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Changes in the Laryngeal Mucosa After Thyroid Surgery: A Rat Model

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Abstract. *Background/Aim: Thyroidectomy can cause various airway symptoms affecting the quality of life. We investigated the changes in extracellular matrix (ECM) composition and markers for inflammation and microcirculation of laryngeal mucosa. Materials and Methods: Sixty Sprague-Dawley rats were categorized into control and three surgical groups based on the extent of surgeries, 1) flap elevation (FE) group, 2) thyroid and trachea exposure (TE) group, and 3) thyroid isthmectomy (TI) group. We analyzed the expression of TGF-β1, VEGFR-3, CD31, and MMP- 9 in relation to the inflammatory and microcirculatory changes in the lamina propria on postoperative days (PODs) 3, 7, and 21. ECM composition of hyaluronic acid (HA) and collagen in the subglottic area (SA) was also evaluated. Results: All parameters increased in surgical groups at each postoperative phase except collagen deposition. On POD 3, TGF-β1 expression and SA increased in relation to the surgical extent and decreased over time, but more than the control in all surgical groups on POD 21. Surgical groups had more HA and less collagen composition, causing a higher HA to collagen ratio in relation to the surgical extent. VEGFR-3 and CD31 expression increased with time at all postoperative phases according to the surgical extent. Expression of MMP-9 increased in TI groups compared to TE and FE groups on POD 7 and POD 21. Conclusion: This study demonstrated that thyroid surgery exposing the thyroid and trachea induces an increase in the SA with a higher HA and lesser collagen composition. Furthermore, the markers for acute inflammation and microcirculation with tissue remodeling increased in the laryngeal mucosa.*

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Patients who underwent thyroidectomy have various airway symptoms, such as a foreign body sensation of the throat, dysphagia, and voice change (1-3). Without recurrent laryngeal nerve injury, about 70% of patients complain of voice symptoms, such as fatigue and difficulty with a high pitch or loud voice over time (3, 4). Many studies have described that the causes of symptoms are the mucosal damage caused by bronchial intubation, injury of microcirculation in the larynx, and adhesion between the strap muscles and the larynx and trachea hindering an upper-lower motion disorder in the laryngeal system (2-5).

Thyroidectomy can induce laryngeal and tracheal edema (6, 7). We reported the increased area of tracheal mucosa and mucus secretion with increased mRNA expression of transforming growth factor-β1 (TGF-β1), hypoxia-inducible factor-1α (HIF-1α), and matrix metalloproteinase-9 (MMP-9) after thyroid surgeries in animal models (7, 8). These essential regulators of homeostatic restoration increased in the tracheal mucosa for a considerable period after surgery, in relation to the increased extent of surgery (8).

Lymphatic supply to the subglottic larynx is extensive and bilateral (9). Liu *et al*. examined 18 fresh fetal cadavers and found that the inferior surface of the vocal folds has rich lymphatic vessels and collecting chambers (10). The subglottic lymph fluid leaves the endolaryngeal space ventrally by way of collectors through the cricothyroid ligament and dorsally through the cricotracheal ligament (11). Thereafter, lymphatic fluid from the subglottis drains to the prelaryngeal, pretracheal, paratracheal nodes, and eventually to jugular lymph nodes (12). These are also the first-echelon lymphatics of the cervical trachea. The surgical approaches to the thyroid gland inevitably include dissection

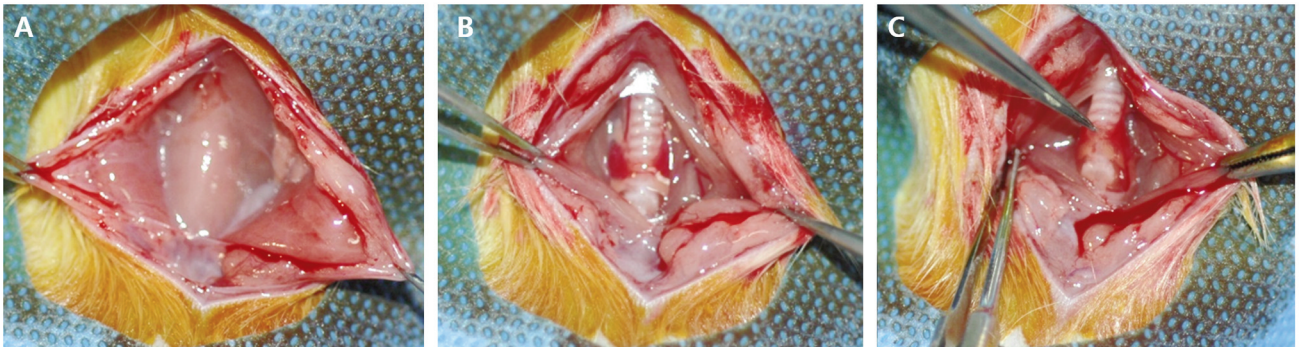

Figure 1. *(a) Treatment in advanced gastric cancer with disseminated intravascular coagulation (DIC). (b) Overall survival (OS) of patients with disseminated intravascular coagulation (DIC) and without DIC. OS in patients with DIC was extremely poor.*

of the superficial cervical fascia and superficial and visceral layers of the deep cervical fascia, which may damage the lymphatics in the fascial layers around the thyroid gland. In other words, surgical dissection of the thyroid glands and trachea might hamper lymphatic drainage, resulting in the lymphatic stagnation of tissue. Furthermore, tissue inflammation can develop as the secondary manifestation of lymphedema induced by changes in the microcirculatory environment due to lymphatic and vascular injuries (13, 14).

Considering these, we hypothesized that surgery's microcirculatory injuries of lymphatics among the neck fascial layers can induce lymphatic stasis of subglottic mucosal tissue as subglottic edema and tissue inflammation leading to the alteration of mucosal extracellular matrix (ECM) composition. This study investigated the changes in the subglottic area (SA), the ECM composition, such as collagen and hyaluronic acid (HA), and the markers for tissue inflammation and microcirculation of the subglottic mucosa in the rat model that underwent thyroid surgeries.

Materials and Methods

Animals and surgical procedures. Sixty female Sprague–Dawley rats weighing 200-250 g were purchased from KOATECH (Pyeongtaek, Republic of Korea) and bred in a specific pathogen-free animal facility. The rats were divided into four groups, with 15 rats in each surgical group, as follows: (a) normal control group, (b) flap elevation (FE) group, (c) trachea exposure (TE) group, and (d) thyroid isthmectomy (TI) group. Anesthesia was induced *via* intramuscular injection with Zoletil®50 (Virbac Laboratories, Carros, France) (0.1 ml/kg body weight) and Rompun® (Bayer Korea, Seoul, Republic of Korea) (0.1 ml/kg body weight) without intubation. The neck was prepared with an aseptic technique using a povidone-iodine solution in the supine position. After a vertical incision about 2 cm over the midline of the neck, the superficial layer of the deep cervical fascia and the strap muscles were exposed and dissected laterally, allocated to a flap elevation (FE) group. Further dissection exposed the thyroid gland and the whole length of the cervical trachea; these were the trachea exposure (TE) group. Then, the isthmus of the thyroid gland (2 mm width) was elevated from the trachea and cut off with a cold instrument for the thyroid isthmectomy (TI) group (Figure 1). Each group consisted of five rats, one for each of the following points: postoperative day (POD) 3, 7, and 21, at which point the rats were euthanized. The animal study protocol was approved by the Institutional Animal Care and Use Committee of Dongguk University Ilsan Hospital (IACUC#2017-08166).

Evaluation of the subglottic area. After acquiring an aero-digestive tract between the larynx and cervical trachea, specimens were fixed with 4% neutral buffered formalin for 24 h. The tissue was embedded in paraffin and sliced to a thickness of 4 μm on a microtome (Leica®, Nussloch, Germany). An average of 10 sections was obtained at each group's subglottis level and stained with hematoxylin and eosin Y (Merck KGaA, Darmstadt, Germany) (H&E) with 200-fold images stored and analyzed using an optical microscope. The subglottic area, the area of lamina propria that lies between the overlying epithelium and the underlying vocalis muscle, was measured in each slide.

Evaluation of collagen and hyaluronic acid composition. The ECM composition of collagen and HA was evaluated at the lamina propria of the subglottis using the Trichrome and Alcian blue stain kit (ScyTek Laboratories, Logan, UT, USA). The proportion of stained area in the lamina propria was measured using ImageJ software (version 1.50i, National Institute of Health, Bethesda, MD, USA) with the ratio of HA to collagen expression.

Immunohistopathological evaluation. To check for the expression of transforming growth factor-β1 (TGF-β1), hypoxia-induced factor (HIF-1α), vascular endothelial growth factor-3 (VEGFR-3), endothelial cell adhesion molecule (CD31), and matrix metalloproteinase-9 (MMP-9) in the subglottis, all the slides were deparaffinized with an application of 3% fetal bovine serum. Antibodies for TGF-β (Abcam, Cambridge, UK), HIF-1α (ab82932 Abcam), VEGFR-3 (Abcam), interleukin CD-31 (Abcam), and MMP-9 (Invitrogen®, Minneapolis, MN, USA) were diluted at 1:100 and reacted at 4˚C. We obtained microscopic images and photographs of each slide at 400× magnification. All the detected antibodies were collected and calculated using ImageJ software.

Statistical analyses. All the data are presented as mean±standard error of the mean values. The statistical analyses were performed

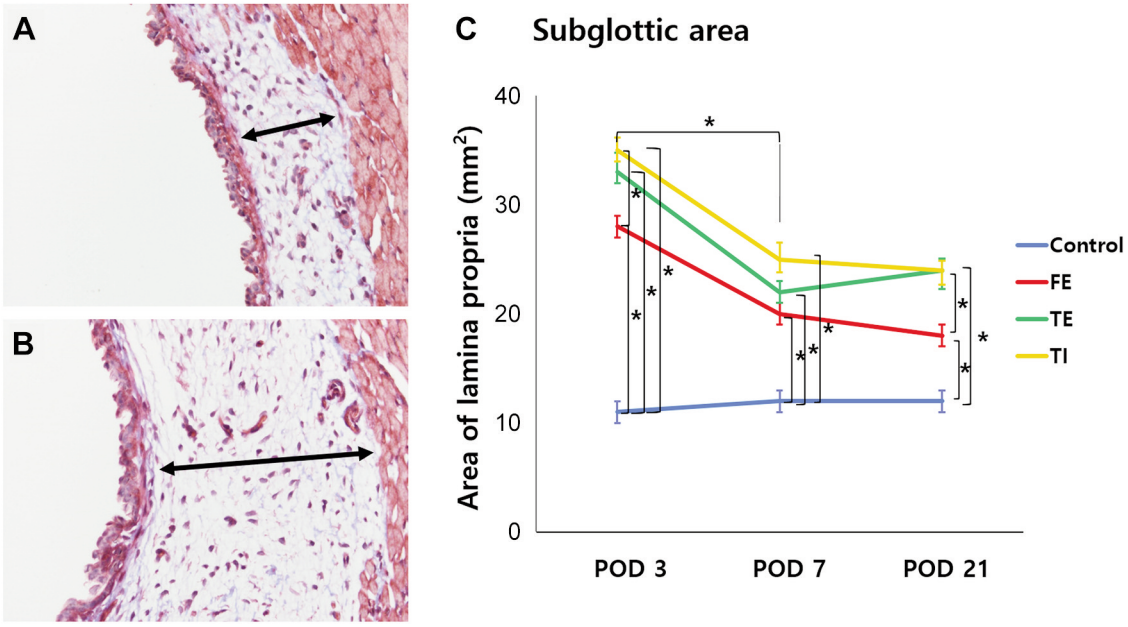

Figure 2. *Histological findings of the subglottis after thyroid surgery. The area of lamina propria in the subglottis increased in the surgical group (B) compared to that in the control group (A), which decreased with time (C) (200× magnification).*

using the Statistical Package for the Social Sciences for Windows, Version 18.0 with the Mann–Whitney *U*-test. The criterion for statistical significance was set at $p<0.05$.

Results

Subglottic area. The SA of the lamina propria in all surgical groups significantly increased compared with that in the control group at all postoperative periods, proportioning to the surgical extent ($p<0.05$), and decreased with time. On POD 7, the TE and TI groups had more SA than the FE group (Figure 2).

Collagen and hyaluronic acid composition. The representative images of collagen and HA composition in the lamina propria are presented in Figures 3A and B. All surgical groups had significantly more HA and lesser collagen composition than the control group at all postoperative phases, which was not significantly different among the surgical groups (Figure 3C and D). The ratio of HA to collagen in the surgical groups was significantly higher than that in the control. TE and TI groups exhibited a higher composition ratio than the FE group, meaning that as the surgical extent increased, the percentage of HA to collagen composition increased (Figure 3E).

Protein expression for inflammation and microcirculation. Figure 4 shows the representative images of TGF-β1, HIF-1α, VEGFR-3, CD-31, and MMP-9 at POD 21. TGF-β1 levels increased in the surgical groups more than those in the control group at all postoperative periods ($p<0.05$). On POD 7, they were significantly higher in the TI group than in the FE group. TGF-β1 expression decreased with time in all the surgical groups except in the TI group, which showed significantly higher expression than in the other groups on POD 21 ($p<0.05$) (Figure 5A). HIF-1α expression increased in surgical groups ($p<0.05$). It was significantly higher in the TI and TE groups than in the FE group at all periods ($p<0.05$) (Figure 5B). TI and TE groups had a significantly increased VEGFR-3 expression than the control group. Further, the surgical groups exhibited a significant difference; the more the surgical extent, the higher its expression ($p<0.05$). In contrast, there was no difference between the control and FE groups (Figure 5C). CD-31 expression increased significantly in the surgical groups compared to that in the control group ($p<0.05$). On PODs 7 and 21, CD-31 expression in the TI group was higher than that in the FE group ($p<0.05$) (Figure 5D). All surgical groups had more MMP-9 expression than the control group ($p<0.05$). On PODs 7 and 21, it was significantly higher in the TI group than that in the TE and FE groups ($p<0.05$) (Figure 5E).

Discussion

This study evaluated the changes in the subglottic mucosa of the larynx after thyroid surgeries, demonstrating the mucosal edema, increased HA to collagen composition ratio, and high

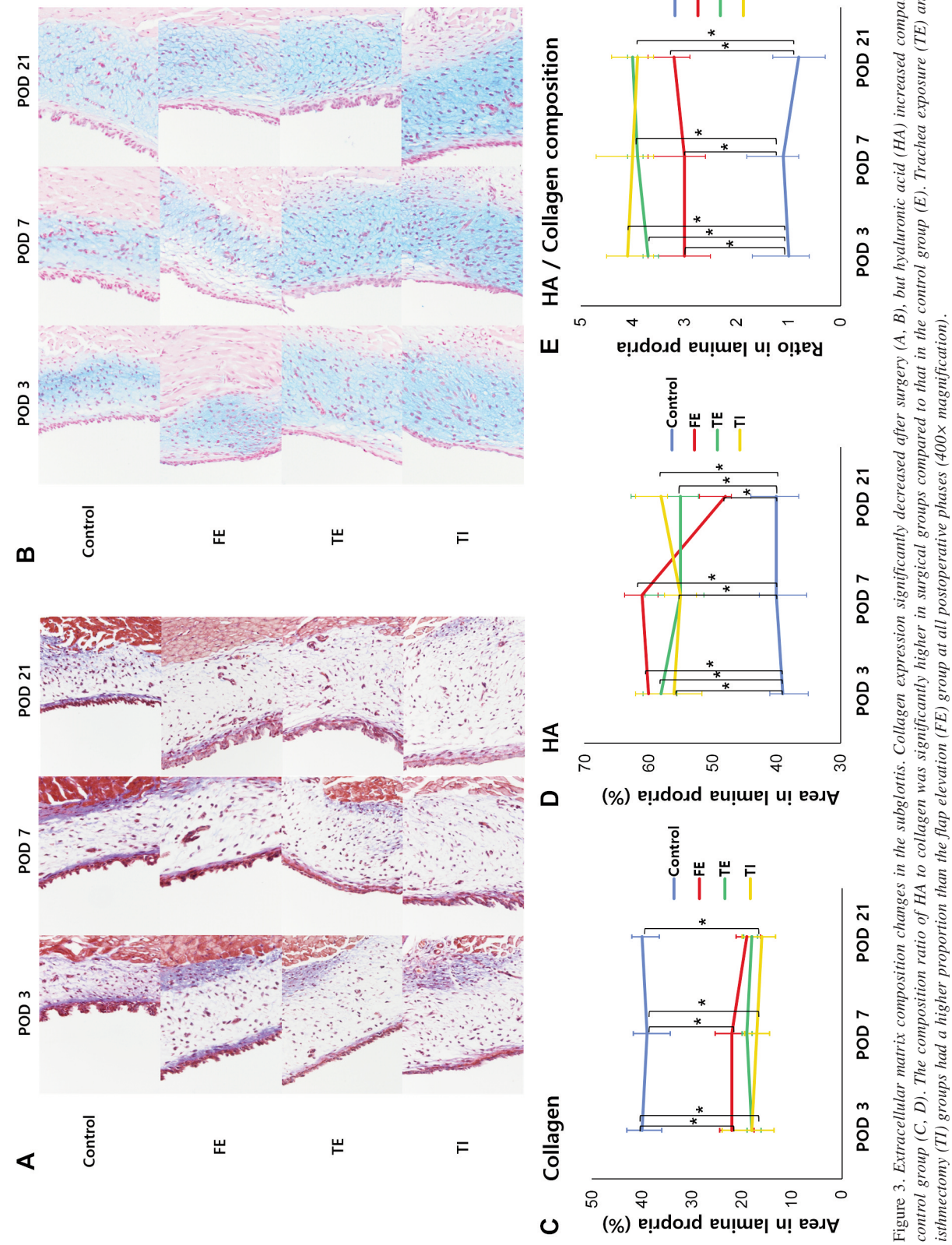

Figure 3. *Extracellular matrix composition changes in the subglottis. Collagen expression significantly decreased after surgery (A, B), but hyaluronic acid (HA) increased compared to the control group (C, D). The composition ratio of HA to collagen was significantly higher in surgical groups compared to that in the control group (E). Trachea exposure (TE) and thyroid isthmectomy (TI) groups had a higher proportion than the flap elevation (FE) group at all postoperative phases (400× magnification).*

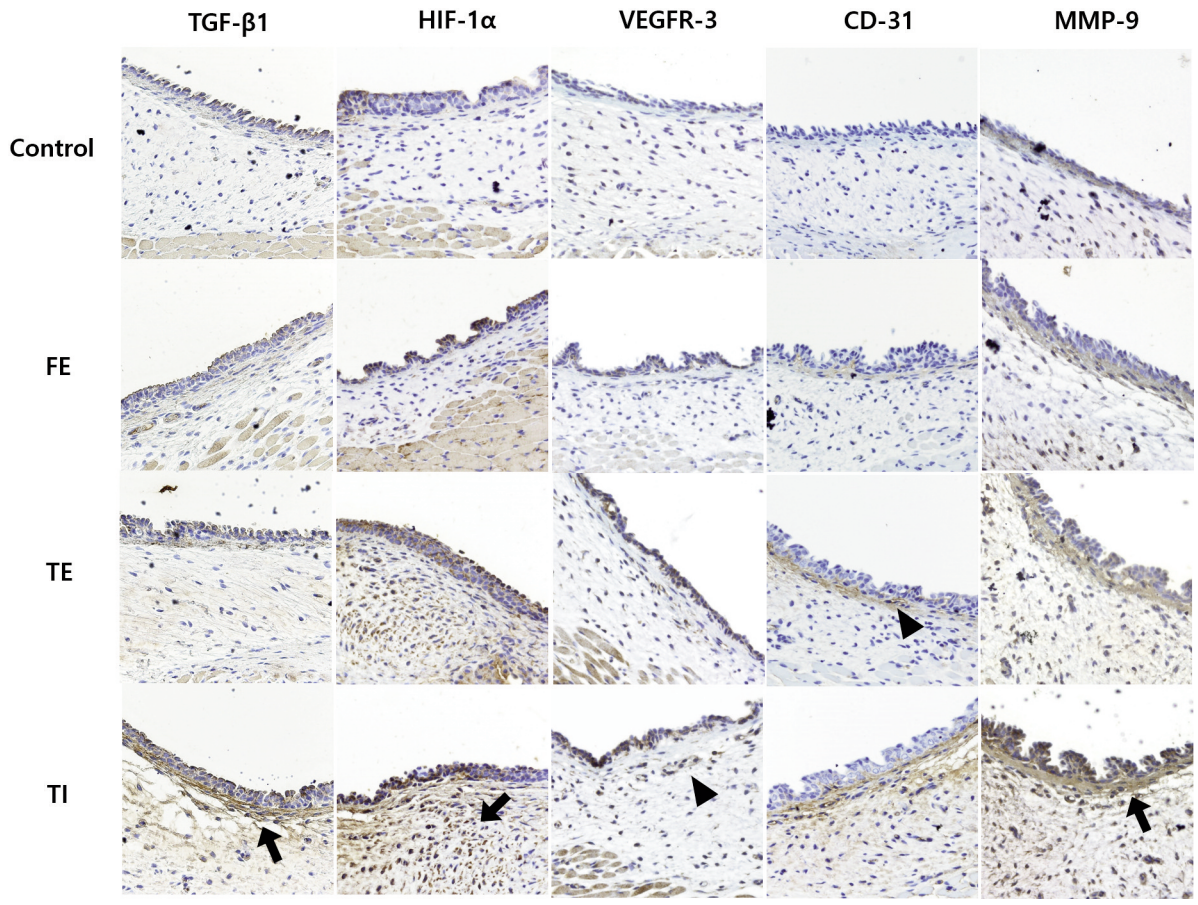

Figure 4. *Representative images of immunohistochemistry findings of the subglottic laryngeal mucosa at postoperative day (POD) 21. TGF-β1, HIF-1α, and MMP-9 expression increased in the TI group compared to the control group (arrow), with increased VEGFR-3 and CD31 expression at the lamina propria (arrowhead) (400× magnification).*

protein expression for inflammation and microcirculation. This research was performed based on the following rational inferences: a surgical approach exposing the thyroid gland and trachea *via* the midline dissects the superficial and deep cervical fascias and would inevitably disrupt the lymphatic vessels that drain the interstitial fluid from the airways. Some mechanisms can alter physiologic lymphatic drainage by occluding the draining lymph collectors or lymph nodes, such as tissue infections, radiotherapy, previous surgical approaches, and tumor growth (11, 15). In other words, the dissection of fascial layers surrounding the larynx and trachea might hinder the lymphatic drainage from the airway. It can induce lymphatic stasis and changes in the subglottic mucosa, leading to increased SA and alteration of the microenvironment of vocal folds. These are similar to our previous reports about the changes in the respiratory mucosa of the trachea after surgery.

Subglottic edema is the most common finding of the laryngopharyngeal reflux disease, one of the chronic inflammatory diseases of the airway, which can cause vocal fold edema with the voice change (16). The increase in SA indicates the tissue edema of the subglottis that the lymphatic stasis may trigger after surgery. The subglottis is a well-known subunit of the larynx famous for its abundant lymphatic channels to the neck. In contrast, the glottis has sparse lymphatic communication with neck spaces. The lymphatic fluid in the subglottis flows into the pretracheal and paratracheal areas exposed in the TE and TI groups through the surgical approaches. We believe this is why both TE and TI groups have more significant changes than the FE group, and there was no significant difference between each other.

Tissue edema can further induce a secondary inflammatory reaction that can cause a change in ECM composition in the lamina propria (13, 15). HA is produced by fibroblasts and macrophages and is essential in tissue hydrodynamics, cell movement, and proliferation. The turnover is rapid within a 3-5-day half-life inside the lamina propria, and its increase can cause tissue fibrosis (17-19).

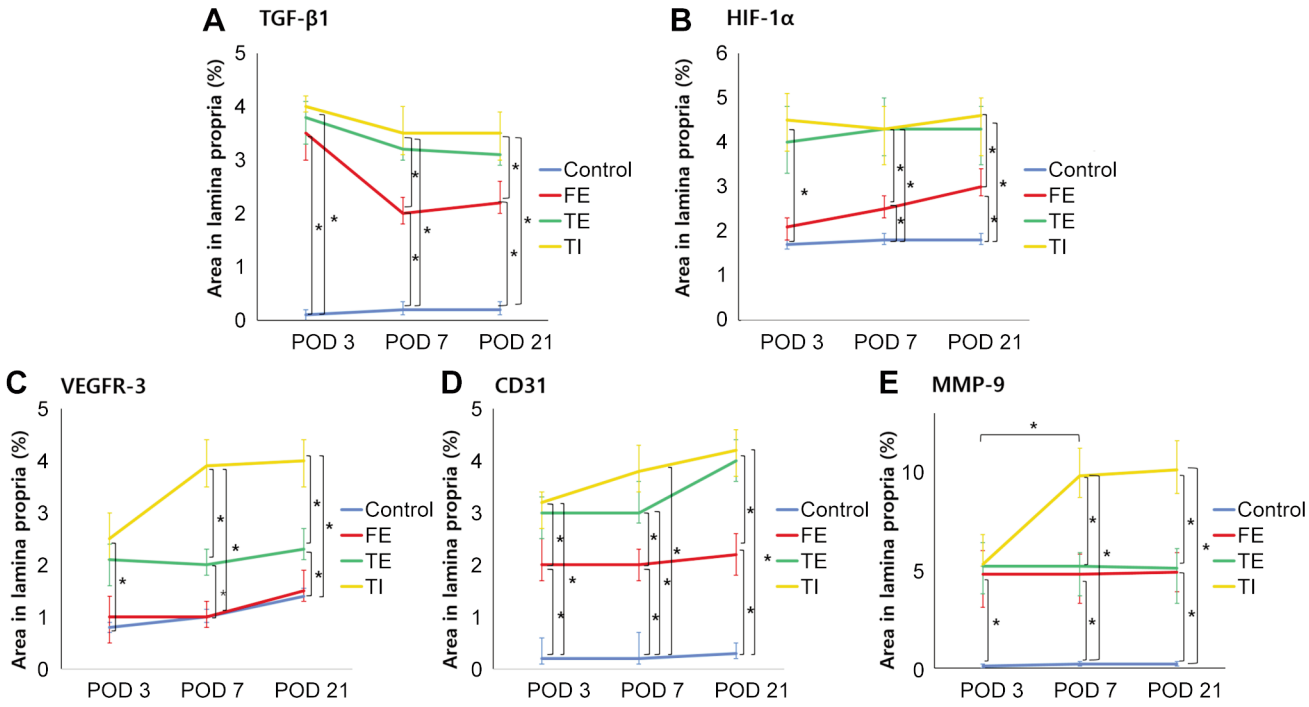

Figure 5. *Expression of inflammatory and microcirculatory factors of vocal fold after surgery. TGF-β1 increased in all surgical groups ($p<0.05$) and decreased with time except in thyroid isthmectomy (TI) group (A). HIF-1α increased after surgery ($p<0.05$), and it was significantly lower in the flap elevation (FE) group than in other surgical groups at all periods ($p<0.05$) (B). VEGFR-3 and CD31 expression increased with surgery ($p<0.05$), and there was a significant increase with surgical extent ($p<0.05$) (C, D). Surgical groups had more MMP-9 expression than the control group. It was significantly higher in the TI group than those in the trachea exposure (TE) and FE groups on postoperative days (PODs) 7 and 21 ($p<0.05$) (E) (400× magnification).*

CD44, the primary receptor of HA, enhances TH2 cell activation, which recruits fibroblasts and induces the proliferation of myofibroblasts. Fibrosis is associated with a significant delay in lymphangiogenesis and impaired lymphatic function, resulting in lymphedema (20-22). As per several reports, hyaluronidase injection in the vocal fold in case of vocal fold diseases, such as Reinke's edema and vocal fold polyp, can help relieve voice symptoms because lower molecular HA fragments reduce the expression of CD44 (17, 22). Collagen provides tensile strength and maintains tissue integrity; this contributes to the viscoelastic properties of the vocal fold (23, 24). It is also known that HA reduces the amount of collagen deposit during the remodeling phase of the healing process, although its precise mechanisms are not entirely understood (25). Surgical groups exhibited increased HA and decreased collagen composition compared with the control group. The high HA to collagen composition ratio occurred in proportion to the extent of dissection of the cervical fascial layer. According to the body-cover theory of the vocal folds, increased HA and decreased collagen composition indicate an imbalance of fluid-structure interaction, resulting in vocal fold diseases, such as vocal fold polyp and Reinke's edema, the polypoid degeneration of vocal folds within the superficial lamina propria or Reinke's space (26, 27).

Tissue injury may cause tissue remodeling, summarized as stages of platelet agglutination, inflammatory change, growth, and remodeling. Neutrophils and monocytes recruited to the tissue secrete variable cytokines, which cause inflammatory changes. In the early inflammatory stage, TGF-β1 is initially identified as a potent chemotactic cytokine to initiate inflammation and plays a significant role in wound healing (28, 29). At POD 3, the TGF-β1 expression increased, which means that TGF-β1 acts as a proinflammatory cytokine that induces various inflammatory cells to invade the tissue. The onset of proinflammatory cytokine amplifies the inflammatory response, and metabolic demands of tissue increase due to inflammatory reactions, causing insufficient tissue perfusion and hypoxia (28). The TGF-β1 induces the expression of HIF-1α in the event of tissue hypoxia, which stimulates angiogenesis (30). In this study, surgical groups had more TGF-β1 expression than the control group during all postoperative periods. HIF-1α expression increased in the surgical groups and was significantly higher in the TI and TE groups than in the FE group.

Angiogenesis and lymphangiogenesis occur in the phase of growth and tissue remodeling (31, 32). Lymphedema can be characterized by a chronic progressive disease that results from congenital abnormalities, obstruction, injury, or infection of the lymphatic system (13, 15). Secondary lymphedema, including iatrogenic injury of the lymphatic system, is a more common cause of lymphedema. In general, lymphangiogenesis accompanies angiogenesis during tissue remodeling, and VEGFR-3, expressed in the endothelial cells in the neonatal lymph vessels, is one of the primary markers of lymphatic channel neoplasms (15). In separating the fascia and connective tissue around the thyroid gland during thyroid surgery, tissue damage to lymphatics that travel around the fascia occurs, which can cause lymphatic stasis. In this study, on PODs 7 and 21, the VEGFR-3 expression significantly increased with the surgical extent, indicating that the greater the extent of surgery, the more the injury to the lymphatic drainage system. It seems that increased VEGFR-3 expression is a manifestation to restore homeostasis and relieve lymph stasis. CD31, expressed in the endothelial cells of the new blood vessels, is a specific marker for new blood vessels (33). Surgical groups had increased CD31 expression compared to the control group, which proportionally increased with the extent of surgery.

MMP-9 is usually expressed during tissue recovery and serves to break down collagen deposits in the degradation of the ECM (34). Also, it mediates continuous inflammatory reactions in tissue remodeling by activating TGF-β1 (35). In this study, MMP-9 expression increased in the surgical groups compared to that in the control group. On PODs 7 and 21, MMP-9 expression was higher in the TI group than in the TE and FE groups, reflecting the increased tissue remodeling in the group with the greater extent of surgery.

The limitations of this study are the relatively small sample size and lack of long-term data. However, each group's overall average of 10 sections was obtained for data reliability. Our experimental data consistently demonstrated that thyroid surgeries induced changes in the laryngeal mucosa, consistent with our previous reports of postoperative mucosal edema and increased inflammatory and tissue remodeling signals in the tracheal mucosa (7, 8). Additionally, the lifespan of rats is not the same as that of humans and reflects a more extended period. Even though this study could not find a complete recovery of the tissue changes until three weeks, we believe these postoperative changes will recover and get homeostatic over time through the physiologic wound-healing processes.

In conclusion, thyroid surgery dissecting the pretracheal fascial layer of deep cervical fascia can cause subglottic edema and increase the HA to collagen ratio in the lamina propria, which might be associated with postoperative voice symptoms. Further, it could induce acute inflammation and a hypoxic condition, leading to angiogenesis, lymphangiogenesis, and tissue remodeling.

Conflicts of Interest

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

Authors' Contributions

Y.S.L., S.W.K., S.W.P., and J.H.P. conceived and designed the study. Y.S.L., S.W.K., and J.Y.K. performed animal experiments. Y.S.L., S.W.K., B.H.K., J.Y.K., C.G.C., and J.H.P. analyzed the data. Y.S.L., S.W.K., B.H.K., and J.Y.K. drafted the manuscript. All Authors revised the article for important intellectual content, reviewed the data and their analyses, and approved it.

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