Endoscopic sealing with a polyglycolic acid sheet for restoration of vocal fold mucosa in dogs

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Short running title: PGA sheet sealing after vocal cordectomy

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Abstract

Objective: Voice outcomes of cordectomy for early glottic cancer are often poor due to vocal fold scarring and tissue defects. Improvements in this aspect could make cordectomy a more acceptable treatment option than radiotherapy. We hypothesized that a polyglycolic acid (PGA) sheet could be used to cover vocal fold defects. The present study aimed to prevent vocal fold scarring after cordectomy using the PGA sheet.

Study Design: Animal experiment

Methods: Nine male beagles were divided into three groups including a control group (n=3). Following cordectomy, the vocal fold defect was covered with the PGA sheet plus fibrin glue (PGA group, n=3) or with the PGA sheet plus fibrin glue containing basic fibroblast growth factor (bFGF) (PGA-bFGF group, n=3). Vocal folds were chronologically observed, and larynges were removed six months after surgery. Mucosal amplitude was measured using a high-speed camera, and histological analysis was performed.

Results: The re-epithelialization process was delayed in PGA and PGA-bFGF groups compared to the control group. The mucosal amplitude was significantly more normalized and the thickness ratio significantly higher in PGA and PGA-bFGF groups compared to the control group. The PGA-bFGF group had the highest elastic fiber density, followed by the PGA group and then the control group, with a significant difference between the PGA-bFGF and the control groups.

Conclusion: The PGA sheet plus fibrin glue could serve as an effective regenerative scaffold for reconstructing vocal fold morphology and function after cordectomy, with the potential benefit of establishing an endoscopic sealing method for vocal fold defects.

Level of evidence: NA

Introduction

Vocal fold resection (cordectomy) for malignant or benign lesions is one of the most common causes of vocal fold scarring.[1] Postoperative voice quality is generally poorer with deeper resection,[2-5] which is consistent with greater glottal insufficiency due to tissue defects as well as smaller glottal vibration due to vocal fold scarring. Since oncologic outcomes are comparable between cordectomy and radiotherapy,[6–8] voice quality after cordectomy for early glottic cancer is often an important consideration when choosing the treatment modality. That radiotherapy yields superior voice outcomes has been the dominant view in the treatment of early glottic cancer.[2, 9] Therefore, improvements in voice outcomes might make cordectomy a more acceptable option for early glottic cancer.

Optimal glottal vibration depends on proper viscoelasticity and volume of the lamina propria in the vocal fold mucosa. After removing the entire layer of the lamina propria, viscoelastic reduction is caused by fibrous tissue, with a decrease in elastin and increase in collagen content.[10-12] Thus, scar formation increases the stiffness of the vocal fold, resulting in severe hoarseness during phonation.[13] The management of this condition is challenging for laryngologists given the lack of effective therapies. To date, several approaches have been used to address vocal fold scarring, including autologous fat implantation,[14] mucosal grafting,[15] and tissue engineering.[16-18] The principle of tissue engineering holds that the introduction of a fibroblast population into the scarred tissue of vocal folds could theoretically lead to reconstitution of lamina propria components and reestablishment of normal mucosal waves.

We previously reported that a tissue-engineered cell sheet from oral mucosal cells could successfully restore the morphological and functional characteristics of the vocal fold mucosa in beagles.[19] This reconstruction technique could offer substantial clinical benefits by preventing vocal fold scarring. However, unlike vocal fold injection, which is an established method, the cell sheet transplantation procedure has yet to be generalized. In order to establish a sheet sealing method for covering the vocal fold defect after cordectomy in clinical practice, a simple procedure needs to be developed that could yield a better voice outcome than cordectomy alone.

A polyglycolic acid (PGA) sheet fixed with fibrin glue has been used to cover

wounds and prevent bleeding and leakage during surgery for the liver, pancreas, and lungs, due to its ability to strongly affix to wounds.[20, 21] It is a synthetic compound that degrades completely into glycolic acid, a nontoxic degradation product, under physiological conditions.[22] Recently, there have been reports in some medical fields that the PGA sheet has the potential to minimize scar contracture.[23-25] Fibrin glue has also been examined as both a gel for cell delivery and a vehicle for drug delivery.[26] Growth factors such as basic fibroblast growth factor (bFGF) for tissue regeneration can be incorporated into fibrin glue in drug delivery systems.[27, 28]

After cordectomy, covering the raw surface of the vocal fold and promoting good mucosal regeneration are particularly important for creating a physical barrier that protects the compromised tissue in the acute phase of wound healing. Replacement of the vocal fold mucosa with the PGA sheet is a potential treatment option for preventing vocal fold scarring. The use of fibrin glue with the PGA sheet is also promising as a drug delivery system. This study aimed to clarify the usefulness of PGA sheet sealing for the prevention of vocal fold scarring.

Materials and Methods

Experimental animals and preparation

All experimental protocols used in this study were approved by the Kurume University Animal Care and Treatment Committee. Nine male beagles (KBT Oriental, Saga, Japan) weighing 10.2 to 12.8 kg were caged individually with free access to standard laboratory chow and tap water. Individual cage sizes were 950 millimeters (width) x 1000 millimeters (depth) x 1920 millimeters (height). The experimental animals were divided into three groups: the control (cordectomy alone) group (n=3), PGA group (n=3), and PGA-bFGF group (n=3). The PGA sheet (Neoveil®, Gunze Co., Ltd., Tokyo, Japan) 100 mm x 50 mm in size and 0.15 mm in thickness and fibrin glue (Bolheal®, Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) consisting of fluid-A (fibrinogen solution) and fluid-B (thrombin solution) were prepared for PGA and PGA-bFGF groups, and bFGF (Fibrast spray®, Kaken Pharmaceutical Co., Tokyo, Japan) was prepared for the PGA-bFGF

111   group. Vocal fold mucosa resection and PGA sheet sealing were performed using the

112   procedures described below.

113

Sealing method for the mucosa-deficient vocal fold

115   The experimental animals were anesthetized by intramuscular administration of

116   xylazine (2 mg/kg), midazolam (0.3 mg/kg), and ketamine (5 mg/kg). An endotracheal

117   tube was inserted, and general anesthesia was maintained by intravenous administration

118   of pentobarbital sodium (10-20 mg/kg). After a direct laryngoscope (Nagashima

119   Medical Instruments, Tokyo, Japan) was inserted into the larynx through the mouth, the

120   unilateral membranous portion of the vocal fold was resected according to the

121   cordectomy type II method proposed by the European Laryngological Society in 2000

122   (**Figs. 1a and 1b**).[29] Cordectomy was performed by cutting between the vocal mucosa

123   and vocalis muscle, i.e., resection of the full depth of the lamina propria (**Fig. 1b**). The

124   vocalis muscle was preserved to the extent possible. The resection extended from the

125   vocal process to the anterior commissure. The superior to inferior dimensions of the

126   resection were approximately half of the vocal fold thickness. The PGA sheet was

127   trimmed to a size less than the resected area to prevent it from peeling off (**Fig. 2**) and

128   saturated with fluid-B for the PGA group, and with fluid-B containing 2 ng/μl bFGF for

129   the PGA-bFGF group. After fluid-A was applied to the wound surface of the vocal fold,

130   the PGA sheet was immediately sealed to the wound surface under an operating

131   microscope (**Fig. 1c**). The PGA sheets were successfully transplanted onto the resected

132   portions of the vocal folds in both groups. The antibiotic cefazolin sodium hydrate (20

133   mg/kg/day; to prevent infection) and acetaminophen (10 mg/kg; analgesic) were

134   administered after surgery.

135

Endoscopic evaluation of vocal folds

137   Nine dogs were anesthetized with intramuscular xylazine (2 mg/kg) and

138   midazolam (0.3 mg/kg) without intubation in the supine position. The dogs' vocal folds

139   were observed using a rigid endoscope (A70940A, Olympus Co., Tokyo, Japan), camera

140   head (OTV-S7**,** Olympus Co., Tokyo, Japan), and video processor (OTV-S190, Olympus

141 Co., Tokyo, Japan) at one week, two weeks, one month, two months, and six months
142 after surgery.

143

Functional evaluation of removed larynges

145 Six months after surgery, all experimental animals were euthanized by intravenous
146 injection of pentobarbitone solution (100 mg/kg), and the larynges were removed.
147 Glottal closure was achieved by suturing the bilateral cartilaginous portion of the vocal
148 folds of the larynges. Experimental phonation was artificially induced by blowing air
149 (50-400 ml/sec) through the trachea. Vocal fold vibrations were recorded though a rigid
150 endoscope (A70940A, Olympus Co., Tokyo, Japan) with a high-speed camera system
151 (HAS-U2, DITECT Co., Tokyo, Japan).

152 Image analyses were performed to measure the area of vocal fold vibration using
153 image analysis software (WinROOF® version 5.5, Mitani Co., Ltd., Tokyo, Japan). **Fig.
154 3** shows a schematic of the procedure, where a1/a1' and a2/a2' represent the areas of the
155 glottal gap on the resected side and the healthy side, respectively, divided by the
156 borderline L in the maximum open and closed phases; L is the length from the anterior
157 commissure to the vocal process. Normalized mucosal amplitude (NMA) was
158 determined using the following formula: resection side = $(a1- a1')/L^2$ x 100, healthy
159 side = $(a2-a2')/L^2$ x 100. NMAs of the resected and healthy sides were compared in
160 each group. NMAs of the resected side were also compared between groups.

161

Histological evaluation of removed larynges

163 The removed larynges were then fixed in 4% paraformaldehyde. Coronal laryngeal
164 sections were made at a thickness of 10 μm at the middle membranous portion of the
165 vocal fold. Histological examinations were performed using elastica van Gieson (EVG)
166 staining and alcian blue staining. For each dog in each group, light microscopic images
167 of the stained sections were quantitatively analyzed on a computer (Microsoft Windows
168 10) using WinROOF®, as follows: the perpendicular line from the surface of the
169 thyroarytenoid muscle to the epithelium of the EVG-stained specimen was measured as
170 the maximum thickness of the vocal fold mucosa (**Fig. 4a**), and the maximum thickness

171 of the resected side relative to that of the healthy side was measured as the thickness

172 ratio (%) of the bilateral vocal fold mucosae. Then, the clear border of the wound was

173 traced on the specimens. The EVG-stained area of elastic fibers was minutely plotted

174 using an image binarization procedure and colored in green (**Fig. 4b**). Using the same

175 procedure for the alcian blue-stained specimens, the blue-stained area of acidic

176 polysaccharide was minutely plotted and colored in clear blue (**Fig. 4c**). The area inside

177 the traced border (a) and the total colored area (b) were calculated using WinROOF®.

178 Two authors (R.M. and S.C.) performed blind measurements of areas for each specimen

179 at least three times. After confirming reproducibility, the average of the measured areas

180 was used to calculate elastic fiber density (%) and acidic polysaccharide density (%)

181 (b/a x 100). The thickness ratio, elastic fiber density, and acidic polysaccharide density

182 were compared between groups.

183

Statistical analysis

185 Statistical analysis was performed using StatMate III for Windows (ATMS,

186 Tokyo, Japan). *P* < .05 was considered statistically significant. A paired t-test was used

187 to compare NMAs between the resected and healthy sides in each group. An unpaired

188 t-test was used to compare the NMA of the resected side, thickness ratio, elastic fiber

189 density, and acidic polysaccharide density between groups.

190

Results

Endoscopic evaluation of vocal folds after surgery

193 In the control group, a large amount of granulation tissue was observed on the

194 vocal fold wound one week after surgery. In the PGA-bFGF group, a small amount of

195 granulation tissue was observed on the vocal fold wound beside the PGA sheet at one

196 and two weeks after surgery, disappearing after one month. In the control group,

197 re-epithelialization was completed without any granulation one month after surgery, but

198 after two months, there were marked tissue defects in the treated vocal folds (**Fig. 5a**).

199 In PGA and PGA-bFGF groups, a part of the PGA sheet remained near the center of the

treated vocal folds at one month after surgery, but after two months, re-epithelialization was completed without any noticeable defects (**Figs. 5b and 5c**) in all cases. There were no clear delineations between the region of the absorbed sheet and the surrounding tissue two months after surgery.

Functional evaluation of removed larynges

In the control group, the high-speed camera captured a few, irregular mucosal waves on the resected side and regular mucosal waves on the healthy side. In both PGA and PGA-bFGF groups, mucosal waves were regular on the resected side but slightly smaller than those observed on the healthy side. Image analyses revealed that the NMA was significantly smaller on the resected side compared to the healthy side in the control group (**Fig. 6**), and that the NMA of the resected side was significantly larger in PGA and PGA-bFGF groups compared to the control group. No significant differences were observed in NMA between the resected and healthy sides in PGA and PGA-bFGF groups.

Histological evaluation of removed larynges

The thickness ratios of the bilateral vocal fold mucosae were significantly larger in PGA and PGA-bFGF groups compared to the control group (**Fig. 7a**). No significant differences were observed in the thickness ratios between the PGA group and the PGA-bFGF group. The PGA-bFGF group had the largest elastic fiber density, followed by the PGA group and then the control group (**Fig. 7b**), with a significant difference between the PGA-bFGF group and the control group. No significant differences were observed in elastic fiber density between the PGA group and the PGA-bFGF group and between the PGA group and the control group. The acidic polysaccharide density did not differ significantly among the three groups (**Fig. 7c**).

Discussion

The present study demonstrated that the PGA sheet used in conjunction with fibrin

229 glue could serve as an effective regenerative scaffold in the reconstruction of vocal fold

230 morphology and function after cordectomy. As a drug delivery system, however, the

231 efficacy of bFGF remains unclear, as there were no significant differences in all

232 measured values between PGA and PGA-bFGF groups. This sealing method utilizing a

233 PGA sheet could offer substantial clinical benefit for reducing scar tissue formation and

234 atrophy of the vocal fold mucosa following cordectomy.

235   The PGA sheet consists of a nonwoven membrane of polyglactin acid, a

236 homopolymer having a molecular weight of 100,000 daltons. It has elastic, soft

237 properties that are acquired through a special process.[30] Clinical applications of PGA

238 sheets have been reported not only for preventing mucosal perforation but also

239 minimizing scar contracture.[23-25] However, few studies have reported on the process of

240 healing after wound sealing with PGA sheets *in vivo*. The wound healing efficacy of

241 PGA sheets used in conjunction with fibrin glue has been histologically demonstrated in

242 a rat cranial periosteal defect model.[31] In that study, while PGA-induced scar formation

243 and a foreign-body reaction were observed six weeks after surgery, the PGA group

244 showed better wound healing compared to the control group. Yet, as it generally takes

245 15 weeks for the PGA sheet to be completely absorbed under physiological conditions,

246 histological results over a longer period of time will be needed. The results of the

247 present study showed less scar formation in the lamina propria after wound healing at

248 six months postoperatively in the PGA groups compared to the control group.

249   The viscoelastic properties of human vocal folds depend on the cover that is

250 composed of the epithelium and superficial layer of the lamina propria. The

251 extracellular matrix (ECM) of the superficial layer of the human lamina propria consists

252 mainly of collagen, elastin, fibronectin, and hyaluronic acids,[10-12] and provides

253 mechanical strength and resistance to shear stress. The main features of vocal fold

254 scarring include disorganized collagen and elastic fibers, loss of important ECM

255 constituents, volume deficiency, loss of vocal fold pliability, and glottal

256 insufficiency.[32,33] Disorganized collagen deposition is observed in most cases of deep

257 resection involving the lamina propria, such as type II cordectomy.[29] A study on acute

258 vocal fold injury reported that the vocal fold wound healing process was analogous to

259 wound repair in the skin during the inflammatory and proliferative phases, but differed

260 during the remodeling phase, i.e., the final phase of wound healing.[34] In the remodeling

261 phase, the wound undergoes contraction resulting in a smaller amount of apparent scar

262 tissue[35] and atrophy.

263   In the present study, the re-epithelialization process was slower, with less wound

264 contraction, in the PGA group compared to the control group. Generally,

265 re-epithelialization of a wound involves the migration of epithelial cells from the edges

266 of the wound. During this process, the migration and proliferation of epithelial cells

267 depend on the interaction of epithelial cells with subepithelial fibroblasts and ECMs.[36,37]

268 The PGA sheet might modify the development of subepithelial microenvironments and

269 their interaction with ECMs. Mori et al. reported that the PGA sheet covering an

270 artificial ulcer after esophageal submucosal dissection exerted anti-inflammatory effects

271 and prompted the creation of rich granulation tissue during the healing process.[44]

272 Simultaneously, the PGA sheet prompted the migration of epithelial cells over the rich

273 granulation tissue and accelerated the activity of fibroblasts to form collagenous tissue

274 with a scar. Following these processes, the PGA became almost completely absorbed

275 within approximately 15 weeks. In another report, early anti-inflammatory effects and

276 rich granulation tissue formation were shown to play a role in protecting the ulcer floor

277 from exogenous materials and factors.[24] The slower tissue regeneration including slow

278 re-epithelialization appears to lead to the stabilization of the morphological structure

279 and function, while suppressing the production of collagen and increasing the density of

280 elastic fibers in the remodeling phase. The PGA sheet, when used in conjunction with

281 fibrin glue, can create a physical barrier that protects the compromised tissue of the

282 subepithelium after cordectomy and prevents infection.

283   Fibrin glue is necessary for the sealing of the PGA sheet. Fibrin glue itself is

284 amorphous and cannot be used as a scaffold for regenerating vocal fold defects. There

285 have been reports on the use of fibrin glue as a drug delivery system.[26, 38] To investigate

286 whether the combination of fibrin glue with the PGA sheet could extend its potential as

287 a drug delivery system, the present study used fibrin glue containing bFGF. The

288 usefulness of bFGF has recently been reported in studies concerning vocal folds.[39, 40]

289 However, the benefits of sealing the PGA sheet with fibrin glue containing bFGF could

290 not be demonstrated in the present study.

A high-speed camera can provide a slow-motion view of vocal fold vibration. It is the key tool used to analyze voice function after cordectomy. In a retrospective analysis of patients who underwent laser cordectomy for early glottic cancer, postoperative amplitude and mucosal wave patterns were reduced proportionally to the amount of cordal tissue removed.[2] Another study showed that larger glottal gaps, scarring, and decreased mucosal waves were more frequently observed in transmuscular and extended cordectomies compared to subepithelial or subligamental ones.[41] On the other hand, Kishimoto et al. reported that, while there were individual variations in temporal changes of mucosal wave amplitude and glottal gap, both parameters appeared to stabilize roughly six months after cordectomy.[42] Thus, abnormalities in vocal fold vibration are commonly associated not with only the depth of resection but also the postoperative period or severity of scar tissue formation after cordectomy.

The present study demonstrated the efficacy of endoscopic sealing using the PGA sheet in conjunction with fibrin glue in the regeneration of the resected vocal fold. To date, no reports have described an endoscopic sealing method for resected vocal folds using a sheet-like artificial material. However, there are some limitations and ethical issues that must be resolved before this method can be applied in human clinical trials. First, there is a possibility that the PGA sheet might peel off the vocal fold and enter the airway, posing a risk of airway foreign body. However, the PGA sheet will eventually be absorbed due to its biodegradability, or in most cases, it will be removed via the digestive tract or excreted from the mouth. Second, canine models frequently used in laryngeal research[43] are not ideal for all voice experiments due to the lack of vocal ligaments. Finally, studies with a larger sample size will be necessary in order to further assess the benefits and risks of this method as a potential treatment option for vocal fold scarring when standard approaches are not effective. We believe our endoscopic sealing method with the PGA sheet will lead to successful clinical application in the future.

Conclusion

The PGA sheet used in conjunction with fibrin glue could serve as an effective regenerative scaffold in the reconstruction of vocal fold morphology and function after cordectomy, with the potential benefit of establishing an endoscopic sealing method for

322   resected vocal folds. The efficacy of a drug delivery system using bFGF still remains

323   unclear. The sealing method with the PGA sheet could offer substantial clinical

324   advantages over cordectomy alone for reducing scar tissue formation and atrophy of the

325   vocal fold mucosa following the procedure.

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327 **Author's Note**

328   Ryota Mihashi and Shun-ichi Chitose equally contributed to this article.

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454

Figure Legends

Fig. 1. Sealing method using a polyglycolic acid sheet with fibrin glue following cordectomy

Endoscopic surgical views (left) and coronal schematic views (right) of the vocal folds are shown. (**a**) Vocal folds were observed under a direct laryngoscope. (**b**) The unilateral membranous portion of the vocal fold was resected to the full depth of the lamina propria, preserving the vocalis muscle. (**c**) A polyglycolic acid (PGA) sheet was grafted to the wound surface using fibrin glue. Asterisks: vocalis muscle. Arrow: coronal resection area in the schematic. Arrow heads: sealed PGA sheet.

Fig. 2. Polyglycolic acid sheet: An original sheet 0.15 mm in thickness trimmed to a size less than the resected area of each vocal fold.

Fig. 3. Measurement of normalized mucosal amplitude (NMA) of vocal fold vibration with a high-speed camera. L: the length from the anterior commissure to the vocal process. NMA of the resection side = (a1- a1')/L^2 x 100, NMA of the healthy side = (a2-a2')/L^2 x 100.

Fig. 4. Histological measurements of removed larynges

(**a**) The perpendicular line from the surface of the thyroarytenoid muscle to the epithelium of the specimen stained with elastica van Gieson (EVG) was measured as the maximum thickness of the vocal fold mucosa. The maximum thickness of the resected side relative to that of the healthy side was measured as the thickness ratio of the bilateral vocal fold mucosae. (**b**) The clear border of the wound was traced on the EVG-stained specimen. The stained area of elastic fibers was minutely plotted and then colored in green. (**c**) On the alcian blue-stained specimen, the blue stained area of acidic polysaccharide was minutely plotted and then colored in clear blue. The area inside the traced border (a) and the total colored area (b) were calculated. The average of the measured areas was used to calculate elastic fiber density and acidic polysaccharide density (b/a x 100).

486 **Fig. 5. Endoscopic laryngeal findings at one week to six months after surgery.**

487 Three representative cases from the (**a**) control, (**b**) PGA, and (**c**) PGA-bFGF groups are

488 shown. (**a**) Re-epithelialization was completed without any granulation at one month

489 after surgery, but there were marked tissue defects in the resected vocal fold (arrows)

490 after two months. (**b** and **c**) A part of the PGA sheet remained near the center of the

491 resected vocal fold at one month after surgery (arrow heads), while re-epithelialization

492 was completed without any noticeable defects after two months. PGA: polyglycolic acid,

493 bFGF: basic fibroblast growth factor

494

495 **Fig. 6. Vibratory function.** (**a**) High-speed digital imaging of vocal fold vibration of a

496 representative case in the PGA group (the right side (R) represents the surgical site). The

497 mucosal movement was almost normal, with complete glottal closure. (**b**) Measurement

498 results of normalized mucosal amplitude (NMA). NMA of the surgical side was

499 significantly smaller than that of the healthy side in the control group (paired t-test).

500 NMA of the surgical side was significantly larger in PGA and PGA-bFGF groups

501 compared to the control group (unpaired t-test). *$P < .05$, †: closed phase, ††: maximum

502 open phase.

503

504 **Fig. 7. Histological image analysis**

505 (**a**) Thickness ratios were significantly larger in PGA and PGA-bFGF groups compared

506 to the control group. Thickness ratios did not differ significantly between the PGA

507 group and the PGA-bFGF group. (**b**) The PGA-bFGF group had the highest elastic fiber

508 density, followed by the PGA group and then the control group, with a significant

509 difference between the PGA-bFGF group and the control group. (**c**) Acidic

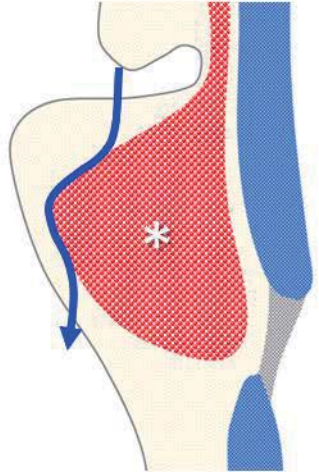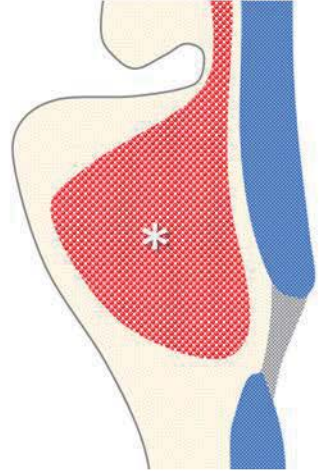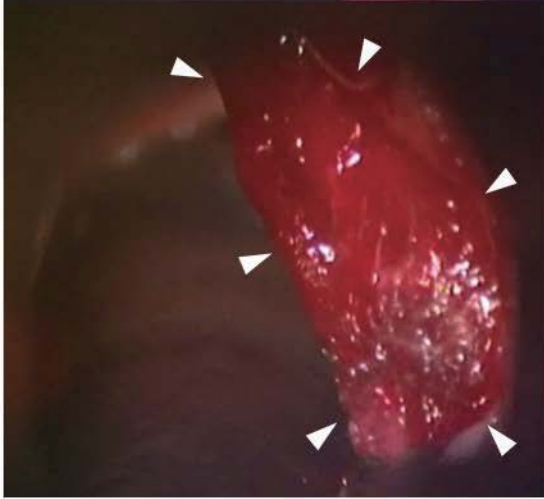510 polysaccharide density did not differ significantly among the three groups. *$P < .05$,

511 NS: not significant
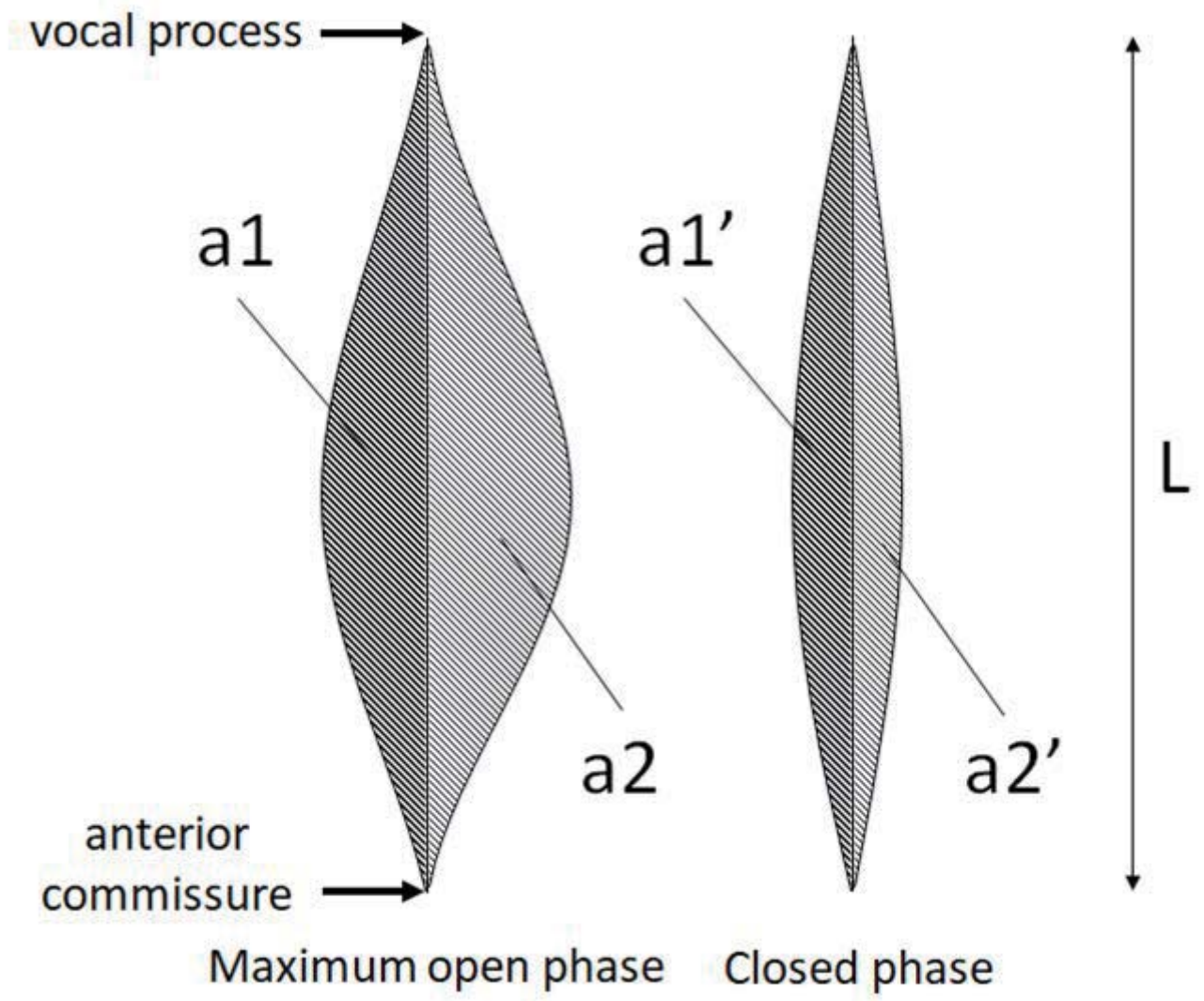
(a)

(b)

(c)

vocal process
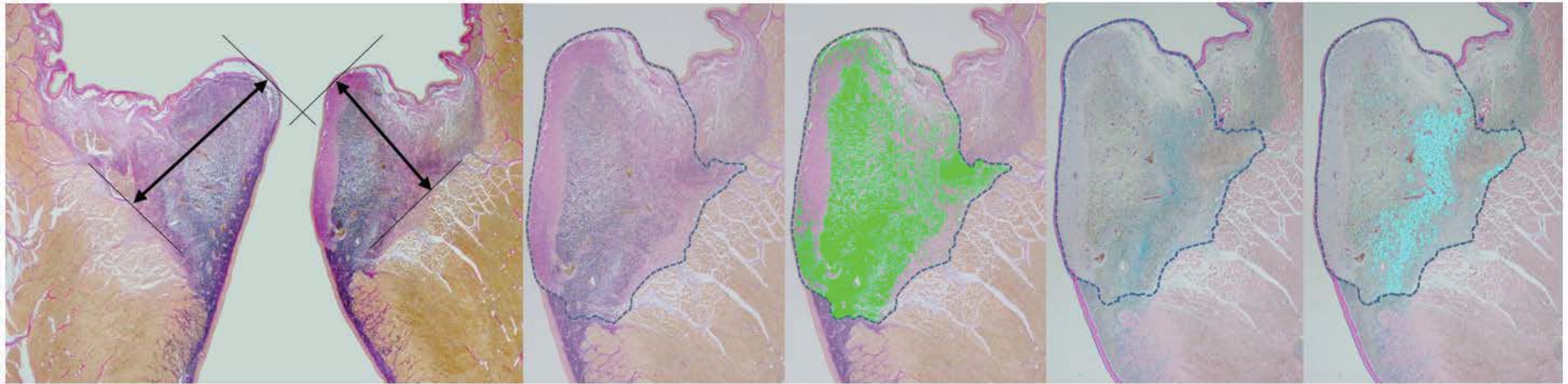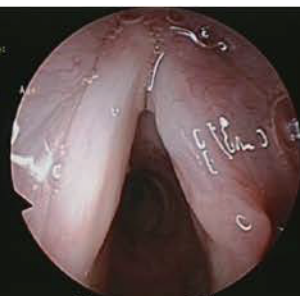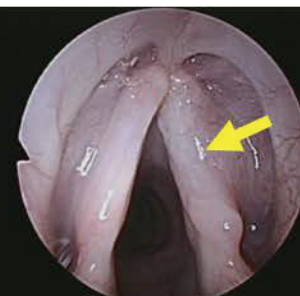
a1

a1'

a2

a2'

anterior
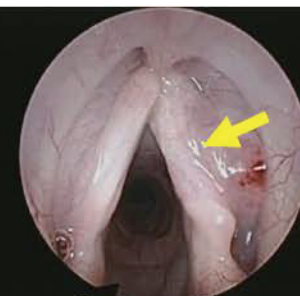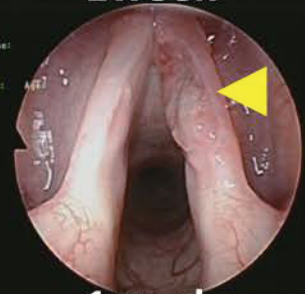commissure

L

Maximum open phase
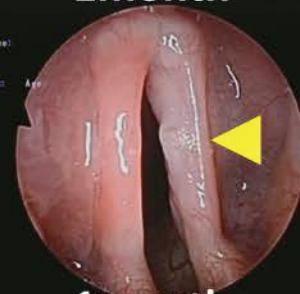
Closed phase
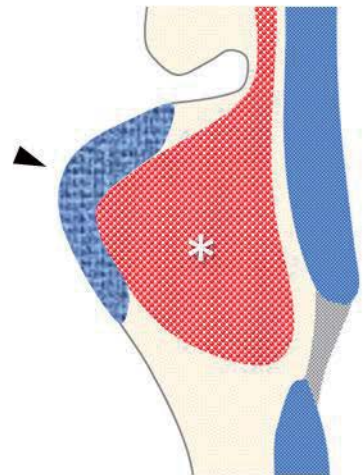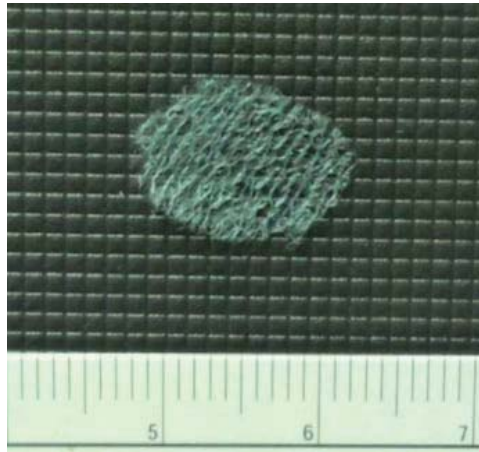
(a) (b) (c)
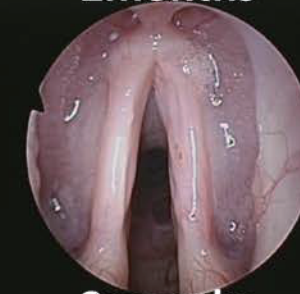
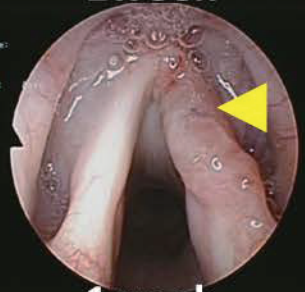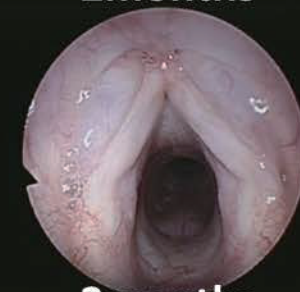(a) 1week 2weeks 1month 2months 6months

(b) 1week 2weeks 1month 2months 6months

(c) 1week 2weeks 1month 2months 6months

(a)

(b)

■ Surgical side ■ Healthy side

(a)

(b)

(%)

100
90
80
70
60
50
40
30
20
10
0

*

control   PGA   PGA-bFGF

(c)

(%)

100
90
80
70
60
50
40
30
20
10
0

NS

control   PGA   PGA-b

control   PGA   PGA-bFGF