Format for ANSWERING REVIEWERS

April 18, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 7781-review.doc).

Title: Prevention of esophageal strictures after endoscopic submucosal dissection

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The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

Reviewer

(1) **Comment:** Can you set some subtitles for this review paper to make the skeleton of manuscript more logistic?

Response: Some subtitles were set in the autors' review paper and the manuscript was reconstructed.

Breifly, 3 subtitles were set in the ANTI-INFRAMMATORY APPROCHES. Eight subtitles were set in the TISSUE ENGINEERING APPROACHES. Moreover, some sentences were added for having a smooth sentence flow.

Page 6 lane 6

ANTI-INFLAMMATORY APPROACHES

Anti-inflammatory approaches for preventing esophageal strictures after ESD are based on the concept that subsequent strictures may be suppressed by the inhibition of the infiltration of inflammatory cells, the hyperplasia of granulation, and the fibrosis of the remaining submucosal layer on the ulcer sites.

Page 6 lane 15

Endoscopic intralesional injections of steroids

Endoscopic intralesional injections of steroids are applied based on the concept that inflammation and fibrosis after esophageal ESD are inhibited by the direct administration of steroids to the ulcer

site.

Page 7 lane 16

Systemic steroid therapy for preventing the esophageal strictures after ESD

The efficacy of systemic steroid therapy on preventing esophageal stricture after ESD has been confirmed by Yamaguchi and colleagues[36].

Page 8 lane 18

Potential drugs to target fibrotic formation

There are several studies on specifically preventing excessive fibrotic formation to avoid the various adverse effects of steroid therapies.

Page 9 lane 18

Scaffold-based therapies

Temporary scaffolds made from biodegradable materials support tissue and protect esophageal strictures.

Page 10 lane 16

Cell-based therapies

Cell-based therapies are expected to have trophic effects on the host as the transplanted cells release cytokines and growth factors and interact with other host cells. The direct injection of primary cells into the host organ has two major disadvantages: low viability and quick diffusion from the host sites after transplantation. Because transplanted cells are difficult to engraft at the target site, there have been attempts to engineer tissues in vitro to effectively engraft objective cells at the target site.

Page 10 lane 25

The technology of autologous oral mucosal epithelial cell sheets

In our laboratory, epithelial cell sheets of oral mucosa without any scaffold are fabricated on temperature-responsive culture inserts that are grafted with poly-N-isopropylacrylamid (PIPPAm).[49]

Page 11 lane 16

Preparing the autologous oral mucosal epithelial cell sheets for clinical application

For clinical applications, oral mucosal epithelial cell sheets must be fabricated in a cell processing center (CPC) (Figure 2).[54, 55]

Page 12 lane 12

Transplanting autologous oral epithelial mucosal cell sheets into the artificial ulcer sites after ESD

Before the transplantation of the autologous mucosal epithelial cell sheet into the artificial ulcer site after esophageal ESD, an esophageal endoscopic mucosal resection (EEMR) tube (Create Medic, Tokyo, Japan) is inserted into the patient's esophagus.

Page 13 lane 4

Clinical study using autologous oral mucosal epithelial cell sheets

The safety of transplanting autologous oral mucosal epithelial cell sheets into artificial ulcer sites has been demonstrated in a phase I study[58].

Page 13 lane 15

Future topics for standardizing treatments using epithelial cell sheets

Cell-based therapy using autologous oral mucosal epithelial cell sheets has several disadvantages compared with scaffold-based therapy and anti-inflammatory drug therapy.

Page 14 lane 25

Adipose-derived stem cells

Adipose-derived stem cells (ADSCs), which are similar to bone marrow-derived stem cells, have various biological features including growth factor secretion, multiple differentiation potency, immunosuppression of inflammatory cells, angiogenesis promotion, and wound healing enhancement[68-70].

Page 15 lane 14

Future prospects of treatments using tissue engineering for esophageal stricture therapy

Tissue engineering approaches have great potential for treating various damaged tissues and organs. Currently, tissue engineering treatments must include safety and quality control and require careful observations after transplantation.

(2) **Comment:** Can you make a table for drugs used in anti-inflammatory approaches for preventing esophageal stricture to make the manuscript easy to be understood?

Response: A table for drugs in anti-inflammatory approarches was made. Successful and potential drugs were described with their pharmacological effect, administration, and advantage/disadvantages.

(3) **Comment:** This paper, which concerns indeed an important topic, like stricture prevention after sub mucosal esophageal resection, is a literature review, mainly concentrated on tissue engineering approaches, which is discussed more extensively. In this last part likely the Authors have a deeper

experience. Regarding the argument of the anti-inflammatory pharmacologic approach, it is really discussed in a slightly superficial way.

Response: Several sentences have been added in the manuscript following the comments. Additional explanation has been especially discussed about anti-inflammatory approaches.

(4) **Comment:** In the Introduction, the authors define "preventive" and "conventional" Endoscopic Ballooon Dilatation (EBD) according to the different interval time. Though the definition may be intuitive, a better explanation approach is required.

Response: As suggested by the reviewr, "preventive EBD" was changed to the multiple sessions of EBD before esophageal strictures and "conventional EBD" was changed to EBD after esophageal strictures. Ezoe et al. firstly reported the effectiveness of preventive EBD after esophageal ESD [Ezoe Y. at al. Efficacy of preventive endoscopic balloon dilation for esophageal stricture after endoscopic resection. J Clin Gastroenterol. 2011;45(3):222-7.]. In the literature, Conventional EBD is performed after the symptoms of esophageal strictures developed. The time interval of conventional EBD is depend on the symptoms of esophageal strictures. On the other hand, preventive EBD is defined in the article as premeditated and frequent EBD during the period of mucosal healing before symptoms of esophageal strictures developed.

Page 5 line 18

The occurrence of strictures after esophageal ESD is more common in EBD performed after esophageal strictures (92%) than in multiple sessions of EBD performed weekly before esophageal strictures (59%)[30]. Furthermore, the duration of EBD after esophageal stricture is generally shorter in patients who undergo multiple sessions of EBD before developing esophageal strictures (29 days) than in those who undergo EBD after esophageal strictures form (78 days). Although multiple sessions of EBD before esophageal strictures can prevent esophageal strictures after ESD, such frequent dilatation treatments are problematic because of their high invasiveness and cost. Therefore, less invasive approaches are desired.

(5) **Comment:** About triamcinolone injection the methods used in literature are often different and the results still conflicting. It would be important to explain in details the different concentration, the amounts and the techniques of injection. All these aspects can determine differences in the outcome. Indeed, optimal dose, frequency, and best application (where exactly are the injections done?) for local management by triamcinolone have to be established.

Response: There are only 2 literatures regarding the prevention of esophageal strictures after ESD with triamcinolone injection. The methods of the literatures are summarized in Table 2.

Triamcinolone injection after Esophageal ESD is found to be effective and safety for preventing strictures. However, there is a case report of delayed perforation after triamcinolone injection (Yamashina T at al. Endoscopy. 2013; 45 Suppl 2)

There are 4 reports regarding triamcinolone injection to benign esophageal strictures. Esophageal strictures sometimes develop after the combinational treatments of dilatation and triamcinolone injection to benign esophageal strictures. However, triamcinolone injection to benign esophageal strictures reduces the sessions of EBD and delays the occurrence of strictures (supplemental data). Therefore, triamcinolone injection may be insufficient to prevent esophageal strictures to large size mucosal defects or the fibrotic tissues.

Preventing esophageal strictures after ESD would be carefully compared with treating benign esophageal strictures. Preventing esophageal strictures are treated before fibroblasts activate and collagens deposit. On the other hand, the treatments after esophageal strictures are required the suppression of activated fibroblasts, which are called as myofibroblasts, and the inhibition of the redeposition of collagens

These comments were added in the paragraph of *Endoscopic intralesional injections of steroids and* Table. 2.

Page 6 line 22

Subsequently, triamcinolone acetonide has been used to prevent esophageal strictures after ESD (Table 2.). Hashimoto et al. reported that the local injection of triamcinolone acetonide into the ulcer site prevents esophageal strictures after ESD [35]. Twenty-one patients were treated with local injections of triamcinolone acetonide at 3, 7, and 10 days after ESD. The total dose of triamcinolone acetonide was 18 to 62 mg in each injection session. The stricture rate in the patients who were given the local injection (19%, 4/21) was lower than in the control patients (75%, 15/20) ($P = 0.03$). Furthermore, the frequencies of dilatation in the patients who were given a local injection (mean 1.7, range 0-15) were significantly lower than the frequency of control patients (mean 6.6, range 0-20) ($P > 0.001$). Hanaoka et al. also reported that local injection of triamcinolone prevented esophageal strictures after ESD using a single injection of triamcinolone acetonide immediately after ESD. The total dose of triamcinolone acetonide was 100 mg [36]. In Nagasaki University Hospital, 3/4-circumferential ESD cases are generally treated with this local injection therapy. Fifty mg of triamcinolone acetonide is endoscopically injected in submucosal layer 1 or 2 times for 3 weeks and generally results in a satisfactory outcome. It is recommended that triamcinolone acetonide doses of approximately 18 to 100 mg are injected several times into the ulcer site in the early phase after ESD to prevent esophageal strictures. However, triamcinolone injection might be insufficient to prevent esophageal strictures for large mucosal defects because esophageal strictures can develop after the combinational treatments of dilatation and triamcinolone injection for benign esophageal strictures [31, 32, 37]. The patients with circumferential ESD were excluded in the clinical studies of local injections of triamcinolone [35, 36].

(6) **Comment:** Regarding systemic administration of steroids, although the issue is mainly addressed to stricture prevention after sub mucosal dissection, the authors should at least cite the conflicting results in the literature about the systemic use of corticosteroids in other types of strictures (caustic, post-infection etc.) Corticosteroids cannot be recommended in these patients. Two important articles should be cited about the argument:

Pelclová D, Navratil D. Do corticosteroids prevent oesophageal stricture after corrosive ingestion? Toxicol Rev 2005; 24: 125-129

Fulton JA, Hoffman RS. Steroids in second degree caustic years of human data: 1956-2006. 2007; Clin Toxicol (Phila) 45: 402-08

Response: In the systemic reviews of articles recommended by the reviews, systemic steroids therapy has no benefit in preventing esophageal strictures after the caustic injury of esophagus. In two respects, it is difficult to compare systemic steroids therapy after caustic esophageal injury with one after esophageal ESD.

First, most patients in that study are children, even though most patients with esophageal squamous cell carcinoma and adenocarcinoma are adults.

Second, preventing esophageal strictures after ESD should carefully be compared with treating benign esophageal strictures. Preventing esophageal strictures are treated before fibroblasts activation and collagens deposition. On the other hand, the treatments after esophageal strictures are required the suppression of activated fibroblasts, which are called as myofibroblasts, and the inhibition of the re-deposition of collagens.

Third, caustic esophageal injury gives a very long esophageal mucosal and submucosal layer, even though the part of esophageal mucosa and submucosa are eliminated in the endoscopic resections of esophageal squamous carcinoma and adenocarcinoma.

Preventing esophageal strictures after ESD, the systemic administration of steroids might be insufficient to prevent esophageal strictures after the very long segmental circumferential ESD as suggested by the reviewer. Several cases after long segmental circumferential ESD (over 10 cm in length) developed actually severe esophageal strictures in the authors' experience unless systemic administration of steroids was applied.

These comments were described in the paragraph of *Systemic steroid therapy for preventing esophageal strictures after ESD*.

Page 9 line 5

Although systemic administration of steroids prevents esophageal strictures after extensive ESD, systemic administration of steroids might not prevent esophageal strictures after a very long segmental circumferential ESD. This occurs because systemic steroid therapy does not prevent esophageal strictures after major injuries of the esophagus involving a long length of circumferential mucosal defect [41, 42].

(7) **Comment:** Regarding mitomycin, this drug has deleterious adverse effects, especially if systemic absorption occurs across the intact mucosa. A recent systematic review indicated encouraging results in the long term [Berger M recurrent esophageal strictures: hype or hope? Eur J Pediatr Surg 2012; 22: 109-116], but prospective studies are clearly mandatory to determine the most effective concentration, duration and frequency of application. The theoretical risk of secondary long-term malignancy should also be taken into account.

Response: In the literature recommended by the reviewer, mitomycin is effective to refractory esophageal strictures. Moreover, locoregional mitomycin injection reduces 5 cases of refractory esophageal strictures, which are steroid-resistant-strictures. Mitomycin may cause several local adverse events such as delayed mucosal healing, ulcer formation, and perforation. In a long term observation, the secondary malignancy should also be taken into account, because mitomycin induces DNA damages.

These comments were added in the paragraph of *Potential drugs to target fibrotic formation*.

Page 9 line 14

Mitomycin C is also effective for the treatment of refractory esophageal strictures which include caustic, surgical, and peptic strictures [44]. Additionally, because mitomycin C injection has an anti-proliferative effect on fibroblasts it also prevents not only refractory esophageal strictures but also esophageal strictures. Although the injection of mitomycin C is suggested for preventing esophageal strictures after ESD, it has poor reproducibility in an animal model[45]. Mitomycin C might cause several local adverse events such as delayed mucosal healing, ulcer formation, and perforation. In long-term studies, secondary malignancy should be examined because mitomycin induces DNA damage [46, 47].

(8) **Comment:** The part regarding the tissue engineering approaches is better written and clearly the Authors have more experience in these methods. As a simple suggestion, the paper could only be based on these considerations, without mentioning the pharmacologic approaches to prevent strictures. It must also be considered that in the literature reported, most patients underwent to balloon dilatation, making thus difficult to distinguish among the effects of dilatation and of pharmacologic prevention. The small and different number of patients reported, as well as the different methodologies of the papers make also more difficult any definite conclusion.

Response: The part of tissue engineering is explained for understanding new treatments easily by using tissue engineering methods, because the literatures of Pittsburgh University and Tokyo Women's Medical University have been reported in various medical fields, gastroenterology, cell biology, biomaterials, and regenerative medicine. For connecting the literature of various fields, the part of tissue engineering systematically describes the authors' experiences.

The part of tissue engineering is also described based on common questions from clinicians and reviewers. *Transplanting autologous oral epithelial mucosal cell sheets into artificial ulcer sites after ESD* and *Future prospects of treatments using tissue engineering for esophageal stricture therapy* are especially required the detailed descriptions and the autors' opinions by the previous reviewer.

No standard procedure has been established for preventing esophageal strictures after ESD expect EBD. Treatment outcomes of anti-inflammatory approaches and tissue engineering approaches

have been evaluated by undergoing no dilatation, reducing the times of EBD, and shortening the periods of EBD in the single center experience.

Biodegradable stents were experienced in only two cases. The scaffold based therapy by using extracellular matrix (ECM) biologic scaffold is required to use an expanding stent for attaching the scaffold to the ulcer. As suggested by the reviewer, the clinical evidence of scaffold therapies is insufficient to show its effectiveness to prevent esophageal strictures after ESD, although the safety was shown in the literatures.

The pharmacologic approaches to prevent esophageal strictures has several possible adverse events, even though the pharmacologic approaches has been shown to prevent esophageal strictures in a single center study. For reducing or eliminating the adverse events, endoscopists in Nagasaki University, who are experts in the fields of preventing esophageal strictures by using systemic steroid therapy, initiated a joint study with scientists in Tokyo Women's Medical University, who are experts in the felids of preventing esophageal strictures by using epithelial cell sheets.

These comments were added in the paragraph of TISSUE ENGINEERING APPROACHES and CONCLUSION.

Page 10 lane 25

Long length (8 to 13 cm) of circumferencial resection were performed. Esophageal strictures after endoscopic resection and were improved by only a few sessions (0 to 9) of endoscopic dilatation even though temporary stent support preventing strictures. Surprisingly, the small perforation site healed at 18 days by covering biological scaffold and stent. Although a scaffold provides an ECM, supports strictures, and also promotes cell migration, it may be insufficient to cover an extensive mucosal defect after esophageal ESD. Because the acellular scaffold of the esophageal mucosa includes key proteins for producing a basal membrane, the acellular scaffold of the esophageal mucosa provides a suitable environment that facilitates cell adhesion and proliferation[55]. Consequently, the risk of local recurrence after scaffold transplantation may be higher than that at normal ulcer sites because the scaffold itself is also a good environment for the engraftment of unwelcome malignant cells. The transplantation of biomaterials is also associated with the risk of developing a local infection, which affects the microenvironment of wound healing. This issue is especially important for esophageal mucosal healing because both the esophageal lumen and the oral cavity are constantly exposed to microorganisms. At this time, scaffold-based therapies are not enough clinical evidence and potential risks to prevent the esophageal strictures. In the future, the development of novel materials and the advancement of biological science will enable these problems to be solved.

(9) **Comment:** In the paragraph regarding "Clinical study using autologous oral mucosal epithelial cells", describing the results reported by reference 58, it has been written "the transplantation of oral mucosal epithelial cell sheets prevented esophageal strictures after ESD". It is not clear what this does mean: no dilatation, fewer dilatations? All these details should be explained.

Response: In the clinical study using autologous oral mucosal epithelial cells, esophageal strictures after ESD were prevented in 8 of 9 cases. In 8 cases, there are no dysphagia and strictures after esophageal ESD, and no additional treatments for complications are required. Only one cases required balloon dilatation 24 times to the esophageal stricture after ESD. These comments were added in the manuscript.

Page 14 lane 11

In the eight successful cases, there was no dysphagia and strictures after esophageal ESD and no additional treatments for complications were required. Only one cases required balloon dilatation of the esophageal stricture after ESD.

(10) **Comment:** Finally, I would be more cautious in the conclusions. Tissue engineering cells approach should be considered still in the experimental phase, although it is an exciting treatment. Any too optimistic conclusion should be avoided.

Response: The authors agree that tissue engineering approach is still an experimental therapy for preventing esophageal strictures after ESD. There is insufficient clinical evidence to assess the tissue engineering approach for preventing esophageal strictures. The comparative study will be required at least.

These comments were added in the paragraph of CONCLUSION

Page 17 lane 13

This review reports several strategies for preventing esophageal strictures after extensive ESD with a focus on anti-inflammatory, scaffold-based, and cell-based treatments. Anti-inflammatory treatments, which are mainly local and systemic steroid therapies, have shown positive outcomes in small comparative clinical studies. However, the clinical evidence of scaffold-based and cell-based treatments is still insufficient, and their efficacy needs to be confirmed in comparative studies because they are potentially new technologies for tissue engineering and novel treatment strategies for wound healing. To establish a truly minimally invasive treatment using endoscopic surgery, improvements to all of these methods are needed. Nonetheless, these three strategies will eventually become available as a combined therapy in the future.

Table 1. Anit-inflammatory drugs for preventing esophageal strictures after endoscopic submucosal dissection

	Action	Administration	Advantages	Disadvantages and limitations
Clinical study				
Corticosteroids	Steroidal	Oral intakes	Strongly inhibits the infiltration of inflammatory cells, the hyperplasia associated with granulation, and the fibrosis of the remaining submucosal layer	General side effects (severe infection, peptic ulcer, hyperglycemia, psychiatric symptoms, and osteoporosis) Delayed wound healing
Triamcinolone acetonide	Steroidal	Local injection	Inhibits the infiltration of inflammatory cells, the hyperplasia associated with granulation, and the fibrosis of the remaining submucosal layer	Risk of ulcer formation due to accidental injection into the muscularis Delayed wound healing
Pre-clinical study				
Mitomycin C (MMC)	Inhibiting DNA synthesis	Local injection	Inhibits the proliferation and activation of fibroblasts	An effect has not been shown for the prevention of esophageal strictures, although MMC improves recurrent dysphagia or restenosis after the dilatation of esophageal strictures The risks of perforation and the secondary malignancy
N-acetylcysteine	An antioxidant molecule	Oral intakes	Anti-fibrotic effect without the inhibition of wound healing	Insufficient effect in an animal model of severe esophageal stricture

Table 2. Intraregional triamcinolone injection for preventing esophageal strictures after Endoscopic submucosal dissection (ESD)

Author	Resection size		The methodology of triamcinolone injection						Treatment outcomes		
	*Circumference	Length (mm)	Ingection needle	Concentration (mg /ml)	Single dose (ml)	Times of punchures (/ session)	Total amounts (mg)	Sessions	The rate of strictures	The number of EBD	Observation periods
Hashimoto[35]	> 3/4	54 (28 - 60)	25 G, 4mm	10	0.2	9 - 31	18 - 62	*Three times	19% (4 /21)	1.7 (0 - 15)	1 year
Hanaoka[36]	> 3/4	58 ± 11	25 G	5	0.5-1	20 - 40	100	**Single	6.6% (3 / 30)	0 (0 - 2)	2 months

* The cases of whole circumferential ESD are excluded

** Three sessions of locoregional triamcinolone injection are performed at 3, 7, and 10 days after ESD.

*** Only single session of locoregional triamcinolone injection is performed immediately after ESD.

G: gaze

EBD: endoscopic balloon dilatation

Supplemental data. Triamcinolone injection therapy with esophageal dilatation to benign esophageal strictures

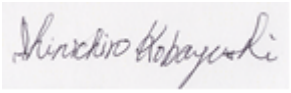
Author	PMID	Ingection needle	Concentration (mg/ml)	Single dose (ml)	Times of punctures	Total amounts (mg)	Sessions	The rate of strictures	The number of EBD	observation periods
Kochhar R	10202068	23 G, 5 mm	10	0.25	4-16	Unknown	Single (immediately before or after dilatation)	82.3% (14/17)	3.6 ± 2.57 (0-10)	10.5 ± 5.58 (4-21) months
Kochhar R	12447293	23 G, 5 mm	40	0.5	8	160	Single (immediately after dilatation)	Unknown	3.8 (0-30)	9.78 (1-70) months
Altintas E	15610312	21 G, 5 mm	8	1	4	32	Single (immediately after dilatation)	100% (17/17)	5.3, 2–12	24 ± 12.75 (6–39) months
Ramage JI Jr	16279894	22 G	40	0.5	4	80	Single (immediately after dilatation)	13% (2/15)	Unknown	12 months

PMID: Pubmed ID

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



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