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Effect of Low Dose Aspirin on Chronic Acid Reflux Esophagitis in Rats

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Acknowledgments: This study was technically supported in part by Department of Pathology, the Jikei University School of Medicine.

Disclosure Statement: All authors have read the journal publication policy and have no relevant financial disclosures and conflicts of interest with regards to this paper.

ABSTRACT

Background: Clinical role of low-dose aspirin (LDA) in pathogenesis of gastroesophageal reflux disease is by far controversial. This can be attributed to the paucity of basic research detailing the mechanism of LDA-induced esophageal mucosal injury (EI) on underlying chronic acid reflux esophagitis (RE).

Aim: The aim of this study was to clarify the effect of LDA on chronic RE in rats.

Methods: Esophagitis was induced in 8-week-old male Wistar rats by ligating the border between forestomach and glandular portion with a 2-0 silk tie and covering the duodenum with a small piece of 18-Fr Nélaton catheter. Seventy-eight chronic RE rat models were divided into 5 treatment groups, consisting of orally administered vehicle (controls), and aspirin doses of 2, 5, 50 or 100 mg/kg once daily for 28 days. EI was assessed by gross area of macroscopic mucosal injury, severity grade of esophagitis and microscopic depth of infiltration by inflammatory cells.

Results: Area of esophagitis in animals with aspirin dose of 100 mg/kg/day showed a 36.5% increase compared with controls, although it failed to achieve statistical significance ($p=0.812$). Additionally, the rate of severe EI was increased in animals with aspirin dose of 100 mg/kg/day as compared with controls ($p<0.05$). The grade of severity correlated with the depth of inflammation ($r_s=0.492, p<0.001$).

Conclusions: Maximal dose aspirin (100 mg/kg/day) contributed in exacerbating pre-existing

EI. LDA (2 and 5 mg/kg/day), on the other hand, did not affect chronic RE in this model. LDA seems to be safe for use in patients with chronic RE.

Keywords

GERD; rat; aspirin; gastroesophageal reflux; low-dose aspirin; chronic acid reflux

INTRODUCTION

Gastroesophageal reflux disease (GERD) is caused by the reflux of gastric contents back into the esophagus, and is characterized by esophageal mucosal injury (EI) and typical symptoms such as heartburn and acid regurgitation [1]. The prevalence of GERD is increasing in Japan and, it is now recognized as a common gastrointestinal disorder [2].

Low dose aspirin (LDA) is one of the most widely used drugs in the world. Through its anti-platelet effects, LDA plays an important role in atherosclerotic diseases, including cerebrovascular atherosclerosis and coronary artery disease [3]. Despite its benefits, upper gastrointestinal mucosal injury appears to be a common side effect. Furthermore, recent case reports and clinical studies have reported a potentiality of small bowel injury with chronic use of LDA [4, 5]. With ageing population in Japan, the number of LDA users is expected to rise, and therefore LDA-induced gastrointestinal mucosal injury is expected to become a serious problem [6].

Some clinical studies have reported for LDA to be one of the risk factors for EI. Taha et al. [7] reported that the patients taking LDA exhibit more severe esophagitis than those on acetaminophen. In another study, Yeomans et al. [8] followed-up the patients receiving prophylactic LDA for 26 weeks and found an increase in the number of cases with erosive esophagitis. However, despite these studies, controversy remains in the proven role of aspirin as a risk factor for EI. Several clinical studies failed to find any association between LDA and

EI [6, 9]. Furthermore, there has been no basic research detailing the mechanism of LDA induced EI on a backdrop of chronic acid reflux esophagitis (RE). The aim of the present study was to clarify the effects of LDA on chronic RE in an animal model.

METHODS

1) Animals

Specific pathogen-free, 7-week old male Wistar rats, weighing 150-170 grams, were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and were allowed to acclimatize for 1-week at the animal facility of the Jikei University School of Medicine, before admitting them for the experiment. They were housed at a uniform temperature (22 ± 2 °C) and relative humidity (55 ± 10%) in cages with an automatically controlled 12:12-hour light/dark cycles (light on at 7:00 a.m.), and they had free access to CE-2 diet (CLEA Japan, Inc. Tokyo, Japan) and water. The experimental protocol of the current study was reviewed and approved by the Institutional Animal Care and Use Committee of the Jikei University (No. 2015-126) and conformed to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan (2006).

2) Induction of acid reflux esophagitis

Animals were deprived of food for 24-hours prior to surgery. Anesthesia was administered using isoflurane 4% for induction and 2% for maintenance. Animals were given a subcutaneous injection of 2 mg/kg butorphanol to prevent surgical pain, immediately following induction of anesthesia, and on post-operative day-1. Under anesthesia, acid reflux esophagitis was induced using the method reported by Omura et al [10]. This model is

established for surgically induced chronic RE, and is commonly used in a wide range of studies such as for evaluation of the pathophysiology or the investigation of drug efficacy [11–14]. To briefly summarize the surgical procedure, a 2-cm midline incision was made in the upper abdomen, the border between the forestomach and the glandular portion (limiting ridge) was ligated with a 2-0 silk tie, and the duodenum near the pyloric ring was covered with a 2-mm wide piece of an 18-Fr Nératon catheter (Figure 1). The sham operation included a midline laparotomy alone without further surgical intervention. Post-operatively, the animals were deprived of food for 48-hours but were allowed free access to water.

3) Experimental groups and design

LDA is generally defined as a dose of 75–325 mg/day in human subjects. The maximal dose of aspirin in Japan is 4,500 mg/day. We determined the relative dosage in rats, taking in account their subjective difference in the body weights. Accordingly, sham-operated rats were divided into 5 treatment groups as follows (n=5–6 per group):

Group Sham I: No aspirin administration (control)

Group Sham II: aspirin administration- 2 mg/kg/day (equivalent to low dose)

Group Sham III: aspirin administration- 5 mg/kg/day (equivalent to low dose)

Group Sham IV: aspirin administration- 50 mg/kg/day (equivalent to high dose)

Group Sham V: aspirin administration- 100 mg/kg/day (equivalent to maximal dose)

Similarly, the rats in chronic RE model were divided into 5 treatment groups (Group I–V, n=11 to 16 per group) based on the same aspirin administration protocol.

Aspirin is not easily dissolvable in water, and thus it was used as a suspension in 0.5 ml distillated water. From the next day following surgery, aspirin suspension was administered orally, using a feeding catheter, once daily for 28-days. In control group, the same volume of distillated water was administered in an identical fashion.

The animals were euthanized on post-operative day 29. The entire esophagus, stomach and proximal duodenum were removed en-bloc, opened longitudinally, and fixed in 10% formalin for 24-hours. The samples were photographed for macroscopic examination, and the area of mucosal injury was measured. The samples were then dehydrated in 100% ethanol solution for 24-hours, embedded in paraffin and were cut into 3-μm thick sections. The sections were stained with hematoxylin and eosin for histopathological examination.

4) Classification of esophagitis severity

The gross area of the esophagitis was measured, and was classified into two grades of severity as follows:

Mild EI: presence of esophagitis limited to <60 mm^2 area

Severe EI: esophagitis area ≥60 mm^2 or esophageal perforation caused by GERD

5) Statistical analysis

The data are presented as the mean ± standard error of mean. All statistical analyses were performed using SPSS version 22.0.0.0 (Armonk, NY, USA). The statistical significance of the mean weight of each group was assessed by one-way analysis of variance (ANOVA) followed by post-hoc analysis with Tukey's HSD (honest significant difference) test. The mean gross esophagitis area of each group was analyzed by using one way ANOVA followed by Games-Howell test and Pearson's product-moment correlation. Fisher's exact test and Spearman's rank correlation were used to compare the severity of esophagitis and the histopathological characteristics between the groups. The level of significance was set at p value <0.05.

RESULTS

The number of animals treated in the study and their outcomes are summarized in Figure 2. A total of 78 rats underwent chronic esophagitis-induction surgery. Within the chronic RE model, esophagitis occurred in 87.2% (68/78) of animals. The 4-week survival rate was 100% (11/11) in control group, while there was a survival rate of 75.6% (59/78) within the overall group with 19 deaths occurring due to esophageal perforation secondary to severe esophagitis (n=7), ileus (n=3), aspiration pneumonia (n=6), self-removal of the midline sutures (n=1) and other causes (n=2). The animals without esophagitis and with survival of less than 4-weeks due to complications such as ileus or aspiration pneumonitis, were excluded. The remaining 62 animals were included in the analysis for this study.

1) Change in body weight

The mean weight of animals in each group which survived for 4-weeks or more is shown in Table 1. A total of 55 animals which survived for 4-weeks were investigated. The animals which did not survive for 4-weeks due to esophageal perforation (n=7) were excluded from the calculation. The weight on operative day were similar in each group, however, weight on post-operative day 28 was lower in group II (aspirin dose: 2 mg/kg/day) than the control group ($p<0.05$).

2) Macroscopic appearances of esophagus

All sham-operated animals of the five treatment groups showed macroscopically intact esophagus (Figure 3a). The chronic RE model showed several erosions and ulcers at the middle and lower esophagus with diffuse dilatation and localized stenosis (Figure 3b–f). Figure 3e shows dilatation proximal as well as distal to the stenosis because food did not immediately pass through the stricture. Figure 4a shows the mean gross esophagitis area for rats which survived for 4-weeks or more in each group. Group V (aspirin dose: 100 mg/kg/day) presented a 36.5% increase in area involving esophagitis compared with control group (48.2 ± 9.3 mm^2 versus 35.3 ± 7.4 mm^2), although the difference didn't reach a statistical significance (p=0.812). There was no significant correlation seen between the area and the dose of aspirin administered as well (r=0.206, p=0.132).

3) Severity of Esophagitis

Differences in the severity of esophagitis between groups were investigated (Figure 4b). The rate of severe EI was increased in group V (aspirin dose: 100 mg/kg/day) as compared with control group (**p=0.038**). Furthermore, severity of esophagitis and dose of aspirin demonstrated positive correlation (r_s=0.343, p<0.01).

4) Histopathological Observations

Figure 5 shows the representative histopathological findings of hematoxylin and eosin staining. The intact esophagus exhibited a thin epithelial layer with squamous cells and only a few inflammatory cells in the submucosal layer. In contrast, the reflux esophagitis model showed thickening of the esophageal epithelium with basal cell hyperplasia, elongation of the lamina propria papillae, marked inflammatory cell infiltration (predominantly neutrophils) with erosions, interruption of the lamina muscularis mucosae and increased collagen fibers.

Table 2a shows infiltration depth of inflammatory cells in each group. The main region of inflammatory cells distribution was represented as the depth. Samples of esophageal perforation were counted as the infiltration of adventitia. There was no correlation between the depth of inflammation and dose of aspirin amongst five groups (r_s=0.176, p=0.171). The difference in depth of infiltration by inflammatory cells between mild EI and severe EI are shown in Table 2b. There was a correlation observed between the grade of severity and the depth of inflammation (r_s=0.492, p<0.001).

DISCUSSION

The sole role of aspirin in pathogenesis of GERD has been controversial. GERD is a well-recognized multifactorial disease. Previous studies have reported the factors associated with an increased development of GERD including age [15], diet [16], obesity [17], *Helicobacter pylori* eradication [18], and the presence of hiatal hernia. Therefore, it is difficult to make a fair judgement of the risk of EI induced by aspirin alone through a clinical study. Several randomized, double-blinded, placebo-controlled trials have suggested the role of LDA in increased prevalence of EI, but failed to elucidate the underlying mechanism behind LDA induced EI [8, 19].

Various animal models, such as rabbits and rats, have been used in studying the pathophysiology of GERD. Recently, rodents, especially rats, are the primary animals used in the GERD models. This is because of their stratified squamous epithelium-lined esophagus which is similar to the human esophagus, despite several histological differences between the rodent and human esophagi, such as presence of keratinized epithelium, existence of cornified epithelium, and absence of submucosal glands in rodents [20].

Lanas et al. [21] showed that aspirin increased EI in rabbits with acute esophagitis following perfusion with hydrochloric acid solutions. However, it is unclear whether how closely this experimental model would resemble with the patients with RE on aspirin. In this acute esophagitis model, the esophageal mucosa was serially exposed to acidified aspirin

followed by acidified pepsin, and subsequently acidified saline, at pH of 2 and a duration of 1-hour each. Under physiological condition, esophagus has a shorter period of contact with food, drugs or liquids. Therefore, this model was distinctly dissimilar to the actual clinical situation in human subjects.

Pawlik et al. [22] also demonstrated that aspirin augmented EI in acute esophagitis models in rats. In the study, the animals underwent pretreatment with intragastric administration of vehicle or aspirin (12.5, 25, 50, 100 mg/kg), and then both the pyloric ring and the limiting ridge were ligated with 2-0 silk ties. In the acute esophagitis model, EI was dose-dependently increased in groups with aspirin of 25, 50 and 100 mg/kg at 4-hours following induction of acute esophagitis; however, the dose of 12.5 mg/kg was not seen to be associated with EI.

Nonetheless, due to the use of acute esophagitis models in above 2 studies, the investigators could not extend their observations beyond 4-hours postoperatively. In clinical scenario in humans, LDA is usually taken over a prolonged period of time. Hence, it is prudent to use a chronic esophagitis model to better assess the role of aspirin in EI. The current study is based on such model to mimic the clinical scenario in humans as closely as possible. To the best of our knowledge, this is the first ever reported basic study to look at the effects of LDA on chronic RE.

The ingestion of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, is

related to inhibition of cyclooxygenase enzymes (COX-1 and COX-2) responsible for the production of prostaglandins (PGs) [23]. It is widely accepted that PGs are the factors that are responsible for the protection of gastric mucosa including regulation of mucosal blood flow, and stimulation of mucus and bicarbonate secretion [22, 23]. At low doses (75–325 mg/day), aspirin predominantly inhibits the COX-1 isoform [24, 25] and the consequent reduction of mucosal PGs is believed to cause gastroduodenal mucosal injury [26]. On the other hand, the mechanism of EI induced by LDA still remains to be fully elucidated. A previous study demonstrated that inhibition of COX-1 exacerbated EI in rabbits with chronic RE [27]. However, the rationale behind this mechanism was unclear. Another report [22] which used a rat model with acute RE suggested that the mechanism of EI induced by aspirin could be attributed to the mucosal ischemia caused by the inhibition of PGs synthesis. Further, the mucosal barrier damage initiated by ischemia may be exacerbated in the presence of gastric acid and pepsin.

The present study showed that the EI did not occur in rats receiving aspirin in sham-operated animals. It shows that the mere presence of aspirin does not induce EI in an otherwise healthy esophagus without backdrop of acidic conditions i.e. free from reflux of gastric contents. The histopathological findings in these groups were similar to those reported in previous studies with the same model [10, 28]. Thus, it is to be noted that LDA did not have any effect on the histopathology of EI in chronic RE. However, the risk of severe EI (seen

macroscopically) increased in a chronic RE model with 100 mg/kg/day dose of aspirin (Figure 4b). Table 2b demonstrated that the degree of the depth of inflammation correlated with the severity of EI. It appears that maximal dose aspirin can also be the potential risk factor for subsequent transmural inflammation.

In human esophagus, some investigators have suggested a hypothesis of a locus minoris of resistance to mucosal break at the side of lesser curvature. Edebo et al. [29] reported that esophageal erosions in gastroesophageal reflux were most predominantly visualized at the 3 o'clock region in the distal esophagus on endoscopy. In rats, EI was predominantly observed at 1 to 2 cm proximal to the esophago-gastric junction unlike humans. This is potentially due to the reason that rats always take a body positon lying on the abdomen resulting in refluxate flowing into the middle esophagus.

There were a few possible limitations in the current study. Firstly, the study duration was perhaps short; however, it was difficult to define the ideal duration of study as the adequate period for evaluating the risk of EI on chronic RE. Secondly, the current study had a possible selection bias. Although the animals were divided and grouped into 5 groups based on their close resemblance to each other in terms of age and body weight by cage (2 to 3 animals per cage) to reduce bias, we did not properly randomize our animals. Further, we didn't extend the present study to evaluate inflammatory mediators including PGs in study groups because we couldn't find any relationship between LDA and EI. However, it may help gain further insights

in the pathogenesis of EI. Additionally, we did not measure the pH of gastric juice because the previous studies [30–32] had reported that pH was not affected by LDA. However, if the pH of gastric juice were measured, the mechanism of action of aspirin could have been elucidated more closely. Finally, existence of several histological differences between rats and human esophagi potentially make it difficult to translate the results directly from an animal model to the human subjects [20]. Further study with more appropriate model is warranted.

In summary, the maximal dose of aspirin seemed to contribute to the exacerbation of pre-existing EI, although our main aim was to investigate the effect of LDA on chronic RE. In the present study, there were no differences between LDA (2mg/kg /day and 5mg/kg /day dose) groups and controls, neither macroscopically nor histopathologically. Accordingly, LDA was proved not to be effective on chronic acid reflux esophagitis in this model. Thus, we conclude that the use of LDA seems to be safe in patients with chronic GERD.

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Figure 1

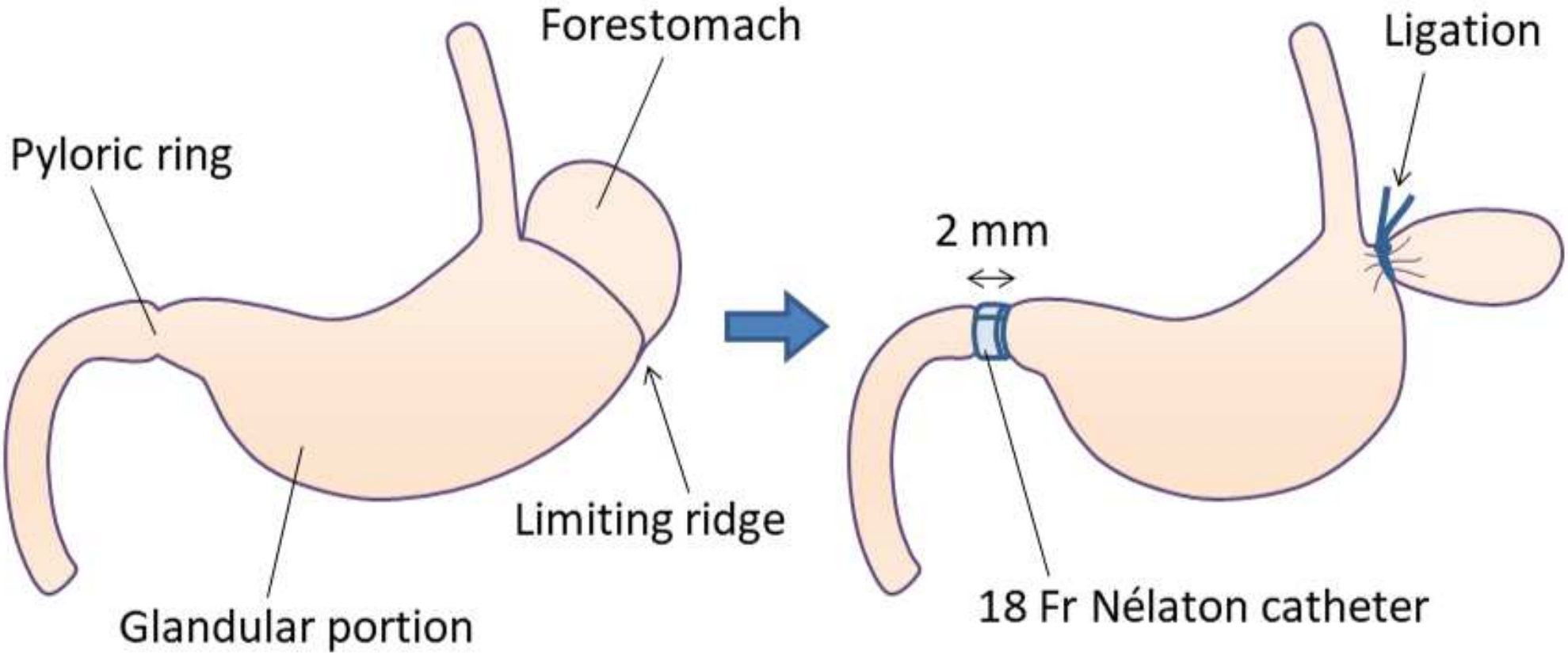
Forestomach
Pyloric ring
Limiting ridge
Glandular portion
Ligation
2 mm
18 Fr Nélaton catheter

Figure 2

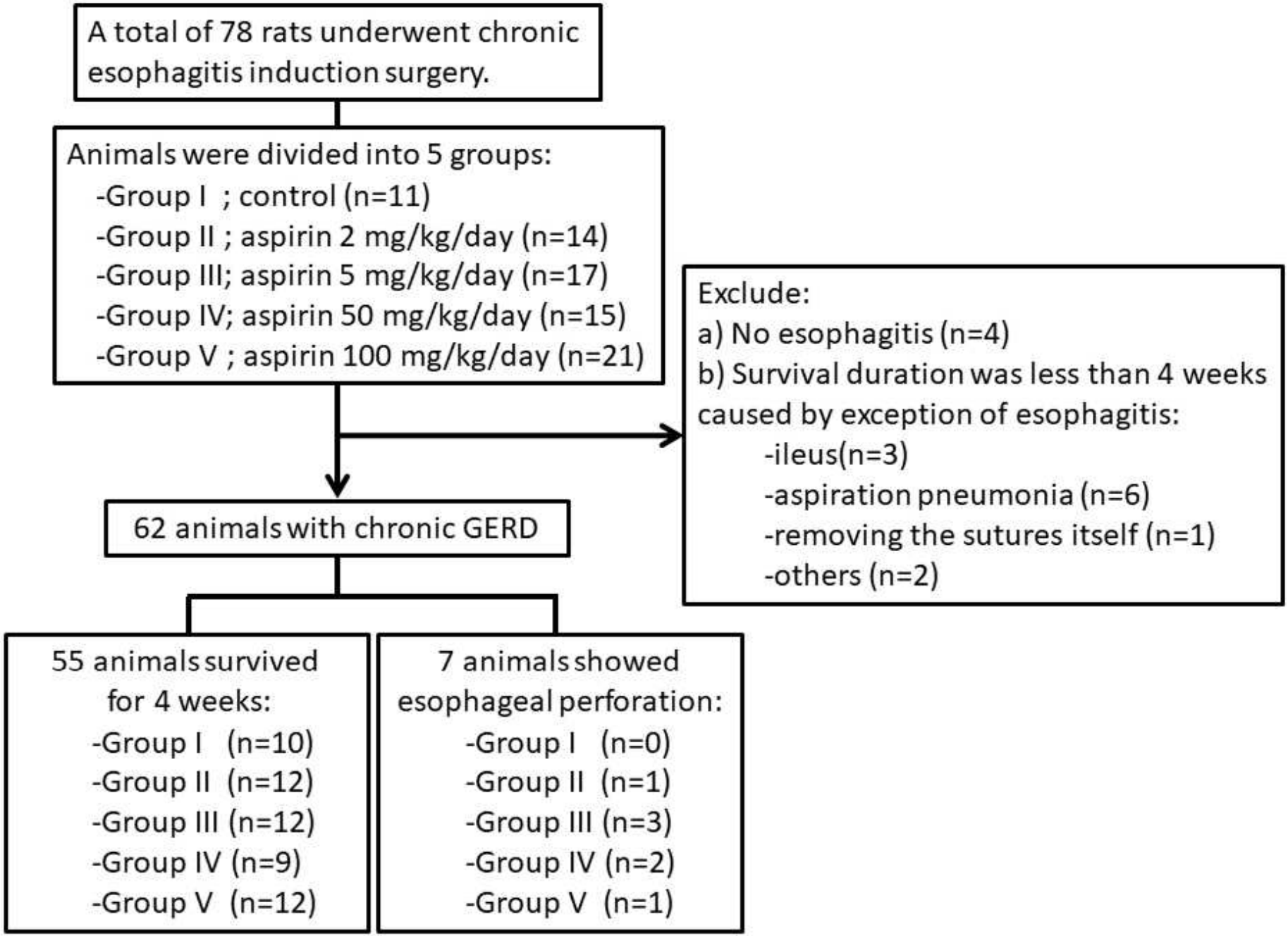
A total of 78 rats underwent chronic esophagitis induction surgery.
Animals were divided into 5 groups:
-Group I ; control (n=11)
-Group II ; aspirin 2 mg/kg/day (n=14)
-Group III; aspirin 5 mg/kg/day (n=17)
-Group IV; aspirin 50 mg/kg/day (n=15)
-Group V ; aspirin 100 mg/kg/day (n=21)
Exclude:
a) No esophagitis (n=4)
b) Survival duration was less than 4 weeks caused by exception of esophagitis:
-ileus(n=3)
-aspiration pneumonia (n=6)
-removing the sutures itself (n=1)
-others (n=2)
62 animals with chronic GERD
55 animals survived for 4 weeks:
-Group I (n=10)
-Group II (n=12)
-Group III (n=12)
-Group IV (n=9)
-Group V (n=12)
7 animals showed esophageal perforation:
-Group I (n=0)
-Group II (n=1)
-Group III (n=3)
-Group IV (n=2)
-Group V (n=1)

Figure 3

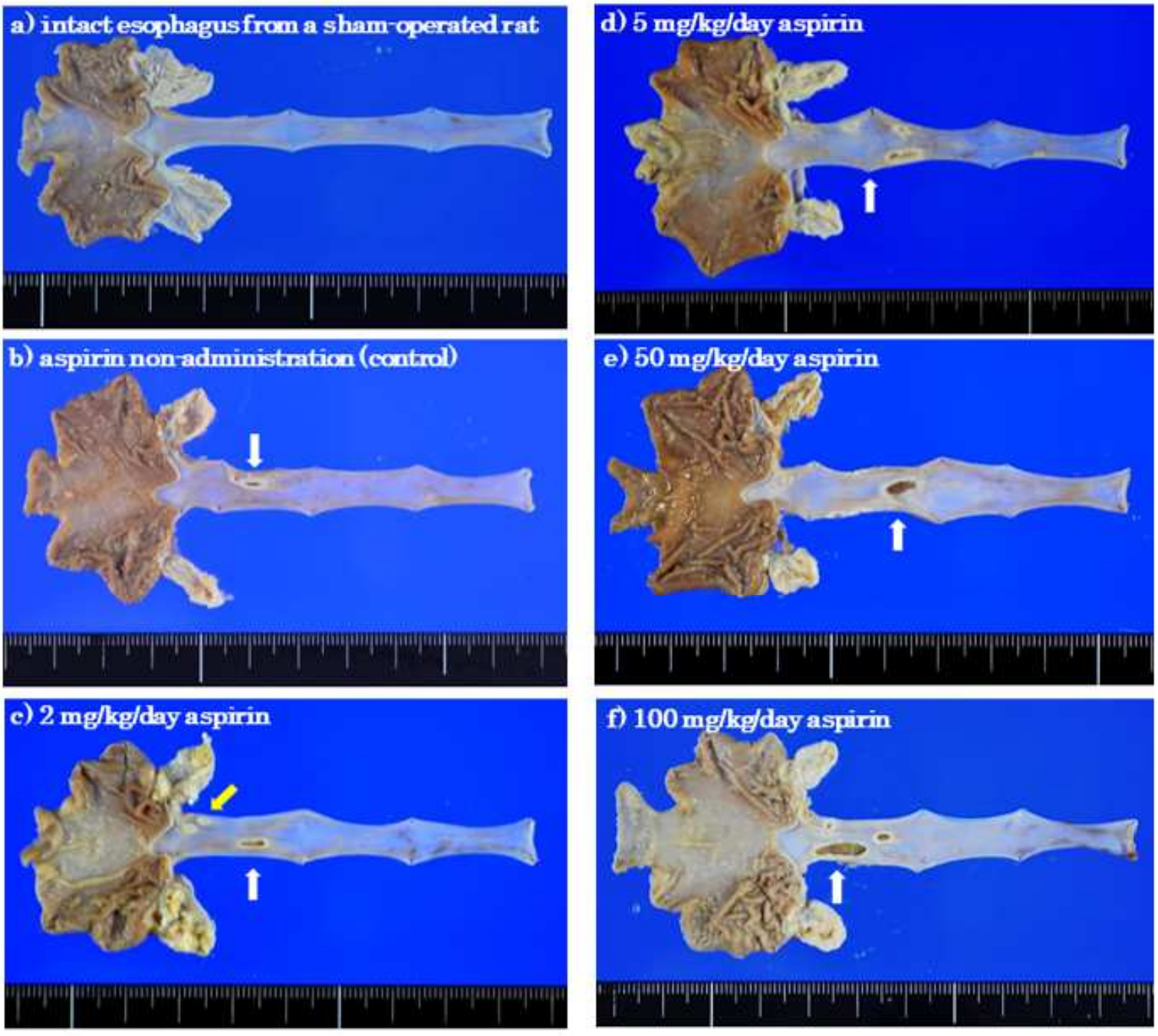
a) intact esophagus from a sham-operated rat
b) aspirin non-administration (control)
c) 2 mg/kg/day aspirin
d) 5 mg/kg/day aspirin
e) 50 mg/kg/day aspirin
f) 100 mg/kg/day aspirin

Figure 4

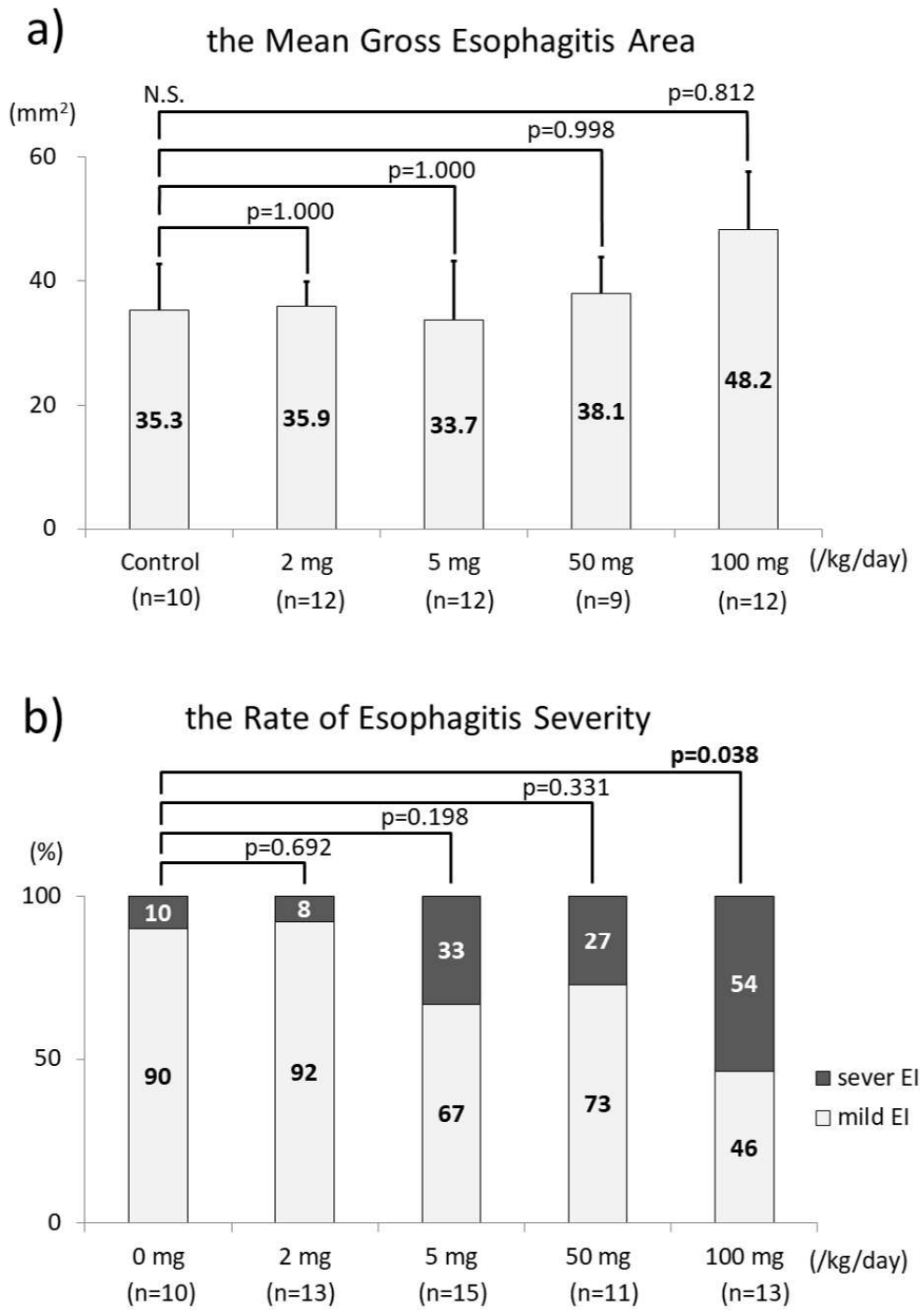
a)
the Mean Gross Esophagitis Area
(mm²)
N.S.
p=0.812
p=0.998
p=1.000
p=1.000
60
40
20
0
35.3
35.9
33.7
38.1
48.2
Control (n=10)
2 mg (n=12)
5 mg (n=12)
50 mg (n=9)
100 mg (n=12)
(/kg/day)
b)
the Rate of Esophagitis Severity
p=0.038
p=0.331
p=0.198
p=0.692
(%)
100
50
0
10
8
33
27
54
90
92
67
73
46
sever EI
mild EI
0 mg (n=10)
2 mg (n=13)
5 mg (n=15)
50 mg (n=11)
100 mg (n=13)
(/kg/day)

Figure 5

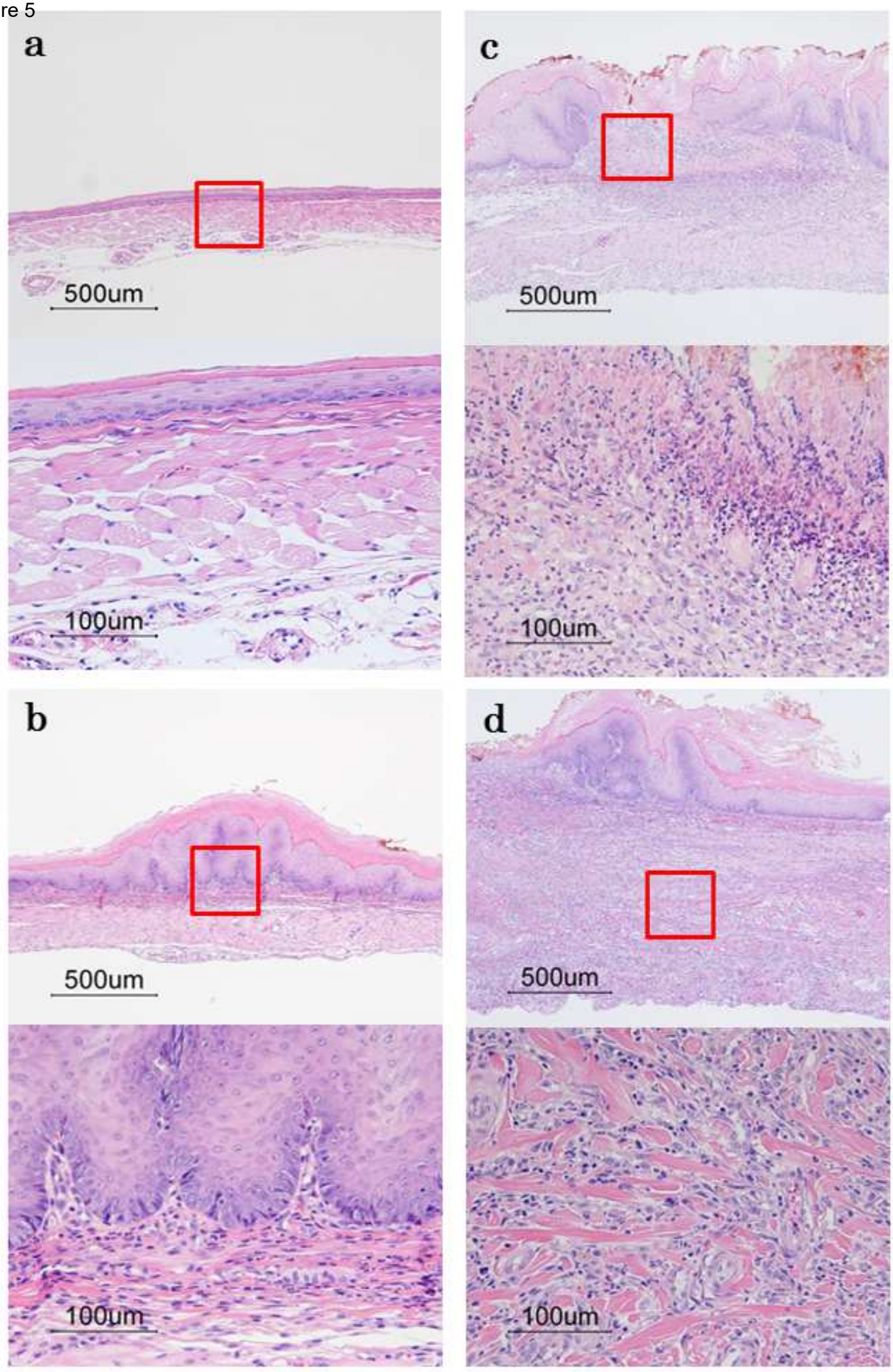

Table 1. The mean weight of esophagitis rats that survived for 4 weeks

			weight (g)	
			preoperative day	post-operative day 28
	Control	(n=10)	187.2 ± 2.2	231.3 ± 5.7
Aspirin	2 mg/kg/day	(n=12)	184.5 ± 1.9	203.1 ± 6.1*
	5 mg/kg/day	(n=12)	179.4 ± 2.3	211.5 ± 7.6
	50 mg/kg/day	(n=9)	178.7 ± 2.1	226.6 ± 4.6
	100 mg/kg/day	(n=12)	178.2 ± 2.5	223.6 ± 6.8

A total of 55 rats which survived for 4 weeks were investigated. The animals which did not survive for 4 weeks due to esophageal perforation (n=7) were excluded from the calculation.

Results are mean ± standard error of mean. * $p < 0.05$ versus control.

Table 2. The depth of inflammation

a) The infiltration depth of inflammatory cells in each group

			infiltration depth of inflammatory cells					
			epithelium	lamina propria	muscularis mucosa	submucosa	muscularis propia	adventitia
Control		(n=10)	0	0	1	2	4	3
Aspirin	2 mg/kg/day	(n=13)	0	2	0	1	4	6
	5 mg/kg/day	(n=15)	2	0	0	1	3	9
	50 mg/kg/day	(n=11)	0	0	0	1	3	7
	100 mg/kg/day	(n=13)	1	1	0	1	2	8

There was no correlation between the inflammatory depth and dose of aspirin (r_s=0.176, p=0.171).

b) The infiltration depth of inflammatory cells between the severity grades of esophagitis

		infiltration depth of inflammatory cells						
		epithelium	lamina propria	muscularis mucosa	submucosa	muscularis propia	adventitia	
Severity	Mild EI (n=45)	3	3	1	6	15	17	**
	Severe EI (n=17)	0	0	0	0	1	16	

The inflammatory depth had a positive correlation with the severity of EI. ** r_s=0.492, p <0.001.