



In vitro model to evaluate effect of acidic pepsin on vocal fold barrier function

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ABSTRACT

The pathophysiology of laryngopharyngeal reflux (LPR) and its impact on the vocal fold is not well understood, but may involve acid damage to vocal fold barrier functions. Two different components encompass vocal fold barrier function: the mucus barrier and tight junctions. Mucus retained on epithelial microprojections protects the inside of the vocal fold by neutralizing acidic damage. Tight junctions control permeability between cells.

Here we developed an in vitro experimental system to evaluate acidic injury and repair of vocal fold barrier functions. We first established an in vitro model of rat vocal fold epithelium that could survive at least one week after barrier function maturation. The model enabled repeated evaluation of the course of vocal fold repair processes. Then, an injury experiment was conducted in which vocal fold cells were exposed to a 5-min treatment with acidic pepsin that injured tight junctions and cell surface microprojections.

Both of them healed within one day of injury. Comparing vocal fold cells treated with acid alone with cells treated with acidic pepsin showed that acidic pepsin had a stronger effect on intercellular permeability than acid alone, whereas pepsin had little effect on microprojections.

This result suggests that the proteolytic action of pepsin has a larger effect on protein-based tight junctions than on phospholipids in microprojections. This experimental system could contribute to a better understanding of vocal fold repair processes after chemical or physical injuries, as well as voice problems due to LPR pathogenesis.

1. Introduction

Laryngopharyngeal reflux (LPR) is common and has a prevalence of between 13 % and 18 % [1]. In LPR, gastric acid and pepsin flow upward from the stomach to the larynx and pharynx to cause various laryngopharyngeal symptoms. In the larynx in particular, these gastric contents can cause laryngitis characterized by tissue edema, ulceration,

polyps and granulations. Patients often complain of hoarseness, chronic cough, and globus sensations [2,3]. Administration of proton pump inhibitors (PPIs) and counseling on lifestyle modifications are widely accepted treatments for LPR [4]. However, 10–30% of patients do not respond to these conservative treatments [5–7].

Damage to vocal fold barrier function is thought to be an important component of LPR pathogenesis [8,9]. Physiologically, vocal fold

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epithelium has two types of barrier functions: a mucus barrier on the epithelial surface [10,11] and tight junctions that affect permeability and form a barrier in cell-cell contacts [8,12]. Mucus is not only a physical protectant of vocal fold mucosa [13], but contains bicarbonate ions that act as a neutralizing buffer against an acidic environment [14]. Mucus is retained by microprojections from vocal fold epithelial cells. Meanwhile cell-cell interactions in the vocal fold involve junctional complexes such as adherens junctions, desmosomes and tight junctions [15]. Among these complexes, tight junctions are thought to control paracellular permeability [16–18]. When epithelial barriers of vocal folds are damaged by gastric contents, the lamina propria can also be affected and lead to a series of vocal pathologies.

There have been several recent reports on the effects of LPR on vocal fold barrier function. The effect of LPR on vocal fold barrier function has been investigated using ex-vivo models [8,19] and in vivo models [20]. However, these studies only examined the injury severity following acid-pepsin exposure. For treatment of LPR-induced vocal problems, a better understanding of the recovery process after exposure to gastric contents is required. In this study we aimed to clarify the effects of gastric acid and pepsin on the epithelial barrier of vocal folds. We first developed a novel in vitro model of vocal fold epithelium using rat vocal fold epithelial cells, and then investigated epithelial recovery processes in terms of barrier function after exposure of these cells to acid and pepsin.

2. Materials and methods

2.1. Animals

Male specific pathogen-free (SPF) Sprague-Dawley (SD) rats (body weight: 160–200g; age: 9 weeks) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Microbiological monitoring items for SPF rats are listed in [Supplementary Table 1](#). The animals were housed under controlled conditions (temperature: 22–26 °C; humidity: 40–60 %; type of bed: wood chips mainly made from domestic cypress). This study was conducted according to the Fundamental Guidelines for Proper conduct of Animal Experiment and related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japanese Government. All experimental protocols were approved by the Animal Committee of the Graduate School of Medicine, Kyoto University (Med Kyo 23113). Animal care was provided under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. Sixteen rats were used in this experiment. All rats were euthanized with carbon dioxide. Larynges of 8 rats were used to create an in vitro model, 4 larynges for immunostaining and remaining 4 larynges for electron microscopy, respectively.

2.2. Isolation of vocal fold epithelial cells

Larynges were treated with 50 U/mL Dispase II (Roche Life Sciences, Indianapolis, IN) in culture medium (see below) at 37 °C for 0.5 h to cleave the fibronectin and type IV collagen of the lamina propria. Following incubation, the epithelial layer of the vocal fold was collected and treated with 0.05 % trypsin-EDTA solution (GIBCO, Grand Island, NY) at 37 °C for 15 min. Additional medium was then added to dilute the trypsin activity, and the isolated cells were collected by centrifugation at 195g (1100 rpm) for 5 min. The collected cells were seeded on collagen-coated 12 well culture plates (Corning, ME).

2.3. Vocal fold epithelial cell culture

Unless otherwise indicated, culture medium was composed of DMEM/Ham's F12 1:1 with L-glutamine, sodium pyruvate, HEPES (Nacalai Tesque, Kyoto, Japan), 10 % fetal bovine serum (FBS, HyClone, South Logan, UT), penicillin-streptomycin solution (penicillin 5000

units/mL, streptomycin 5000 µg/mL, Nacalai Tesque), epidermal growth factor (10 ng/mL, Peprotech, Rocky Hill, NJ), insulin (5 µg/mL, Sigma-Aldrich, Darmstadt, Germany) and adenine (24 µg/mL, Sigma-Aldrich) [21,22].

The harvested cells were cultured on collagen-coated plates, on which feeder cells had been cultured ([Fig. 1A](#)). Plates were coated by incubation at 37 °C for 1 h with 0.5 mL of a 37.5 g/mL collagen solution (Advanced Biomatrix Pure Col, Carlsbad, CA) in each well. The plates were then rinsed with phosphate buffered saline (PBS). Feeder cells were prepared by treating 3T3-Swiss cells (3T3-Swiss Albino, ATCC CCL®-92™, ATCC, Manassas, VA) with 10 µg/mL mitomycin C (Sigma-Aldrich) at 37 °C for 2.5 h, and seeding at a density of 1.0×10^5 cells/cm². Epithelial cells were seeded 1 day after seeding of the feeder cells.

In primary cultures, half the medium volume was exchanged on day 2 of culture and the full volume of the medium was exchanged after day 4. On day 7 of primary culture, the epithelial cells were passaged. The First Passage (PS1) was performed after vocal fold epithelial cells reached about 90 % confluence.

During the passage process, feeder cells and any vocal fold fibroblasts were removed from the plates by treating with a 0.05 % trypsin-EDTA solution at room temperature. Under microscopic observation, immediately after feeder cells and vocal fold fibroblasts were detached from the culture plate surface, the solution in the plate was removed. The remaining epithelial cells were harvested by treating with 0.05 % trypsin-EDTA solution for ≤20 min. The epithelial cells were recovered by centrifugation for 5 min at 195 g (1100 rpm), resuspended in medium, and counted using a hemocytometer.

To seed PS1 epithelial cells, collagen-coated cell culture inserts (Corning Transwell, Sigma-Aldrich, St. Louis, MO) were used to enable separation of epithelial cells and feeder cells ([Fig. 1A](#)). Epithelial cells were seeded on the insert, while mitomycin-C treated feeder cells were seeded on the plate bottom. Both types of cells were seeded at a density of 1.0×10^5 cells/cm².

2.4. Immunofluorescence

To examine the distribution of tight junction and desmosomes associated proteins, vocal fold specimens and cultured cells (PS1 day 7) were fixed for 30 min with 4 % (w/v) paraformaldehyde in PBS at room temperature and washed with PBS. They were then immersed in embedding medium (Optimum Cutting Temperature Compound; Tissue-Tek Inc., Kyoto, Japan) and frozen quickly with liquid nitrogen. Cryostat sections of 10 µm thickness (Leica, CM 1850 Kryostat; Leica Instruments GmbH, Nussloch, Germany) were made. The cutting plane of vocal fold specimen was vertical to the glottis (coronal sections). In vitro section preparation was performed vertical to the membrane. Then they were air-dried and permeabilized using 0.4 % (w/v) Triton X-100 for 30 min, blocked with 1 % (w/v) protease-free bovine serum albumin (Wako, Osaka, Japan) for 30 min and incubated with a primary antibody at room temperature for 1.5 h. The samples were then incubated with the secondary antibody at room temperature for 1 h, washed three times in PBS for 10 min each and mounted using Fluoromount-G (Southern Biotech, Birmingham, AL). Samples images were acquired with a ZEISS LSM 900 confocal microscope ([Fig. 1B–E](#)).

The following primary antibodies were used for immunohistochemistry and immunofluorescence: rabbit monoclonal *anti*-occludin (1:100, Abcam plc, Cambridge, UK) and rabbit polyclonal anti desmoglein 1 antibody (1:100, Protein Tech, Rosemont, IL). The following secondary antibodies (all from Invitrogen) were used: Alexa Fluor 546 goat anti-rabbit immunoglobulin G, Alexa Fluor 488 goat anti-rabbit immunoglobulin G. Cell nuclei were stained with 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI; Life Technologies, Grand Island, NY).

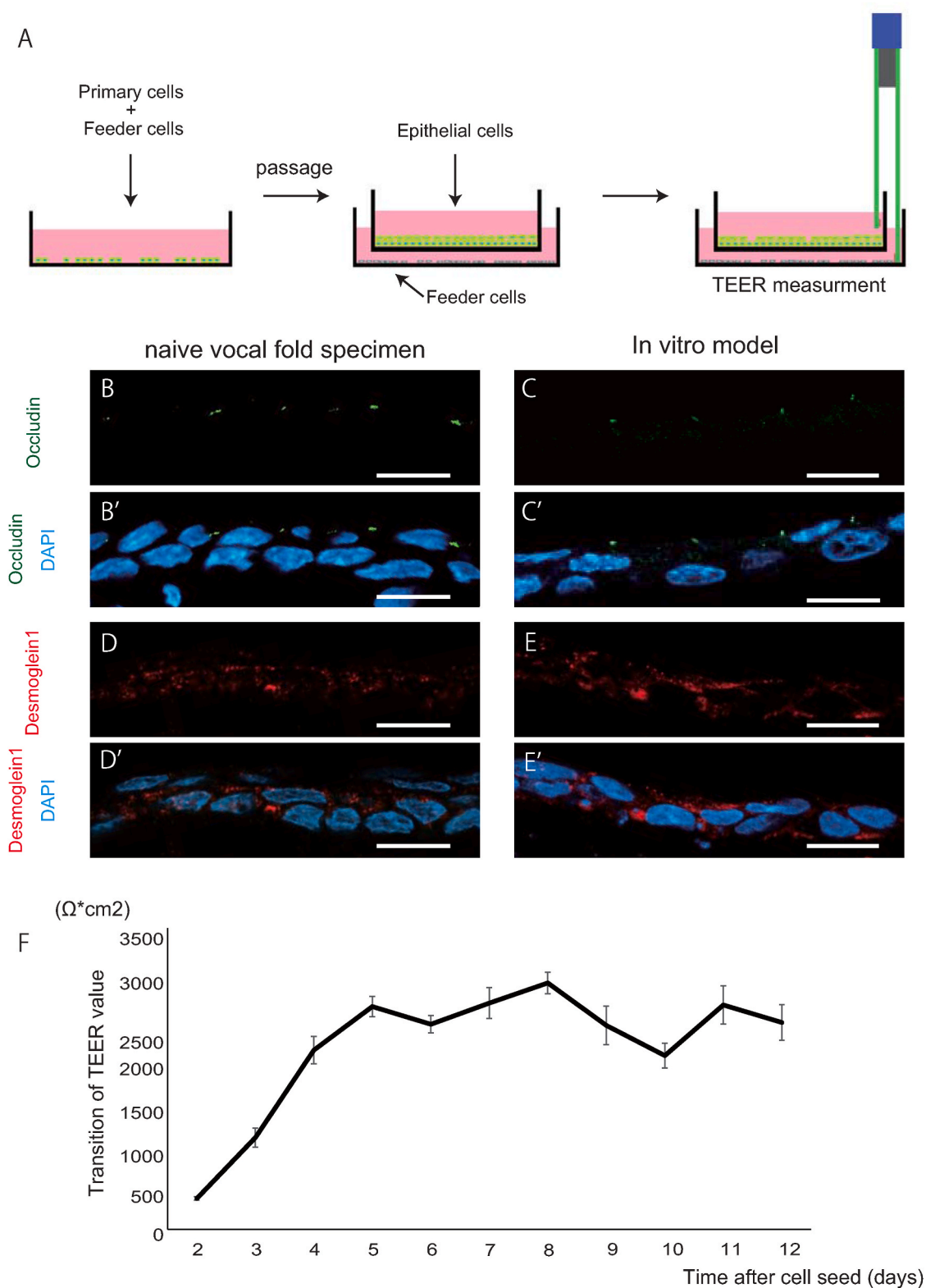


Fig. 1. Development of an in vitro model, driving from rat vocal fold epithelial cells. **(A)** Vocal fold epithelial cells were co-cultured with feeder cells and reached confluence by culture day 7, when epithelial cells were passaged and seeded into inserts. To measure TEER values, the electrodes were placed inside and outside the insert. **(B–E')** The images with apostrophe indicate with DAPI counterstaining. **(B–C')** Occludin expression was measured in vocal fold specimens **(B, B')** and the in vitro models **(C, C')**. The apical lateral borders of adjacent epithelial cells were stained green dots. Scale bar: 10 μm . **(D–E')** Desmoglein 1 expression was measured in vocal fold specimens **(D, D')** and in vitro models **(E, E')**. The expression was scattered at the cell boundaries. Scale bar: 10 μm . **(F)** The transition of TEER values after the second day of PS1 cells was shown. The x-axis represents the time after passage. Values are presented as the mean $\pm$ standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.5. Transepithelial electrical resistance (TEER)

Measurement of TEER is a widely accepted approach for assessing the integrity of epithelial layer barrier function [23–25] as well as for continuous evaluation of epithelial barrier function over time. TEER values decrease immediately after exposure to several irritating substances [26,27].

To measure TEER, feeder and epithelial cells were cultured in the lower and upper compartments, respectively, of culture inserts as described above (Fig. 1A). TEER was measured using an epithelial volt-ohmmeter (EVOM2, World Precision Instruments, Sarasota, FL) equipped with a measurement chamber (World Precision Instruments) to evaluate the resistance across the epithelium [8,28–30]. Before measurement, electrodes were sterilized. Medium in the insert and the lower compartment was renewed with five hundred microliters of fresh culture medium. The final unit area resistance ($\Omega \cdot \text{cm}^2$) was calculated by multiplying the sample resistance by the effective area of the membrane (1.12 cm^2 for 12-well inserts).

2.6. Electron microscopy imaging of ultrathin section

For electron microscopy thin-section imaging analysis, vocal fold specimens and cultured cells (PS1 day 7) on inserts were pre-fixed with 2.5 % (w/v) glutaraldehyde in 100 mM HEPES buffer at 4 °C overnight. The specimens were then post-fixed with 1 % (w/v) osmium tetroxide (OsO_4). After post-fixation, these specimens were dehydrated with a dilution series of ethanol (50 %, 60 %, 70 %, 80 %, 90 %, 99 %, and 100 %) and embedded in EPON 812 (Nacalai Tesque, Kyoto, Japan) resin. Ultrathin sections of 70–90 nm thickness were cut with a diamond knife (Ultra, 45°, DiATOME, Nidau, Switzerland). The sections were picked up on copper grids for transmission electron microscopy (TEM) or collected on the cleaned silicon wafer strip (about 5–6 mm $\times$ 10–15 mm) for scanning electron microscopy (SEM). Those sections for transmission and scanning electron microscopy were stained using uranyl acetate and lead citrate. The sections were imaged using a TEM (JEM-1400 flash; JEOL, Tokyo, Japan) and a SEM (JSM-7900F; JEOL, Tokyo, Japan). Backscattered electron (BSE)-mode [31] was also used for SEM observation.

2.7. Exposure to acidic pepsin

The pH of the medium was measured with a pH meter (D-21, HORIBA, Ltd, Kyoto, Japan) and adjusted with hydrochloric acid. Porcine pepsin (Sigma-Aldrich) having an optimum pH of 2–4 was used. Experiments involving acid-pepsin damage were carried out using 2 mg/mL pepsin at pH 4 as described in previous report on gastrointestinal pH measurements in fasted rats and in previous acid pepsin injury studies. Experiments involving acid-pepsin damage were carried out using 2 mg/mL pepsin at pH 4 as described in previous report on gastrointestinal pH measurements in fasted rats [32] and in previous acid-pepsin injury studies [20,33,34]. Cells that were not treated with acidic pepsin served as normal controls. After exposure to acid-pepsin for 5 min, the medium inside the insert was replaced with medium lacking pepsin and the repair process was evaluated. The medium was fully exchanged before measuring TEER.

2.8. Cell viability and density

Cell viability of three experimental groups was compared: pre-injury, immediately after injury and 1-day post-injury. Immediately after the 5 min treatment to induce injury, the acidic medium was exchanged with normal medium. At the time of the evaluation, the medium was removed again and medium containing staining solution (Ethidium Homodimer III, PromoCell GmbH, Heidelberg, Germany and Hoechst 33342, Nacalai Tesque) was added and incubated for 20 min in the dark at room temperature. After removing the staining solution, the cells were washed

with PBS three times and imaged by microscopy.

Cell density was evaluated by DAPI staining. After exchanging acidic medium with normal medium, cells were fixed with 4 % PFA, and DAPI was added to the PFA. The number of cells in microscopy images was counted for each experimental group in 100 $100 \times 100 \mu\text{m}$ regions in randomly-chosen fluorescent images.

2.9. Scanning electron microscopy (SEM) procedures

Damage to the epithelial cell surface of three experimental groups was also evaluated by SEM imaging: pre-injury, immediately after injury and 1-day post-injury. Specimens from three experimental groups were pre-fixed as described above. After fixation, tissues were dehydrated with ethanol, immersed in t-butanol, and frozen at -20°C . Then the t-butanol was sublimed off. Samples were sputter-coated with a platinum-palladium alloy using an ion coater (JEC-3000FC, JEOL, Tokyo, Japan) and then inspected using a SEM (JSM-7900F; JEOL, Tokyo, Japan). A total of 100 SEM images ($8 \times 8 \mu\text{m}$ at $5000\times$ original magnification) were randomly selected from each group. Otsu's method with ImageJ software (W. Rasband, National Institutes of Health, Bethesda, MD) was used to evaluate SEM images [11]. In this method, an image is treated as a bi-modal histogram and the optimum threshold dividing the two classes is evaluated. The selected threshold is specific to each image, but is calculated in an automated and consistent manner. Each image was classified as either microprojection or background using ImageJ.

2.10. Statistical analysis

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [35]. To compare the relative ratio of dead to total cells, non-parametric Kruskal-Wallis tests followed by Mann-Whitney U tests and Bonferroni correction were applied, because the data distributions deviated from normality (Fig. 3A). Alpha levels were adjusted to 0.005 to control for type I error (0.05/10) using two-tailed P values. To compare the density of DAPI-positive cells, a two-way factorial analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons was carried out. Alpha levels were adjusted to 0.005 to control for type I error (0.05/10) using two-tailed P values (Fig. 3B).

TEER values after injury were compared by calculating a two-factor repeated measures ANOVA. Relative values of TEER measured for each of the three experimental groups were compared with a Bonferroni post hoc test (Fig. 3C). When the assumption of sphericity was violated by Mauchly's test, the Greenhouse-Geisser correction was applied. Alpha levels were adjusted to 0.0167 to control for type I error (0.05/3) using two-tailed P values.

To determine the effect of injury on the density of microprojections, natural log transformation was performed, because the homogeneity was rejected by Levene's test. Log transformation of the density of microprojections followed a normal distribution and homogeneity. It enabled statistical analysis by a two-way ANOVA. Alpha levels were adjusted to 0.017 to control for type I error (0.05/3) using two-tailed P values (Fig. 4D).

3. Results

3.1. Multilayered structures and characterization of cultured epithelial cells

Epithelial cells taken from 1 to 2 mm-long samples of rat vocal fold tissues were grown in primary cultures. On culture day 3, epithelial cell colonies could be confirmed by bright field microscopy. During coculture, epithelial cells proliferated and pushed away the feeder cells. On culture day 7, epithelial cells reached confluence. Stratified epithelia with two or three cell layers could be observed by immunofluorescence

of cross sections of vocal fold specimens and cultured cells (PS1 7-days) (Fig. 1B', 1C', 1D', 1E'). In each of them, the expression of occludin, a marker for tight junctions, were also localized as punctate structures at the apical lateral borders of adjacent epithelial cells (Fig. 1B-C'). Desmoglein 1 is one of the main structural components of desmosome which is located at cell boundaries [36]. The expression of desmoglein 1 were scattered at the cell boundaries, consistent with those of vocal fold specimens.

Electron microscope observation was performed to confirm the fine morphology of the cultured cells and naive vocal fold specimens. In the observation of BSE-mode SEM and TEM images of vocal fold cross section, intercellular spaces and desmosomes which were scattered at the boundaries of adjacent cells, were observed (Fig. 2A and C). Moreover, tight junctions were found at the apical lateral borders of adjacent epithelial cells as same as the immunofluorescence observation (Fig. 2A, B, 2C, 2D). In these observation, the cultured cells had an appearance similar to that of vocal fold specimens. Additionally, SEM images revealed the existence of microprojections, both in superficial layers of vocal folds and in vitro cultured cells (Fig. 2E-G).

Changes in TEER values, a measure of epithelial layer barrier function, during the reconstruction process of rat epithelial cells were next assessed (Fig. 1F). On day 2 of PS1 culture, the TEER values for epithelial cells were approximately $500\Omega\cdot\text{cm}^2$. After day 4, the value rose sharply to approximately $2000\Omega\cdot\text{cm}^2$. After the TEER reached a maximum value, it then plateaued and maintained that value for one week.

3.2. Cell viability and cell density (in vitro injury model)

Cultured cells (PS1 7-days) exhibited mature barrier function, and were then exposed to acid-pepsin treatment. To assess the injury process, we first evaluated the relative ratio of dead cells and cell density, using Live/Dead assay and DAPI staining, respectively. The number of EthD-III positive cells (i.e., dead cells) among Hoechst 33342-positive cells (total cells) was determined and the ratio of dead to total cells was compared among the treatment groups. No significant difference in cell viability was found between the groups ($p = 0.164$, Kruskal-Wallis). Mann-Whitney U tests (Fig. 3A) also demonstrated no significant difference among the groups (0 min: $p = 0.470$, 1-day: $p = 1.000$;

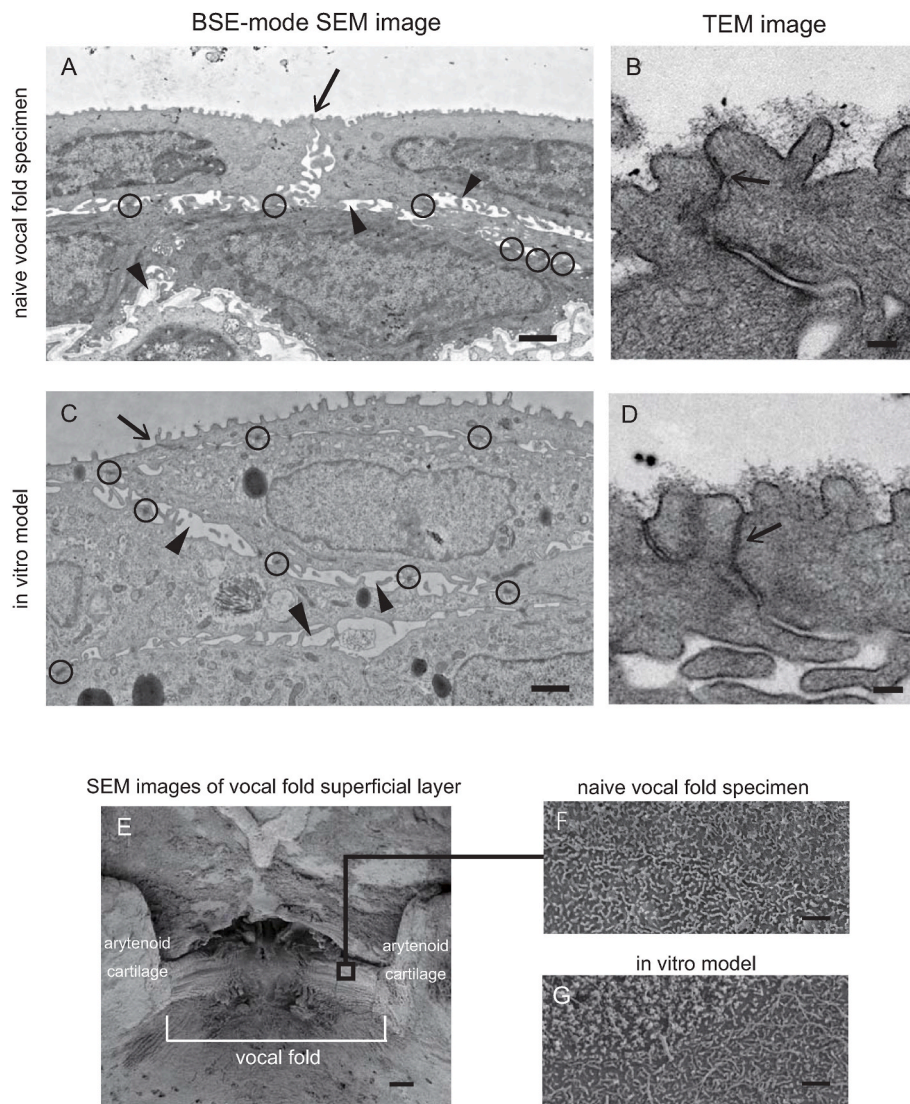


Fig. 2. Evaluation of tight junctions by immunofluorescence and electron microscopy. (A, C) BSE-mode SEM image showing the presence of tight junctions (arrow) at the cell boundaries on the cell surface. The large intercellular spaces (arrowhead) between adjacent cells and desmosomes (circle) were scattered around the cell boundaries in both vocal fold specimens (A) and in vitro (C). Scale bar: 1 μm . (B, D) Representative transmission electron microscopy (TEM) image showing the presence of tight junctions (arrow) in both vocal fold specimens (B) and the in vitro (D). Scale bar: 100 nm. (E) SEM image of a rat larynx specimen which cut open on the back side. Scale bar: 100 μm . (F, G) SEM images of vocal fold superficial layer in vocal fold specimens (F) and in vitro model (G). Scale bar: 1 μm .

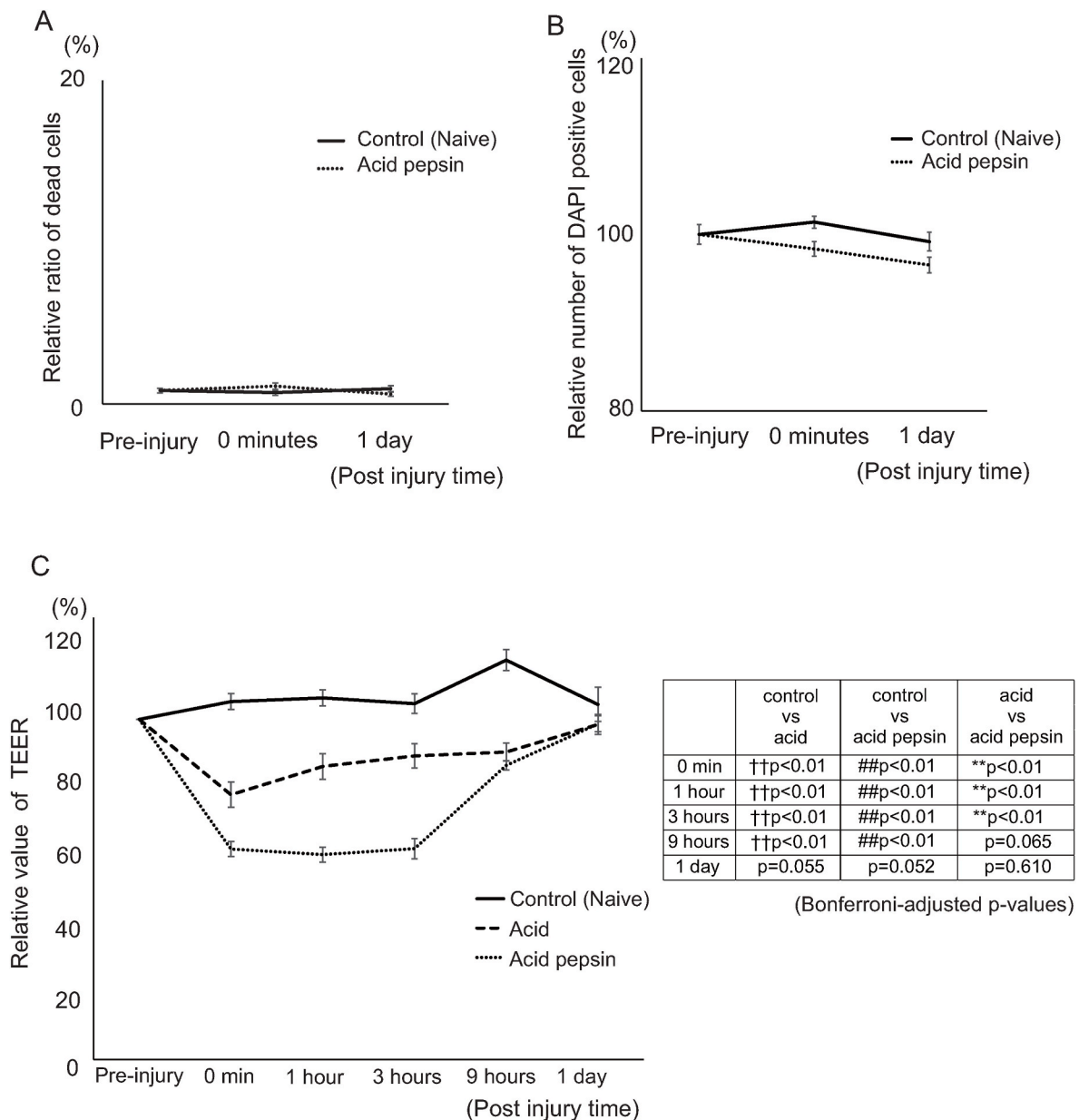


Fig. 3. Evaluation of epithelial cell viability, density and TEER values after damage. (A) The relative ratio of dead cells to the total number of epithelial cells was compared after acid-pepsin injury. No significant difference was found between the groups ($p = 0.164$, Kruskal-Wallis). (B) Cell density was also evaluated using DAPI staining, since dead cells may have been removed during medium exchange. The Pre-injury group was set as 100 %. Two-way ANOVA showed no significant difference among injury type ($p = 0.065$), time ($p = 0.586$) and interaction effects ($p = 0.422$). (C) Change in TEER values after damage. The y-axis shows the percentage of TEER and the pre-injury state was set as 100 %. The x-axis shows the post injury time. A two-factor repeated measures ANOVA after Greenhouse-Geisser correction showed significant differences in the time factor ($p < 0.01$), injury type ($p < 0.01$), and interaction effects ($p < 0.01$). In the two-factor repeated measures ANOVA, the assumption of sphericity was violated by Mauchly's test (time factor $p < 0.01$, interaction effect $p < 0.01$). In each panel, solid, dashed and dotted lines indicate control (pH 6.8 medium), acid (pH 4) challenge and acid-pepsin (pH 4 + 2 mg/mL pepsin) challenge, respectively. †† $p < 0.01$ control (pH6.8) vs acid (pH4); ## $p < 0.01$ control (pH6.8) vs acidic pepsin (pH 4 + 2 mg/mL pepsin); ** $p < 0.01$ acid (pH4) vs acidic-pepsin (pH 4 + 2 mg/mL pepsin). The values are presented as the mean $\pm$ SE.

Bonferroni correction changed the significant p-value threshold from $p < 0.05$ to $p < 0.005$.

As dead cells may have been removed during medium exchange and the staining process of the Live/Dead assay, we also measured cell density with simple DAPI staining (Fig. 3B). A two-way ANOVA showed no significant difference by injury type ($p = 0.065$), time ($p = 0.586$) and interaction effects ($p = 0.422$). An additional Bonferroni test also indicated no significant difference at each time point (0 min: $p = 0.568$, 1-day: $p = 0.799$; Bonferroni correction changed the significant p-value threshold from $p < 0.05$ to $p < 0.005$). There was no significant

difference in cell density with DAPI, and it is highly likely that the number of dead cells washed away by the medium exchange was very small.

3.3. Transition of TEER (in vitro injury model)

Cultured epithelial cells having a TEER value of approximately $1900 \Omega \cdot \text{cm}^2$ were used to evaluate injury and the recovery process after acid exposure. Epithelial cells were treated with acid only or acid-pepsin for 5 min (Fig. 3C). TEER values had significantly decreased at the end of

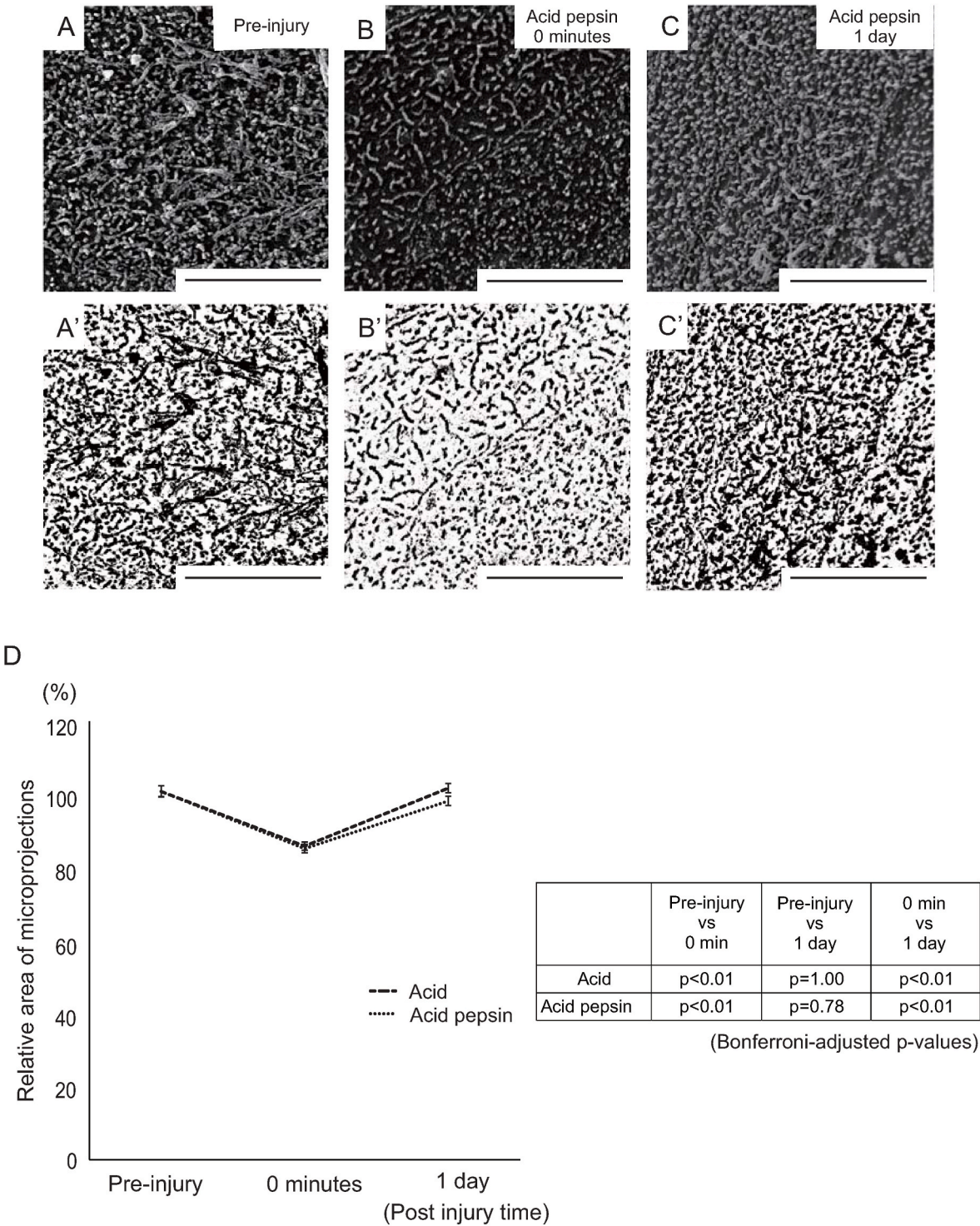


Fig. 4. SEM evaluation of acid-pepsin damage to epithelial cell microprojections. (A–C) Acid-pepsin damage to epithelial cell microprojections was evaluated using SEM. (A', B', C') Binary images were created to assess the microprojections amount. Otsu's method was applied with Image J to create binary images. (A'), (B'), and (C') are converted from (A), (B), and (C), respectively. Scale bar: 5 μ m. (D) Damage to microprojections was evaluated by measuring the area of damage in binary images. The y-axis represents the percentage of relative area of the epithelial surface microprojections, and the average for the pre-injury group was set as 100 %. A two-way ANOVA showed a significant difference in time factors ($p < 0.01$) and no significant difference among injury type ($p = 0.217$) and interaction effects ($p = 0.452$). The dashed line indicates acid (pH 4) challenge and the dotted line indicates acid-pepsin (pH 4 + 2 mg/mL pepsin) challenge. Evaluation was performed pre-injury, immediately after injury, and 1 day post-injury. The values are presented as the mean $\pm$ SE. * $p < 0.05$.

the 5-min treatment period (control vs. acid 0 min after injury period: $p < 0.01$, control vs. acid-pepsin 0 min after injury period: $p < 0.01$; Bonferroni correction changed the significant p-value threshold from $p < 0.05$ to $p < 0.0167$). In observing the recovery process, TEER values

recovered to baseline within 24 h (control vs. acid 1 day after injury: $p = 0.055$, control vs. acid-pepsin 1 day after injury: $p = 0.052$; Bonferroni correction changed the significant p-value threshold from $p < 0.05$ to $p < 0.0167$). Compared to the acid-only condition, TEER values decreased

significantly with acid-pepsin treatment (acid vs. acidic-pepsin 0 min after injury: $p < 0.01$, acid vs. acidic-pepsin 1 h after injury: $p < 0.01$, acid vs. acidic-pepsin 3 h after injury: $p < 0.01$; Bonferroni correction changed the significant p-value threshold from $p < 0.05$ to $p < 0.0167$).

3.4. Scanning electron microscopy analysis of epithelial surface damage (in vitro injury model)

To investigate epithelial surface damage, we examined damage to microprojections on the surface of epithelial cells using SEM. Otsu's method, which was developed to evaluate epithelial surface damage [11], was used. The area of the microprojections was estimated in each image that had been processed with binary coding, (Fig. 4A–C, A'–C'). A two-way ANOVA showed a significant difference in time factors ($p < 0.01$) and no significant difference among injury type ($p = 0.217$) and interaction effects ($p = 0.452$). An additional Bonferroni test was performed. The relative area of the microprojections significantly decreased immediately after injury (Pre-injury vs. 0 min under acidic condition: $p < 0.01$, under acid-pepsin condition: $p < 0.01$; Bonferroni correction changed the significant p-value threshold from $p < 0.05$ to $p < 0.0167$), and recovered within 24 h (Pre-injury vs. 1 day under acidic condition: $p = 1$, under acid-pepsin condition: $p = 0.78$) (Fig. 4D).

4. Discussion

In this study, we successfully established an in vitro experimental rat vocal fold epithelium model that could demonstrate the recovery process of barrier function after exposure to acid-pepsin. Our results revealed that acid damage induces increased paracellular permeability, and the effect was enhanced by the addition of pepsin. However, the presence of pepsin appeared to have only a small effect on epithelial cell microprojections, which are associated with the protective effect provided by mucus. Further, our results confirmed that injury induced in the vocal fold epithelial barrier by a single exposure to acid-pepsin was reversible and healed within one day.

To our knowledge, this is the first report of an in vitro model of acid injury using rat vocal fold epithelium. The study protocol was adapted from techniques used for previous studies using a rabbit model [22]. Compared to rabbit, a rat model has significant advantages from the perspective of a larger body of knowledge of genetic characteristics and a wider selection of immunohistochemical antibodies.

In this model, we confirmed the stratified epithelium and the development of tight junctions and desmosomes by using immunofluorescence and electron microscopic observations, which were similar to the morphology of actual vocal fold epithelium. These findings indicated current in vitro model is well expressing intercellular structures related to barrier function and cell adhesion. Tight junction, which plays an electrical barrier in the epithelium in controlling the permeability of small molecules and ions [16–18], were formed between cells in the uppermost layers in the in vitro model, as in vocal fold tissue. On the other hand, desmosomes, which represent the intercellular adhesion factors of the cadherin family, are not directly involved in the barrier function that controls molecular permeation, but have the function of mechanically adhering adjacent cells. In stratified vocal fold epithelium, there are numerous intercellular spaces between adjacent epithelial cells, and numerous desmosomes are tightly attached to adjacent cells. These structures are thought to contribute to forming a tensile strength structural framework with flexibility against vibration and abrasion [37–39]. These intercellular spaces and desmosomes are well developed in our in vitro models and well represent the structure of actual vocal fold epithelium.

Following the evaluation of the morphological structure, we also carried out the measurement of TEER to evaluate the barrier function of cultured epithelium. For PS1 cells, TEER values reached a maximum on day 5, and tight junction and desmosome structures could be visualized by immunofluorescence on day 7. Both structures were also confirmed

by TEM and SEM imaging. After the TEER reached the maximum value, it plateaued and was maintained for one week. On the other hand, previous reports described assessment of ex vivo vocal fold epithelium up to 30 min after excision [8]. Compared to this ex-vivo culture, the in vitro model described here allows a relatively long-term evaluation. This in vitro experimental model might thus contribute to assessment of the effects of chemical and physical injuries localized to the vocal fold.

This is the first study to evaluate the repair process after LPR damage. We found that acid-pepsin exposure rapidly increased ion permeability without inducing cell death. We further showed that changes in epithelial permeability could be recovered after a single exposure to acid-pepsin. To assess the effects of the presence of pepsin on injury, which would mimic the effect of reflux of gastric contents, an acid-only condition was also evaluated. TEER values were significantly worsened upon exposure to acid-pepsin, compared to acid-only exposure. These results suggest that proteins constituting tight junctions could be damaged by the proteolytic activity of pepsin, and this damage translates to increased permeability between epithelial cells.

SEM images also revealed damage to microprojections, which, like paracellular permeability, recovered relatively rapidly. However, we observed no significant difference between the acid-only condition and the acid-pepsin condition. As microprojections are protrusions in which sphingomyelin, a type of cell membrane phospholipid, is present in large amounts [40], the proteolytic effect of pepsin may have had little additional effect.

This study does have several limitations. First, the cultured cells have limited proliferative capacity and may have variability as they originate from primary cultures and are not cell lines. Second, given the nature of this in vitro study, the experimental model may not fully mimic the physiological condition of vocal fold. For example, factors associated with vascular migration could not be evaluated in this model.

Additionally, the current study was designed to measure the effects of a single exposure to acid, whereas clinically reflux is often repetitive. Thus, experiments with repeated exposure of acid-pepsin on vocal fold epithelium are needed to clarify at which point epithelial barrier function is irreversibly affected. We must also evaluate the mucus-retaining function of microprojections, which would be a more direct measure of barrier capacity.

This new experimental system will be valuable for evaluating the mechanisms associated with repair processes induced after vocal fold epithelial injuries and could be applied to evaluate other types of injuries to vocal fold tissues.

5. Conclusions

The pathophysiology of LPR-induced chronic laryngitis and dysphonia has not yet been fully elucidated. Here an in vitro experimental system was developed to evaluate mechanisms of vocal fold epithelial injury and repair. In this model, samples exhibited the characteristic structure of non-keratinized stratified squamous epithelium, with tight junction and cell surface microprojections. The model was also used to demonstrate that acid-pepsin injury was reversible and healed within one day. The results suggested that pepsin had a strong effect on tight junctions but not on microprojections. This model may allow assessment of changes in vocal fold epithelia over time following other chemical or physical injuries. These results will help elucidate the unique response of vocal fold epithelium to injury and facilitate development of novel treatments for these injuries.

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Ethical statements

All applicable institutional guidelines for the care and use of animals were followed. All experimental protocols were approved by the Animal Committee of the Graduate School of Medicine, Kyoto University (Med Kyo 23113). Animal care was provided under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University.

CRediT authorship contribution statement

Keisuke Kojima: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Tatsuya Katsuno:** Visualization, Validation, Data curation, Conceptualization. **Yo Kishimoto:** Validation, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Masanobu Mizuta:** Supervision, Methodology, Funding acquisition. **Ryosuke Nakamura:** Methodology. **Hiroe Ohnishi:** Methodology. **Koichiro Yamada:** Funding acquisition, Conceptualization. **Yoshitaka Kawai:** Visualization, Supervision, Project administration, Methodology, Conceptualization. **Ichiro Tateya:** Supervision, Funding acquisition, Conceptualization. **Koichi Omori:** Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Masanobu Mizuta, Ichiro Tateya reports financial support was provided by Japan Society for the Promotion of Science. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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