luminal stricture on day 22.
Values are the mean (standard deviation) of 4 animals per group. **P < 0.01 vs. rectum group.

190x254mm (300 x 300 DPI)

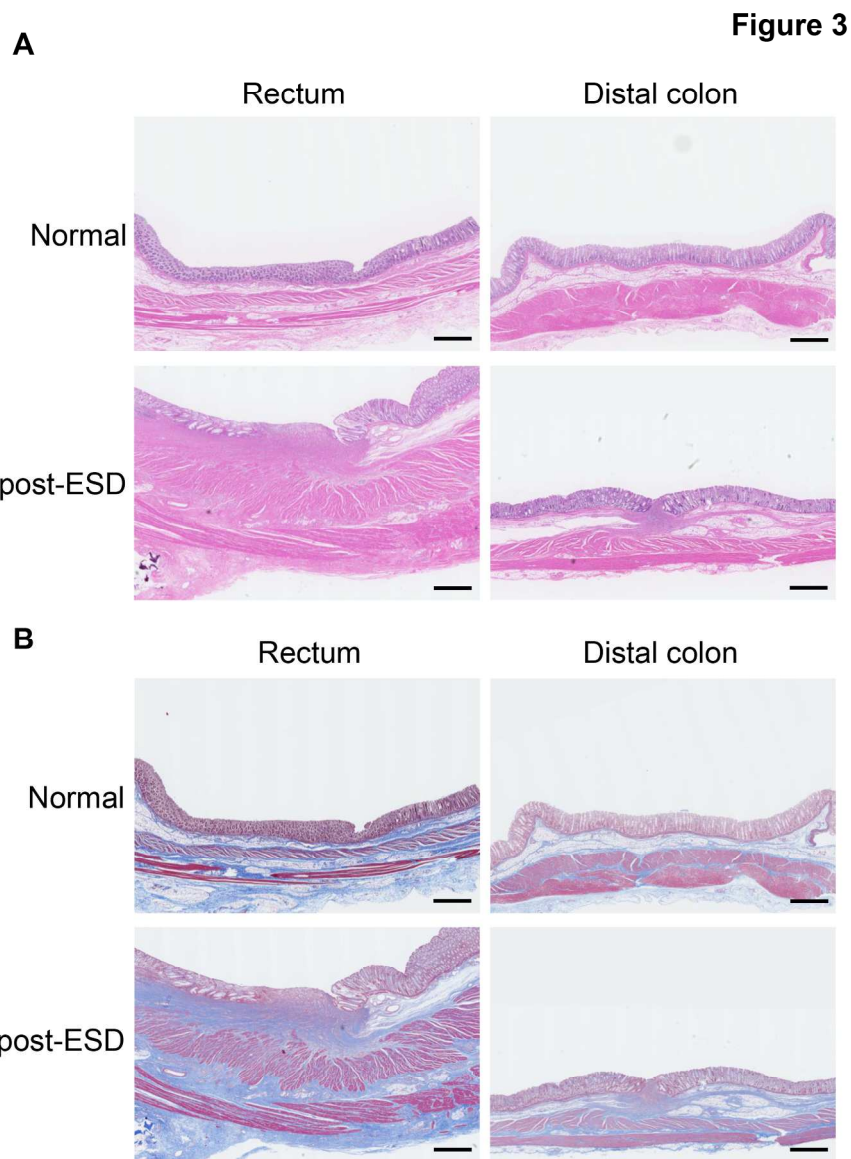


Figure 3. Histological examination of the colorectum before and after endoscopic submucosal dissection (ESD) on day 22.

(A) Hematoxylin and eosin staining. Scale bars, 1 mm.

(B) Masson's trichrome staining. Scale bars, 1 mm.

190x254mm (300 x 300 DPI)

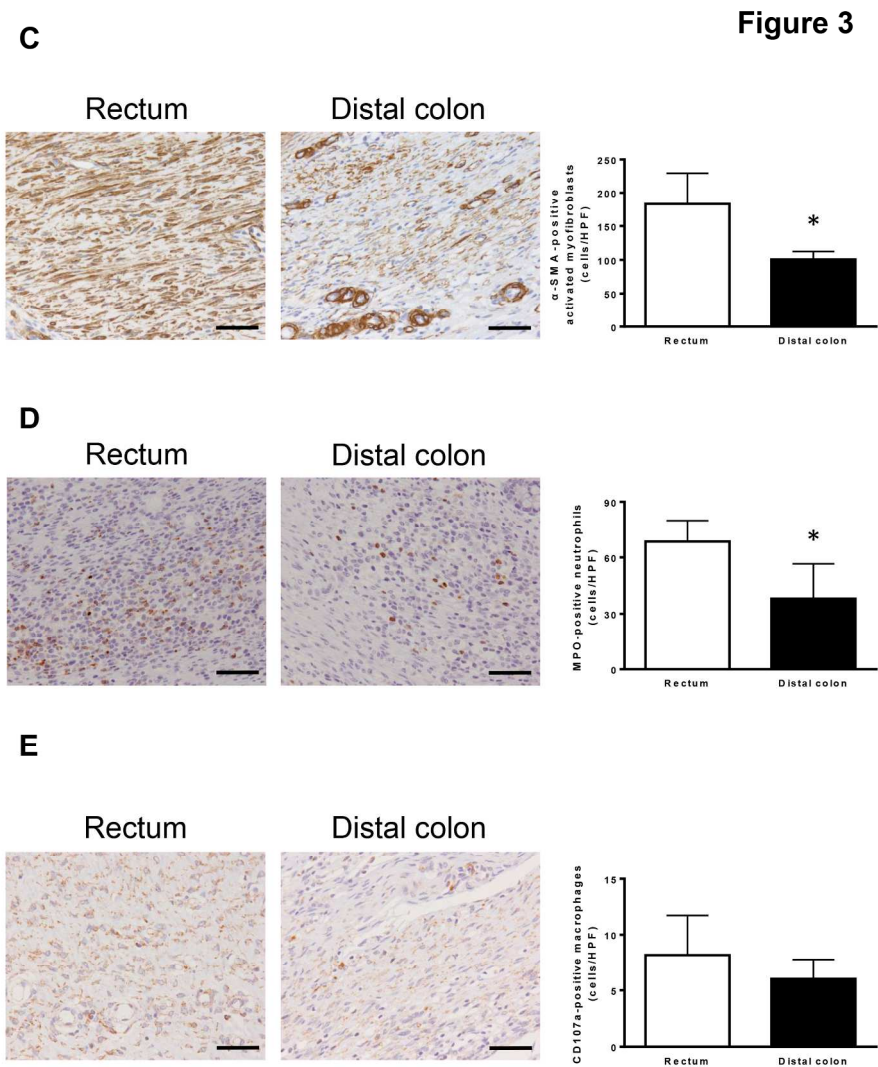


Figure 3. Histological examination of the colorectum before and after endoscopic submucosal dissection (ESD) on day 22. (C) Expression of α -smooth muscle actin (α -SMA). Scale bars, 50 μ m. (D) Expression of myeloperoxidase (MPO). Scale bars, 50 μ m. (E) Expression of CD107a. Scale bars, 50 μ m. HPF; high-powered fields ($\times 400$). Values are the mean (standard deviation) of 4 animals per group. *P < 0.05 vs. post-ESD rectum, respectively.

190x254mm (300 x 300 DPI)

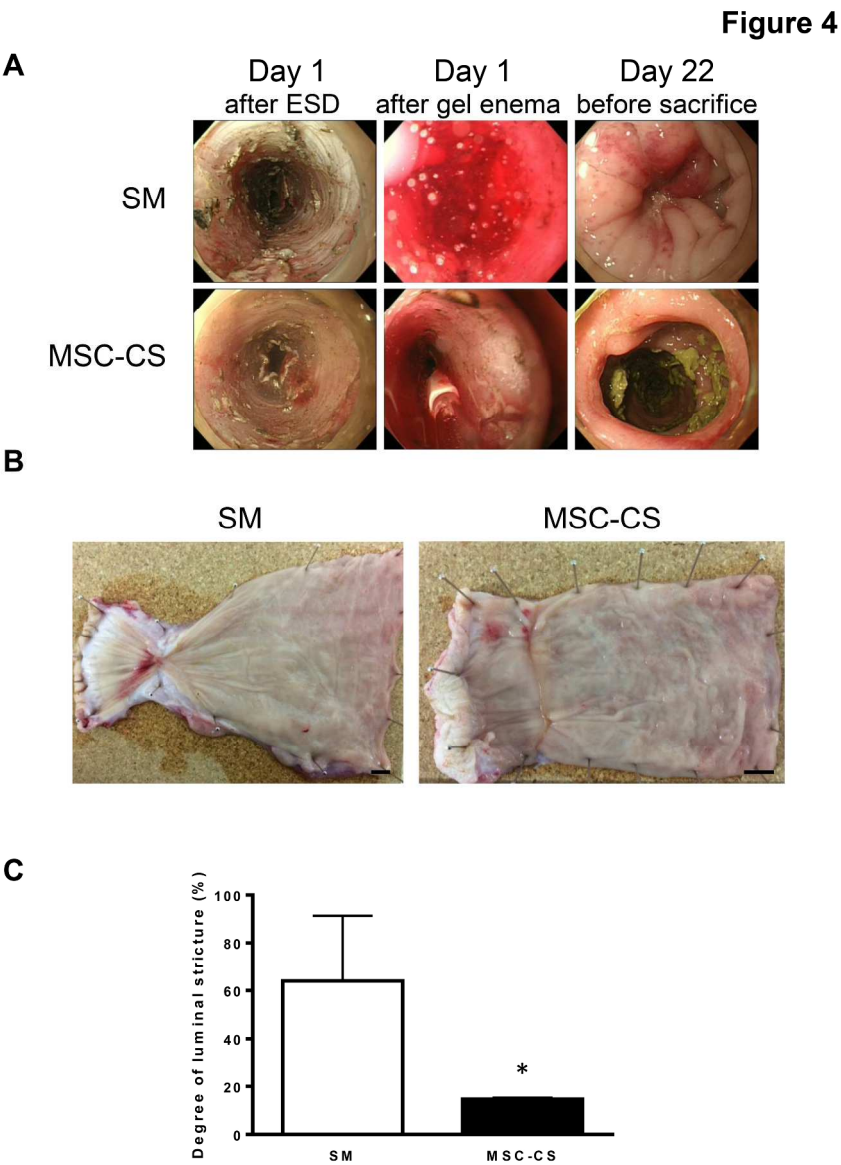


Figure 4. Effect of mesenchymal stem cell-culture supernatant (MSC-CS) gel enema on the prevention of luminal stricture in the rectum after circumferential endoscopic submucosal dissection (ESD) on day 22. (A) Endoscopic findings from the rectum after ESD. (Left) Immediately after ESD, (Middle) Immediately after administration of gel enema, (Right) Immediately before sacrifice on day 22. (B) Excised rectum on day 22. Scale bars, 10 mm. (C) Degree of luminal stricture on day 22. SM, standard medium. Values are the mean (standard deviation) of 3 animals per group. *P < 0.05 vs. the SM group.

254x338mm (300 x 300 DPI)

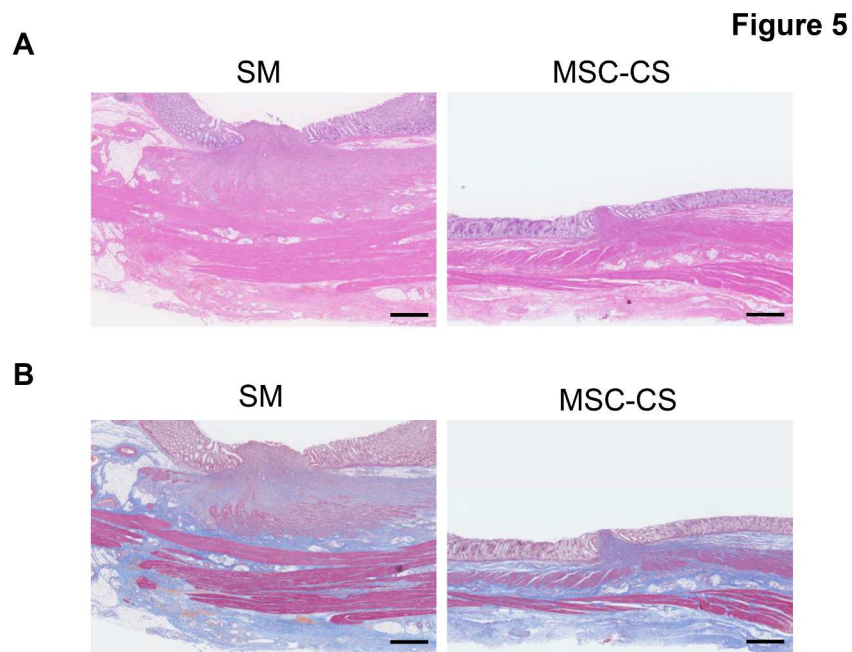


Figure 5. Histological analysis of the rectum after circumferential endoscopic submucosal dissection (ESD) and mesenchymal stem cell-culture supernatant (MSC-CS) gel enema on day 22.

(A) Hematoxylin and eosin staining. Scale bars, 1 mm.

(B) Masson's trichrome staining. Scale bars, 1 mm.

SM, standard medium.

254x338mm (300 x 300 DPI)

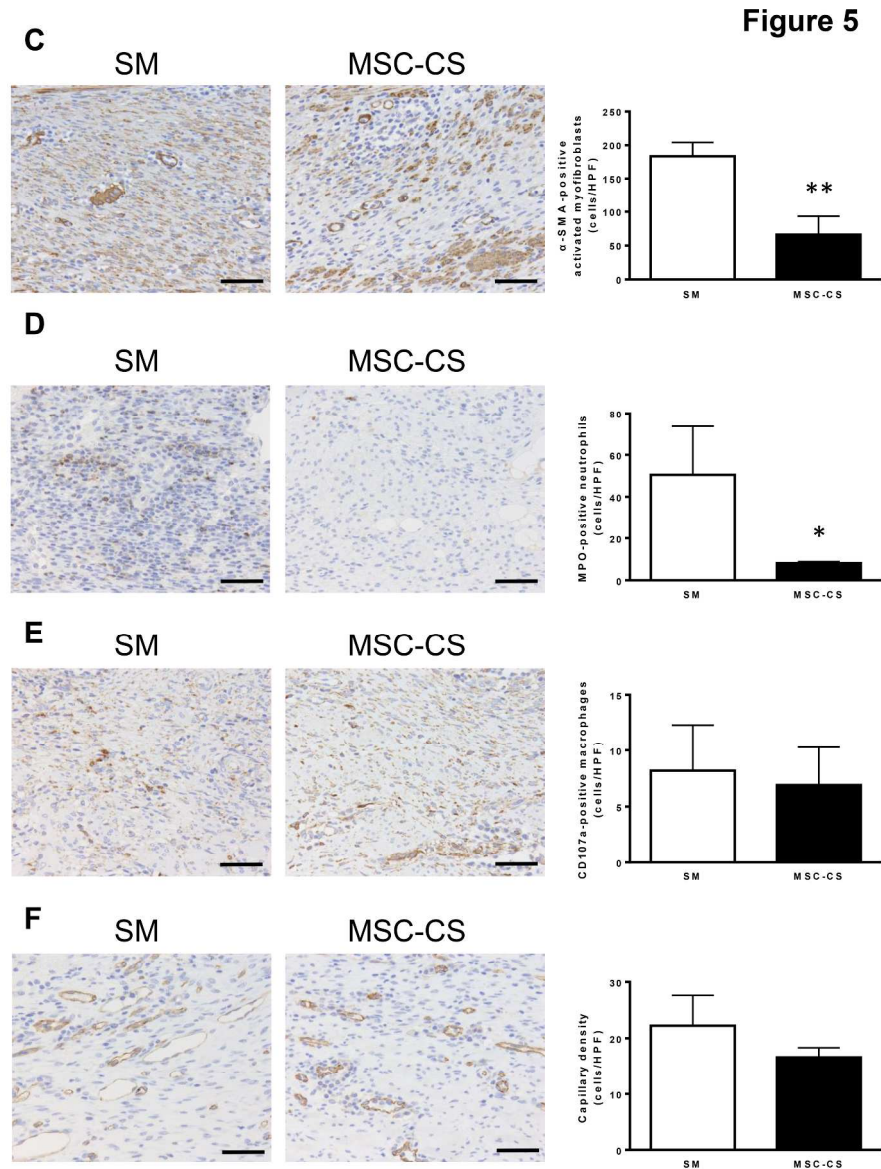


Figure 5. Histological analysis of the rectum after circumferential endoscopic submucosal dissection (ESD) and mesenchymal stem cell-culture supernatant (MSC-CS) gel enema on day 22. (C) Expression of α -smooth muscle actin (α -SMA). Scale bars, 50 μ m. (D) Expression of myeloperoxidase (MPO). Scale bars, 50 μ m. (E) Expression of CD107a. Scale bars, 50 μ m. (F) Expression of CD31. Scale bars, 50 μ m. SM, standard medium; HPF, high-powered fields ($\times 400$). Values are the mean (standard deviation) of 3 animals per group. * $P < 0.05$, ** $P < 0.01$ vs. SM group, respectively.

254x338mm (300 x 300 DPI)

Figure 6

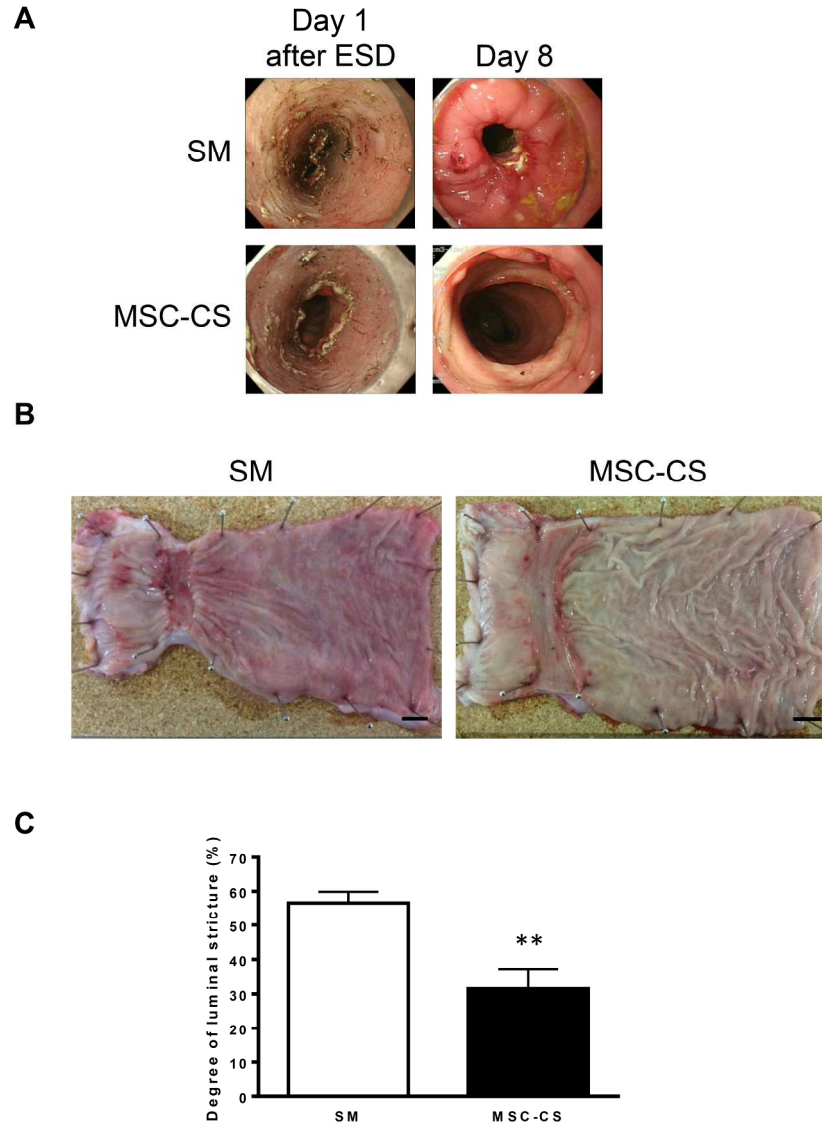


Figure 6. Effect of mesenchymal stem cell culture supernatant (MSC-CS) gel enema after circumferential endoscopic submucosal dissection (ESD) on day 8.

(A) Endoscopic findings from the rectum.

(B) Excised rectum on day 8. Scale bars, 10 mm.

(C) Degree of luminal stricture on day 8.

SM, standard medium. Values are the mean (standard deviation) of 3 animals per group. **P < 0.01 vs. SM group.

254x338mm (300 x 300 DPI)

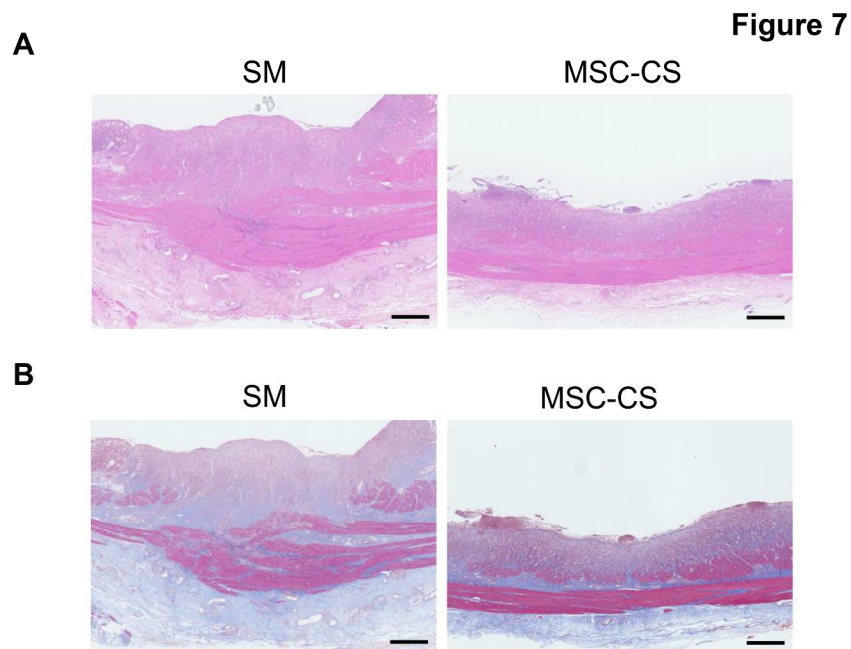


Figure 7. Histological analysis of the rectum after circumferential endoscopic submucosal dissection (ESD) and mesenchymal stem cell-culture supernatant (MSC-CS) gel enema on day 8.

(A) Hematoxylin and eosin staining. Scale bars, 1 mm.

(B) Masson's trichrome staining. Scale bars, 1 mm.

SM, standard medium.

254x338mm (300 x 300 DPI)

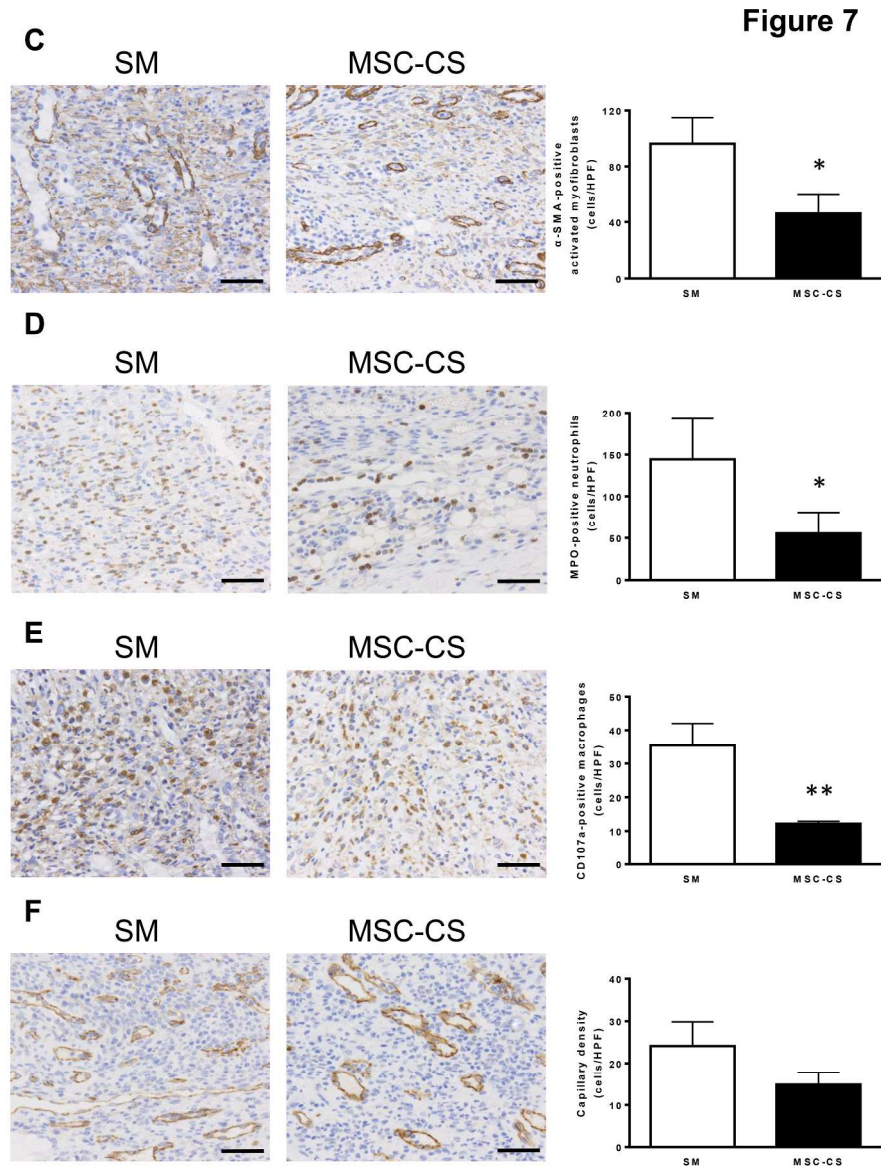


Figure 7. Histological analysis of the rectum after circumferential endoscopic submucosal dissection (ESD) and mesenchymal stem cell-culture supernatant (MSC-CS) gel enema on day 8. (C) Expression of α -smooth muscle actin (α -SMA). Scale bars, 50 μ m. (D) Expression of myeloperoxidase (MPO). Scale bars, 50 μ m. (E) Expression of CD107a. Scale bars, 50 μ m. (F) Expression of CD31. Scale bars, 50 μ m. SM, standard medium; HPF, high-powered fields ($\times 400$). Values are the mean (standard deviation) of 3 animals per group. *P < 0.05, **P < 0.01 vs. SM group, respectively.

254x338mm (300 x 300 DPI)