

Changes in Tracheal Respiratory Mucosa After Thyroidectomy: A Rat Model

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Abstract. *Background/Aim:* This study aimed to investigate changes in the tracheal mucosa after thyroidectomy, that can be a cause of post-thyroidectomy discomfort. *Materials and Methods:* Forty rats were divided into normal controls and 3 surgical groups: (i) thyroid isthmectomy with cauterization, (ii) isthmectomy by a cold instrument without hemostasis, and (iii) sham (exposure of the trachea and thyroid gland without thyroidectomy by dissection through pretracheal fascia). Animals were euthanized at 1 and 4 weeks. Mucosal edema and glandular hyperplasia were measured. Mucin production and basal cell activities were evaluated by mucin 5AC (MUC5AC) and keratin 5 (KRT5) using immunofluorescence staining. *Results:* Larger mucosal areas were observed in all surgical groups at 1 and 4 weeks. More submucosal glandular hyperplasia was noted in the group with isthmectomy without hemostasis. MUC5AC and KRT5 expressions were significantly higher in the surgical groups. *Conclusion:* The tracheal mucosa may change after surgery, which could explain postoperative discomfort after thyroidectomy.

Thyroidectomy is the treatment of choice for thyroid malignancy as well as a viable option for benign thyroid disease. Although life-threatening complications such as hematoma and bilateral vocal-fold paralysis are decreasing, about 80% of patients complain of postoperative discomfort, including vague voice changes, increased thick secretion,

neck paresthesia, and choking sensation even without recurrent laryngeal nerve paralysis (1, 2). Voice changes are usually expected to improve within months, but in approximately 25% of patients, these persist up to 12 months (3). Voice and swallowing symptoms are known to be related to surgical extent and globus sensation, and swallowing difficulty can last many years (3, 4). However, its mechanism is still not fully understood. Many studies have focused on the external environment of the trachea (5). Trauma caused by endotracheal intubation, adhesion of the strap muscle, poor blood supply to the larynx, and laryngotracheal fixation are known possible risks (5, 6).

Inflammation in the airway mucosa can induce respiratory mucosal thickening with increased mucus secretion by hyperplasia of goblet cells and submucosal gland hyperplasia and hypertrophy (7, 8). Basal cells are the progenitor cells of the airway epithelium and are located above the basement membrane (9). They express keratin 5/14 and transcription factor *TP63*, are activated after epithelial injury, and induce pathological airway remodeling (10). The activity of basal cells can be measured by keratin 5 (KRT5) expression (9, 10).

Generally, a wound-healing process is initiated by a local inflammatory reaction and fibrin deposition (11). The structural composition of fibrin can affect the quality of the healing process. If hemostasis is not performed properly during surgery, excessive fibrin deposition can occur and induce disturbance of wound healing through prolonged inflammation (12, 13). We previously reported that excessive fibrin deposition after thyroidectomy can induce inflammation and increase collagen formation in the external environment of the trachea (13).

To our knowledge, there has been no study on the postoperative changes in the respiratory mucosa in a thyroidectomy wound-healing model. We aimed to further understand the discomfort in post-thyroidectomy patients and identify the morphological and functional changes in the trachea. Maeda *et al.* (14) reported that the voice changes after thyroid surgery might be due to airway edema caused by disturbance in the venous and lymphatic drainage. It is also

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known that the first-echelon lymphatics for cervical trachea are the pretracheal and paratracheal nodes *via* the intercartilaginous spaces, next to the deep cervical nodes (15-17).

We hypothesized that thyroid surgery can also influence the internal environment of the trachea, which would exhibit changes in the tracheal mucosa such as mucosal edema and increased secretion. The objective of this study was to investigate the structural and functional changes in the tracheal respiratory mucosa using a thyroidectomy wound-healing murine model with several surgical conditions.

Materials and Methods

Animals and surgical procedures. Forty female Sprague-Dawley rats weighing 200 to 250 g were housed in standard laboratory conditions, under standardized light (12/12-h light/dark cycle), temperature (21-23°C), and humidity (40-60%), with free access to food pellets and water. The study was approved and conducted in accordance with the Institutional Animal Care and Use Committee of Dongguk University Ilsan Hospital (IACUC: 2014-11114).

Forty rats were divided into 4 groups of 10 rats each: normal control (NC), sham, isthmectomy after cauterization of cutting edge of thyroid for hemostasis (I+C+), and isthmectomy by a cold instrument without hemostasis that leads to excessive fibrin deposition (I+C-). Anesthesia was performed by intramuscular injection with tiletamine hydrochloride/zolazepam hydrochloride (0.1 ml/kg body weight, Zoletil 50; Virbac Laboratories, Carros, France) and xylazine (0.1 ml/kg body weight, Rompun; Bayer, Leverkusen, Germany), including rats in the normal control group. In the supine position, the neck was prepared with a povidone-iodine solution. All surgical procedures were performed under aseptic conditions. A vertical incision approximately 3 cm was made over the midline of the neck. After separating the submandibular glands on both sides, the superficial layer of the deep cervical fascia and strap muscles were exposed and dissected laterally. We eventually exposed the thyroid and full length of the cervical trachea, and rats that underwent this procedure were allocated to the sham operation group (Figure 1A). Then, we removed the isthmus of the thyroid (2 mm width), and rats that underwent this procedure were assigned to the I+C+ and I+C- groups, depending on whether they underwent electrocauterization or not. Half of the rats were euthanized using carbon dioxide at 1 week and the remainder at 4 weeks after the operation. The sample size in each group was calculated to provide 90% power for detecting a difference of magnitude $\Delta=0.05 \text{ mm}^2$ of the mucosal area using a 5% level 2-sided test. Preoperative and postoperative thyroid function tests were performed to rule out the effect of hypothyroidism.

Histopathological evaluation. *En-bloc* resection of the aerodigestive tract, including the larynx, trachea, and esophagus with the overlying strap muscles, was performed (Figure 1B). The specimens were fixed in 4% neutral buffered formalin for 24 h. The tissue was then embedded in paraffin and sliced to a thickness of 4 μm . Three sections were prepared for each animal, and 15 slides were evaluated per group in the area of the isthmectomy for each staining. We measured the mucosal and submucosal areas in the hematoxylin and eosin (H&E)-stained slides to determine postoperative mucosal edema. The increase in airway secretion was evaluated by submucosal glandular hypertrophy and hyperplasia.

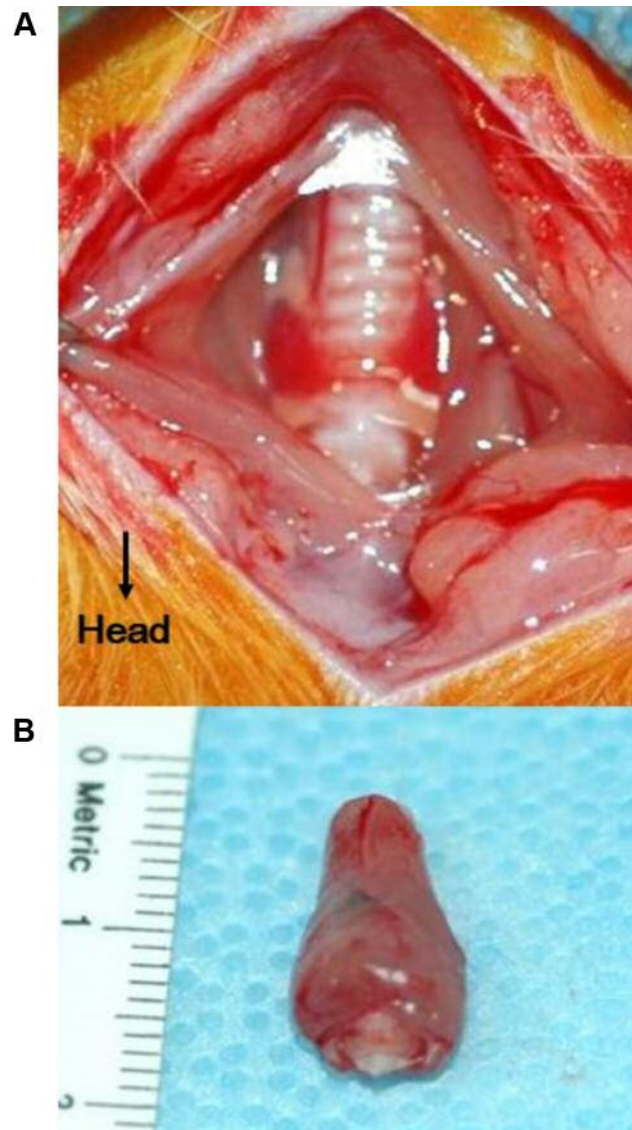


Figure 1. Strap muscles separated along the midline after incision. (A) Visceral fascia with the trachea and thyroid. (B) Postsurgical *en-bloc* resection of the aerodigestive tract.

The number of secretory ductal openings from the submucosal gland to the airway lumen was also evaluated (Figure 2). Photographs at $\times 40$ magnification were taken of each slide that contained views of the upper half of the tracheal mucosa and lumen. The images were measured by Image J software version 1.50i (National Institutes of Health, Bethesda, MD, USA).

To check for expression of mucin 5AC (MUC5AC) and KRT5 in the tracheal mucosa, immunohistochemically-staining antibodies for each specific antigen (mouse monoclonal, ABIN966607, ABIN126702; Antibodies, Davis, CA, USA) were used. MUC5AC expression was evaluated for mucin deposition in goblet cells, and KRT5 detection was performed by immunofluorescence staining (secondary Ab; Alexa Fluor® 488 goat anti-mouse IgG, A11001,

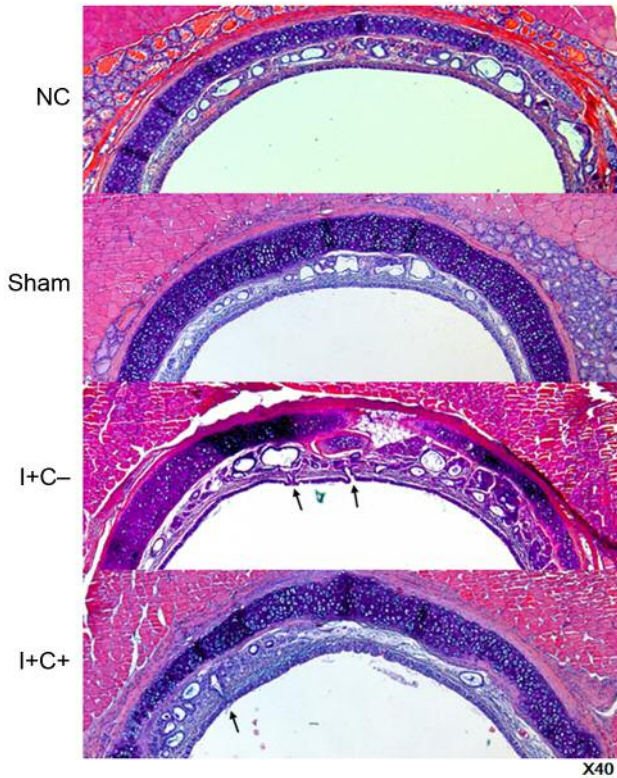


Figure 2. Representative images of the tracheal mucosa at postoperative 4 weeks (H&E stain, $\times 40$). Secretory duct openings are marked by black arrows.

Life Technologies, Carlsbad, CA, USA) to determine basal cell proliferation in the epithelium. Each image was captured by an Olympus BX53 microscope (Olympus, Tokyo, Japan) fixed with a DP73 cooled digital color camera (model DP73-1-51; Olympus, Tokyo, Japan). Captured images were adjusted for brightness and intensity of green fluorescence to a standard level using Photoshop CS6 (Adobe Systems, San Jose, CA, USA). Using the Image J software under magnification ($\times 400$), the count of cells that reacted with each antibody per total epithelial area was converted into percentile, and the mean value was calculated.

Statistical analysis. The Mann-Whitney *U*-test was used to compare differences among the experimental groups. The null hypothesis of no difference was rejected if the *p*-value was <0.05 . All statistical analyses were performed with IBM SPSS Statistics for Windows version 18.0 (IBM, Armonk, NY, USA).

Results

Changes in the tracheal mucosa: H&E staining. All animals tolerated the surgeries and survived the experimental period. Compared to the NC group, the mucosal area significantly increased in all surgical groups at 1 week (sham: 0.16 vs. 0.30 mm², $p=0.013$; I+C-: 0.16 vs. 0.29 mm², $p=0.017$; I+C+: 0.16 vs. 0.39 mm², $p=0.008$) and persisted at 4 weeks

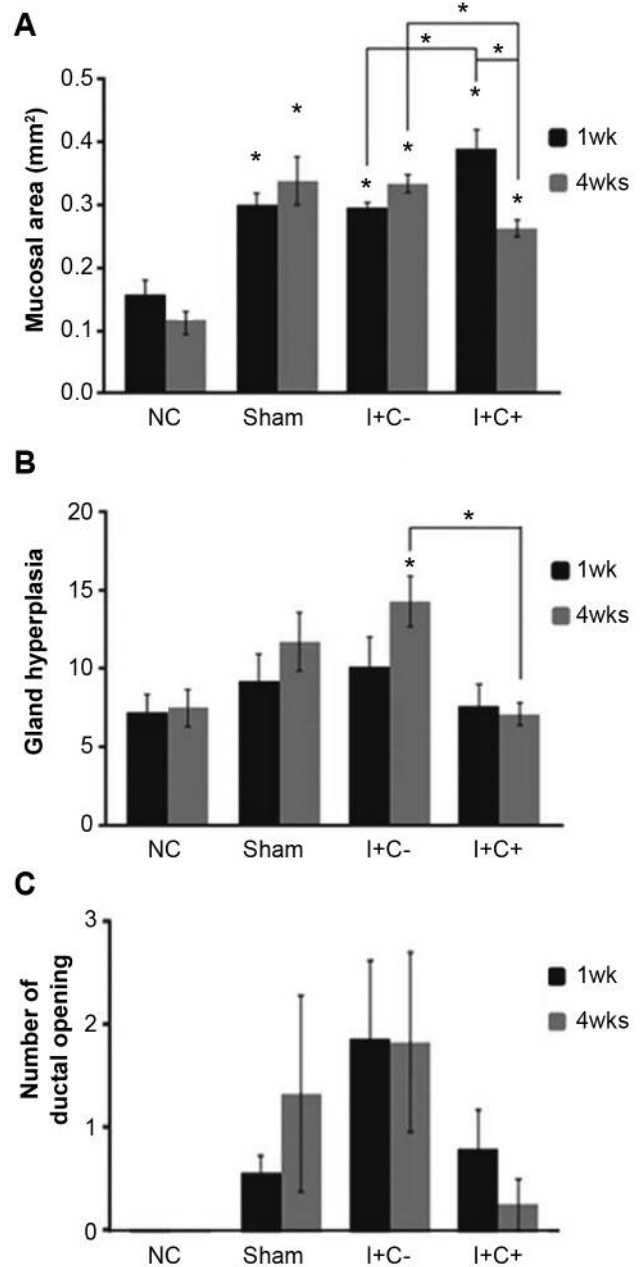


Figure 3. Changes in the tracheal mucosa. (A) The mucosal area significantly increased in all surgical groups compared to that in the NC group at 1 and 4 weeks. (B) Only the I+C+ group exhibited significantly improved mucosal edema with time, which had a lesser extent compared with that of the sham and I+C- groups at 4 weeks. (C) All surgical groups demonstrated ductal openings to the tracheal lumen, whereas the NC group did not. $*p<0.05$.

(sham: 0.12 vs. 0.34 mm², $p=0.039$; I+C-: 0.12 vs. 0.33 mm², $p=0.020$; I+C+: 0.12 vs. 0.26 mm², $p=0.034$) (Figure 3A). Although the mucosal area increased in the sham and I+C- groups at 4 weeks, only the I+C+ group exhibited

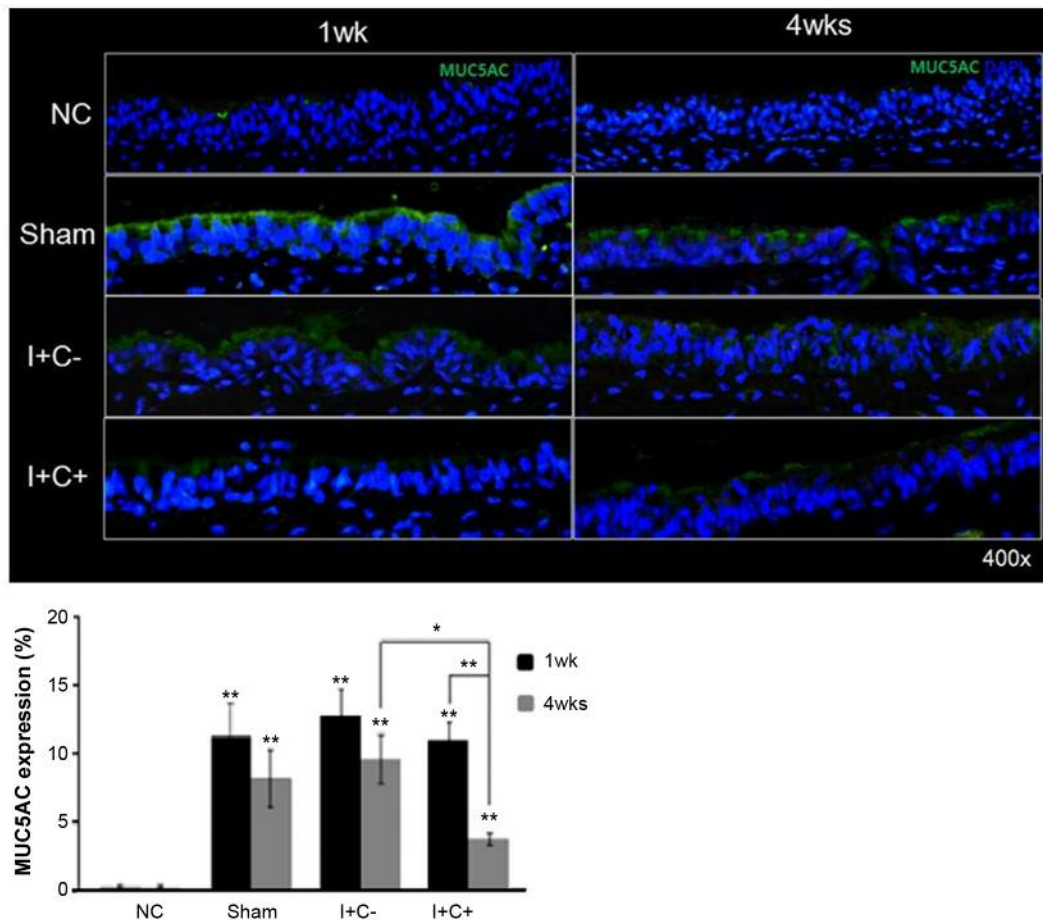


Figure 4. Mucin deposition significantly increased in all surgical groups compared with that in the NC group. Mucin production decreased at 4 weeks in all surgical groups but was only statistically significant in the I+C+ group (immunofluorescence stain, $\times 400$). * $p < 0.05$, ** $p < 0.01$.

significantly improved mucosal edema with time (0.38 vs. 0.26 mm², $p = 0.014$), which had a lesser extent than the sham and I+C- groups at 4 weeks.

The number of submucosal glands significantly increased at 4 weeks in the I+C- group compared to NC (7.5 vs. 14.1, $p = 0.015$), which was higher than that of the I+C+ group (14.1 vs. 6.8, $p = 0.013$) (Figure 3B). Ductal openings to the tracheal lumen were noted in all surgical groups, but not in the NC group. The I+C- group had the highest number of secretory ductal openings at both 1 and 4 weeks, but there was no difference between the surgical groups (Figure 3C). Submucosal gland hypertrophy did not differ among groups.

Changes in MUC5AC and KRT5 expression: immunofluorescence staining. MUC5AC expression significantly increased in all surgical groups compared to the NC at 1 week (sham: 0.2% vs. 11.2%, $p = 0.001$; I+C-: 0.2% vs. 12.8%, $p = 0.001$; I+C+: 0.2% vs. 11.0%, $p = 0.001$) and 4 weeks (sham: 0.2% vs. 8.2%, $p = 0.003$; I+C-: 0.2% vs. 9.6%, $p = 0.003$; I+C+:

0.2% vs. 3.7%, $p = 0.004$) (Figure 4). At 4 weeks, mucin production decreased in all surgical groups, that was statistically significant in the I+C+ group (11.0% vs. 3.7%, $p = 0.008$). Furthermore, the I+C+ group had lesser expression than the I+C- group at 4 weeks (3.7% vs. 9.6%, $p = 0.041$). Basal cell activity by KRT5 expression significantly increased in all surgical groups compared to the NC group at 1 week (sham: 2.3% vs. 12.5%, $p = 0.004$; I+C-: 2.3% vs. 11.5%, $p = 0.006$; I+C+: 2.3% vs. 19.5%, $p = 0.001$) and 4 weeks (sham: 1.3% vs. 11.7%, $p = 0.001$; I+C-: 1.3% vs. 15.8%, $p = 0.001$; I+C+: 1.3% vs. 16.4%, $p < 0.001$) (Figure 5). At 4 weeks, the I+C- and I+C+ groups had higher activities than the sham group (11.7% vs. 15.8%, $p = 0.010$; 11.7 vs. 16.4%, $p = 0.011$).

Discussion

In this study, we found that the surgical procedure of thyroidectomy can induce significant changes in the tracheal respiratory mucosa. Mucosal edema increased in all surgical

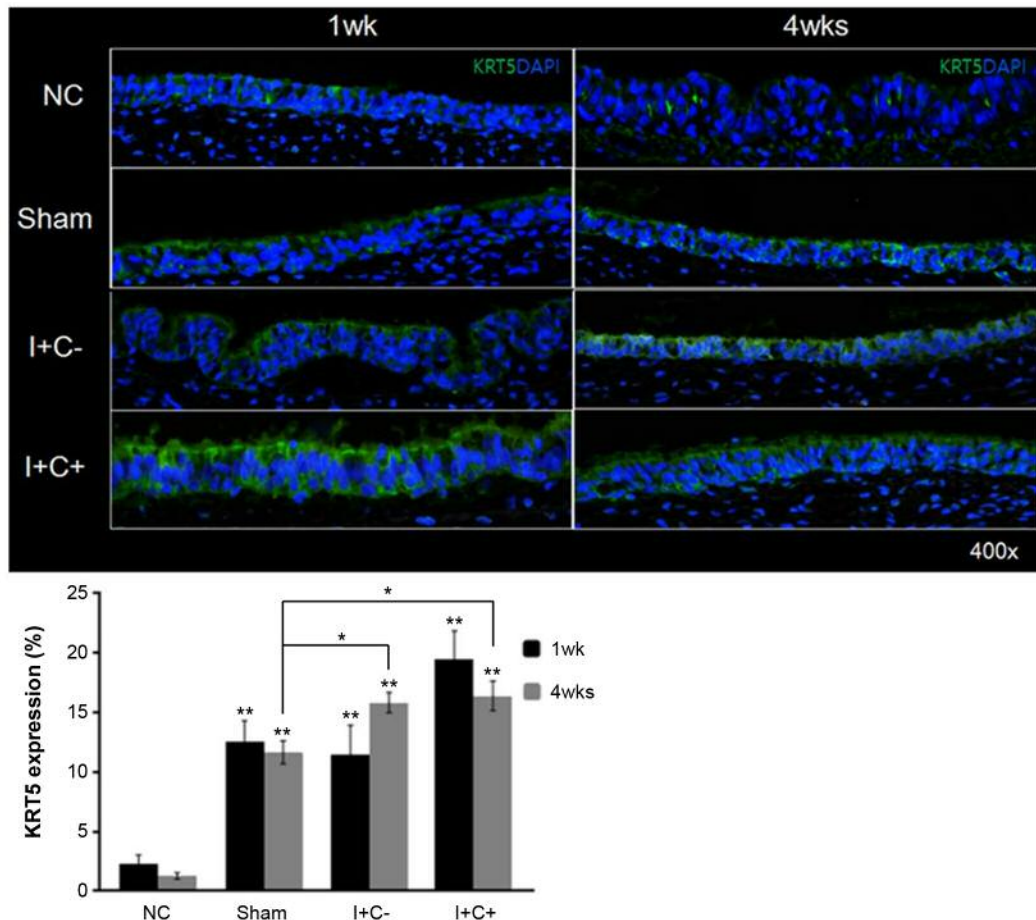


Figure 5. *KRT5* expression increased in all surgical groups compared to that in the NC group. Although *KRT5* expression decreased at 4 weeks in the sham and I+C+ groups, it decreased in the I+C- group (immunofluorescence stain, $\times 400$). * $p < 0.05$, ** $p < 0.01$.

groups at both 1 and 4 weeks after surgery. It improved significantly only in the I+C+ group with time, but persisted in all surgical groups at 4 weeks. Significant submucosal glandular hyperplasia occurred in the I+C- group at 4 weeks, and the number was much higher than that in the I+C+ group. The surgical groups exhibited secretory ductal openings to the lumen, but the NC group did not. The I+C- group had the highest number of openings at both 1 and 4 weeks. These results suggest that surgical procedures including sham operation caused adverse effects to the inner environment of the trachea and induced structural changes in the respiratory mucosa. The excessive fibrin deposition in the outer environment had an adverse impact on the inner tracheal respiratory mucosa.

When the airway mucosa is exposed to respiratory irritants, inflammatory mediators induce peripheral plasma extravasation and cause mucosal edema (18). In diseased lung tissue caused by chronic bronchitis, asthma, and cystic fibrosis, hyperplasia of the submucosal gland, which is more

prominent than hypertrophy, and an increase in mucous cells or inflammatory score are more responsible for sputum production than the other changes that occur (19, 20). Mucus is a viscoelastic gel composed of water and high-molecular-weight glycoproteins called mucin. The major gel-forming mucins in human respiratory tracts are MUC5AC and MUC5B (21, 22). We noted that MUC5AC expression increased in all surgical groups and significantly decreased only in the I+C+ group over time. At 4 weeks, the I+C- group had more MUC5AC expression than the I+C+ group, which suggests that delayed wound healing due to excessive fibrin deposition can lead to increased mucus secretion postoperatively (13).

Basal cells constitute approximately 30% of the pseudostratified ciliated columnar epithelium (23). Although they are a generally undifferentiated state, human airway basal cells are characterized by expression of transcription factors such as cytokeratin 5, 14 (KRT5/14) and transformation-related protein 63 (Trp63) (24, 25). When

trauma occurs, they quickly proliferate and initiate the healing process (26). In our study, basal cell activities of KRT5 expression increased to a high degree at 1 week and persisted in all surgical groups. Additionally, groups with thyroid surgery had higher KRT5 expression than the sham group at 4 weeks. These findings suggest that surgical procedures influenced the internal environment of the trachea, which necessitated the proliferation of basal cells.

The fascias of the neck consist of connective tissue and constitute a potential space containing interstitial fluid, most of which merge into lymphatic flow (27). Deep cervical fascias comprise the superficial, middle, and deep layers. The middle layer of the deep cervical fascia, referred to as the visceral layer, is divided into muscular and visceral layers, which envelope the strap muscles of the anterior neck and the thyroid, esophagus, pharynx, and larynx, respectively (28). The pretracheal and paratracheal spaces contain lymphatic drainage from the cervical trachea (15-17). To expose the thyroid and the trachea, we dissected the superficial and middle layers of the deep cervical fascia of the surgical groups including the sham group through the pretracheal fascia. It is thought that this surgical procedure, which exposes the full length of the trachea in a murine model, would bring about mucosal tissue edema or lymph stasis due to disruption of the lymphatic channel especially in the pretracheal and paratracheal spaces. Since lymphatic drainage of the trachea will be hampered by the disruption of lymphatics in the fascias that would be dissected, it can be reasonably assumed that the exposure of the trachea through the pretracheal fascia is a determinant step that induces these changes. We believe this is the reason the sham group was not an exception to the postoperative changes in the tracheal mucosa. Considering full exposure of the cervical trachea in the sham group, it is thought that additional thyroid isthmectomy just a few millimeters in width could not make much difference in the tracheal mucosa (Figure 1).

Lymphedema results from lymphatic insufficiency and leads to a progressive inflammatory process that manifests as discomfort in the target organ and causes recurrent infections (29). Most of all, these morbidities contribute to a poor quality of life (30, 31). In this respect, it is thought that there would be chronic inflammatory stimuli in the tracheal mucosa that drive inflammatory airway response such as increased secretion as well as tissue edema. Our data suggest that the surgical procedure exposing the visceral layers of the neck overlying the thyroid and trachea can induce changes in the inner environment of the trachea such as mucosal edema and increased mucus secretion.

This animal experiments were performed by the same surgical procedure used for humans and has the advantage of excluding the effect of endotracheal intubation and the imbalance of thyroid hormone compared to thyroidectomized

patients. However, full exposure of the cervical trachea in rats might have brought the more prominent change. Meanwhile, the actual thyroid surgery would be more complicated and can be more extensive if indicated; such as total thyroidectomy with central or lateral neck lymph node dissection. Furthermore, possible postoperative complications have to be considered in patients with a history of previous surgery (32). The more extensively dissected, the more disturbed the lymphatic system would be, which can explain the findings of a previous study that voice and swallowing discomforts related to surgical extent (3). Thyroid isthmectomy in this murine model is a simple thyroid surgery, and did not influence the thyroid function. Our experiments were performed without intubation, and anesthesia was also introduced to the control group. Thus, the effect of anesthesia and endotracheal intubation can be excluded. We believe that this study is helpful in understanding the discomfort of patients who underwent thyroidectomy. It can also lead to further studies on reducing or restoring the changes in the tracheal mucosa after thyroidectomy for a better quality of life.

The limitation of this study is the lack of long-term observation/analysis. However, the lifespan of rats is not the same as that of humans and thus reflects a long period. Even though it is thought that these postoperative changes will recover along with the wound-healing process, these data consistently show that thyroid surgeries influenced the tracheal respiratory mucosa and induced mucosal changes, that are negatively affected by excessive fibrin deposition. Recently, we also noticed significant changes in gene expression in the tracheal mucosa, which explain these findings. Our future research will be focused on changes in the lymphatic system in the trachea and their mechanisms, with long-term analysis.

Conclusion

Airway edema with submucosal gland hyperplasia was noted in all surgical groups with increased mucus production and basal cell activities and it was more pronounced in the group with excessive fibrin. This study demonstrates that thyroid surgeries dissecting the pretracheal space can induce structural and functional changes in the tracheal mucosa.

Conflicts of Interest

The Authors declare no conflicts of interest associated with this manuscript.

Authors' Contributions

Y.S.L., Y.J.C., H.B.K., S.W.P. and J.H.P. conceived and designed the study. Y.S.L., Y.J.C. and H.B.K. performed animal experiments. Y.S.L., Y.J.C., B.H.K., H.B.K., C.G.C. and J.H.P. analysed the data.

Y.S.L., Y.J.C., B.H.K., H.B.K drafted the manuscript. All authors revised the article for important intellectual content, reviewed the data and their analyses and approved this article.

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