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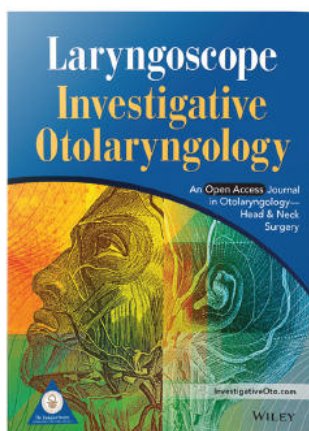


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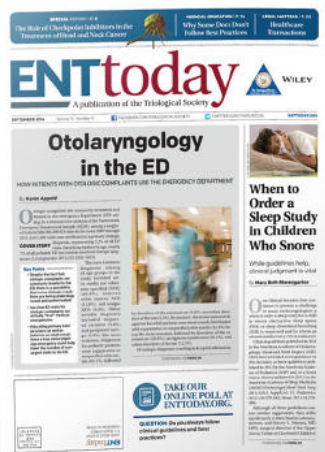


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Prevention of Tracheal Inflammation and Fibrosis Using Nitinol Stent Coated With Doxycycline

Jeong-Seok Choi, MD ; Jeong-Mi Kim, PhD; Ji-Won Kim, MD; Young-Mo Kim, MD; In Suh Park, MD; Su-Geun Yang, PhD

Objectives: This study was conducted to determine whether a nitinol stent coated with doxycycline prevents tracheal inflammation and fibrosis in a rabbit.

Methods: A nitinol stent coated with doxycycline was designed by us. Twelve rabbits were divided into three groups: normal, control (nondoxycycline-coated stent), and doxycycline-coated stent group. The stents were inserted into the tracheal lumen through the oral cavity. Tracheal granulation was evaluated and graded by laryngoscopy. Histological examinations evaluated the inflammatory response and fibrosis. Real-time polymerase chain reaction (PCR) and Western blot assessed the changes to the extracellular matrix (ECM).

Results: Endoscopic findings showed that the nitinol stent coated with doxycycline resulted in lesser granulation tissue in the trachea than the noncoated stent. Histologic examination further revealed that the doxycycline-coated stent was associated with decreased inflammatory cells and reduced fibrosis, compared to the noncoated stent. In PCR and Western blot, the doxycycline-coated stent showed lower expression of ECM components inducing fibrosis.

Conclusion: A nitinol stent coated with doxycycline showed favorable effects in reducing tracheal inflammation and fibrosis in a rabbit model. Further research is required to study the beneficial effects of local application of doxycycline for prevention of tracheal stenosis.

Key Words: Tracheal fibrosis, tracheal inflammation, nitinol stent, doxycycline.

Level of Evidence: NA.

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INTRODUCTION

Endotracheal intubation or tracheal tube insertion is a common procedure in patients with respiratory problems. Tracheal stenosis is the most frequent complication after endotracheal intubation or tracheal tube insertion. The incidence of tracheal stenosis following tracheostomy or intubation ranges from 0.6% to 21%.¹ Surgical procedures, such as segmental resection and anastomosis of trachea and laser excision, are often required; however, these methods often lead to new scar formation and restenosis. Despite surgical improvements, tracheal stenosis is regarded as a challenging problem, and its prevention has been investigated using pharmaceutical agents that modulate the wound healing process.^{2–4}

Wound healing is important for the induction of tracheal stenosis.⁵ Modulation of wound healing helps in

prevention of fibrous scar formation. In a previous study, we showed that in a rabbit model, some modulators such as hyaluronic acid or polyethylene glycol in the extracellular matrix reduce the tracheal stenosis.⁶ Doxycycline is a tetracycline analogue and inhibits the matrix metalloproteinase (MMP) enzymes⁷; it also has an effect on immune modulation and wounding-healing regulation.⁸

We therefore postulated that a tracheal stent coated with the antifibrotic modulator, doxycycline, can reduce fibrosis in the tracheal mucosa and prevent tracheal stenosis. To prove this concept, we designed a new tracheal stent in which we modified nitinol by coating with doxycycline. The macroscopic and microscopic changes, and the expression of messenger RNA (mRNA) and protein of the tracheal mucosa after placement of these stents into the tracheal lumen, were evaluated in a rabbit model to determine the efficacy of our tracheal stent coated with doxycycline for prevention of tracheal stenosis.

MATERIALS AND METHODS

Preparation of Doxycycline-Loaded Core-Shell Nanofiber Stent Using Electrospinning

Doxycycline hyclate (Sigma-Aldrich, St. Louis, MO, U.S.A.), tetrahydrofuran (THF), N,N-dimethylformamide (DMF), and polyurethane (Pellethane 2363-80A, Lubrizol, Wickliffe, OH, U.S.A.) were obtained. Methanol, ammonium acetate, and acetonitrile of high-performance liquid chromatography grade were used. All other chemicals were of analytical reagent grade without further purification.

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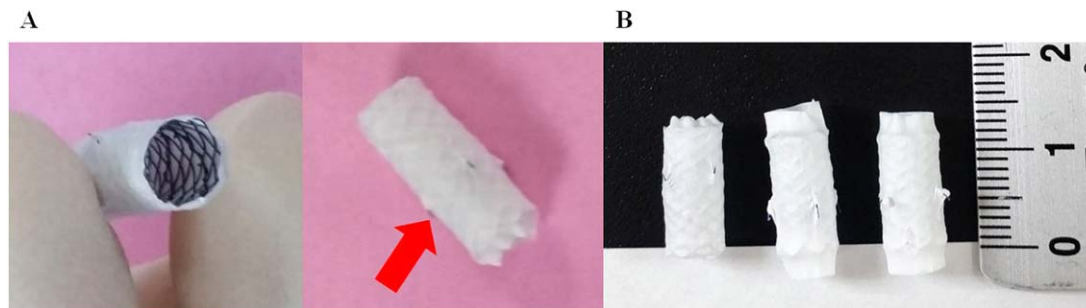


Fig. 1. Nitinol stent coated with doxycycline. (A) Photographs of nitinol stent coated with doxycycline. (B) The size of nitinol stent coated with doxycycline. Arrow represents the hooks on the outer surface of the stent to prevent migration of stent in the tracheal lumen.

For the core layer, doxycycline (30 mg, 10% weight/weight [w/w]) was added to a mixture (0.3 mL) of DMF and THF solutions (1:2 w/w). After complete dissolution of doxycycline, polyurethane (30 mg) was added (10% w/w) and was mixed to get a clear solution. Poly (D, L-lactide) (30 mg, 3.3% w/w) was prepared separately for the shell layer. Air bubbles and polymeric aggregates in the solutions were removed. For electrospinning, the core and shell solutions were simultaneously injected through a dual-metal nozzle at 0.3 mL/hour (outer shell) and 0.9 mL/hour (inner core). The nanofibers were coated onto the stent clipped in a cylindrical rotator at 1,000 revolutions per minute. We designed and prepared the nitinol-based tracheal stent (length: 15 mm, diameter: 6 mm) with hooks on the surface to prevent stent migration in the trachea (Fig. 1). After coating, stents were dried and stored in an airtight container for further experiments.⁹

Animal Model

Twelve New Zealand white rabbits, ranging in weight from 2,500 to 3,500 g, were divided into three groups of four rabbits each. The normal group (n = 4) did not receive a stent. Tracheal stents were inserted in control group (nondoxycycline-coated stent; n = 4) and the doxycycline coated group (n = 4). This study was approved by the Animal Ethics Committee of Inha University Hospital, and the animals were cared for in accordance with established institutional guidelines. Intramuscular ketamine and xylazine (35 mg/kg and 5 mg/kg, respectively) were used for inducing anesthesia. The circumference of the tracheal mucosa was consistently scraped 10 times with a knife, 1 cm from the caudal end of the vocal cord; the length of injury inflicted was 1.5 cm.¹⁰ Using a laryngoscope, stents were inserted into the tracheal lumen through the oral cavity. A pediatric 3 mm-diameter rigid scope was used for the safe insertion and accurate placement of the stent. These procedures were performed by a well-trained practitioner. Animals were observed daily for a period of 4 weeks.

Endoscopic Evaluation

Four weeks after the injury, rabbits were anesthetized and secured to an operating platform in the supine position. After gentle removal of the stent, a rigid laryngeal endoscope (Storz, Tuttlingen, Germany) was inserted orally and fixed in position to provide the best view of the trachea. Tracheal mucosal granulation was evaluated and recorded using an endoscopy recording system.

Histomorphologic Examination

After endoscopic evaluation, the animals were humanely euthanized, and the trachea was harvested. Tracheal specimens

were fixed in 4% paraformaldehyde and sectioned transversely. The samples were then embedded in paraffin, sectioned at 4 μ m thickness, and stained with hematoxylin and eosin and Masson's Trichrome (MTC) using standard pathology department protocols. Slides from each group were examined by a single pathologist, blinded to the group designation of the tissue. Tracheal sections were observed under a light microscope (Nikon, Tokyo, Japan). Each cross-section was evaluated thrice, and their average value was determined. The inflammatory response was measured by counting the number of inflammatory cells (i.e., leukocytes including neutrophils, monocytes, lymphocytes, and plasma cells) in three random fields per tissue section. Fibrosis was calculated using the Image J software by measuring the relative area of fibrosis (fibrosis area/total area \times 100) by MTC staining.

mRNA and Protein Expression

Real-time polymerase chain reaction and Western blot were carried out to assess the changes within the extracellular matrix (ECM). Sense and antisense primers were used, specific for collagen-1 (Forward 5'-CGGCAACCTCAAGAAGTCCC-3', Reverse 5'-CGACATCGATGATGGGAGG-3'), Fibronectin (Forward 5'-CTGGCACTTCTGGTCAGCAG-3', Reverse 5'-ATACGTC TCCCTGGGAACG-3'), MMP-2 (Forward 5'-GCCCTGACC AAGGTTACAGC-3', Reverse 5'-CCTGTGACAGGCGGAAGTTC-3'), TIMP-2 (Forward 5'-TGAGCAGCAGCAGAAGAAG-3', Reverse 5'-TCTCCGTGACCCAGTCCATC-3'), TGF- β 1 (Forward 5'-GACCTGGCCACCATTCACAG-3', Reverse 5'-AGTCAATGTAC AGCGCTGCCGC-3'), smad-3 (Forward 5'-CGAGAACACCAAC TTCCCCG-3', Reverse 5'-ATCGGATTCGGGGACAGGTT-3'), or GAPDH (Forward 5'-GATTCCACCCACGCAAGT-3', Reverse 5'-GGGATCTCCTCTCCTGGAAG-3'). Western blots were probed with primary antibodies to MMP2, transforming growth factor (TGF)- β 1, β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), collagen (Abcam, Cambridge, U.K.), and fibronectin (Cohesion Biosciences, London, U.K.).

Statistical Analysis

Data analysis was performed using the Graph Pad Prism 5 package (GraphPad Software Inc., La Jolla, CA, U.S.A.). The statistical significance of differences between the stents was evaluated using the Kruskal-Wallis test followed by a post hoc test (Dunn's Test). Null hypotheses of no difference were rejected if *P*-values were less than 0.05.

RESULTS

The surgical procedure in all animals was considered successful, with no reports of mortality or acute

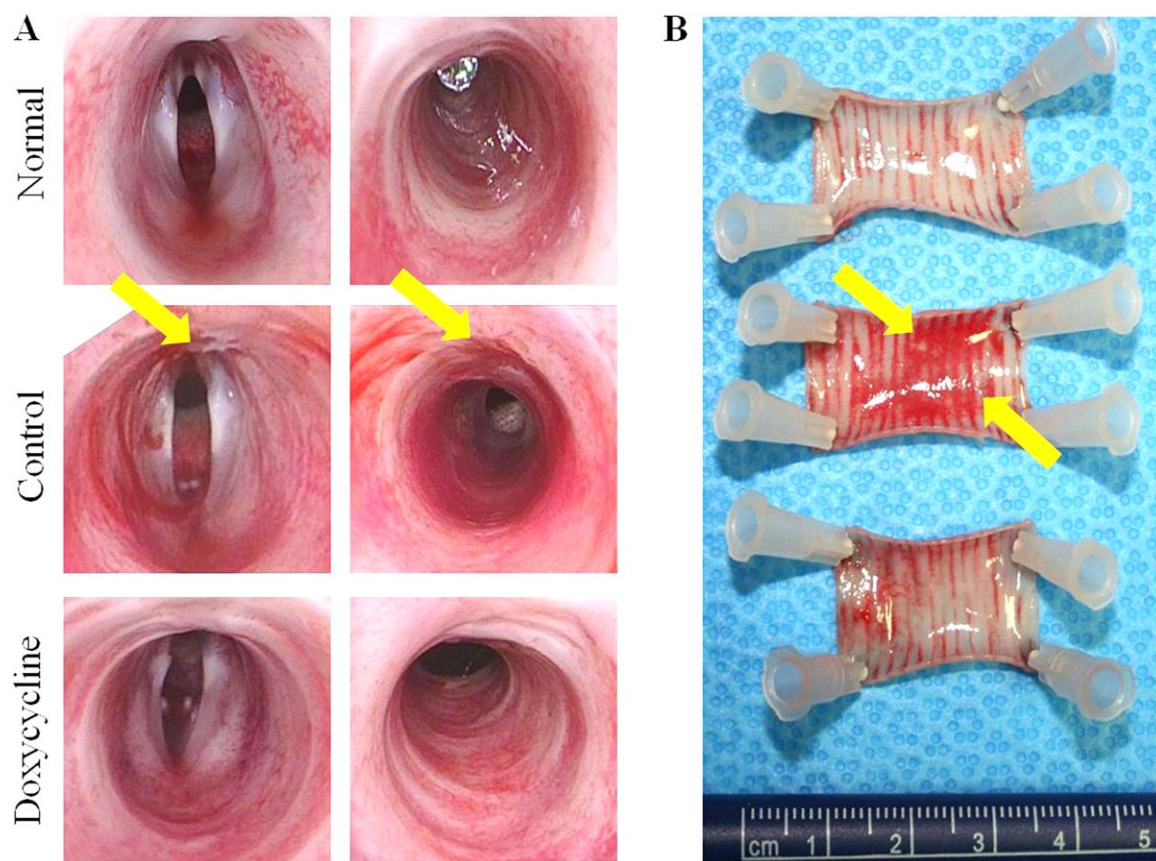


Fig. 2. Gross morphology of tracheal mucosa. (A) Endoscopic findings of tracheal mucosa. (B) Unfolded tracheal mucosa. Arrow represents granulation of tracheal mucosa.

respiratory problems during the 4-week period. Endoscopic findings and gross morphology of the tracheal mucosa are presented in Figure 2. The doxycycline-coated stent group had less flared tissue and granulation in the trachea than the noncoated control group (henceforth referred to as *control group*).

Figure 3A shows the inflammatory responses and fibrosis of the tracheal mucosa, as seen in the histologic examinations. The control group showed more inflammatory cells than the normal group ($P < 0.05$) (Fig. 3B), whereas the doxycycline-coating group showed fewer inflammatory cells than the noncoated stent (normal) group ($P < 0.05$) (Fig. 3B). The results of MTC staining showed significantly greater fibrosis in the control group than in the normal group ($P < 0.05$) (Fig. 3A and 3B), whereas the doxycycline-coating group showed lesser fibrosis than the normal group ($P < 0.05$) (Fig. 3A and 3B).

To investigate the changes of ECM components, the mRNA expressions of collagen 1, MMP2, fibronectin, tissue inhibitor of metalloproteinases (TIMP)2, TGF- β 1, and smad3 were compared among the groups (Fig. 4). Compared to the normal group, the mRNA expressions of collagen 1, MMP2, fibronectin, TIMP2 and TGF- β 1 were increased in the control group and decreased in the doxycycline-coated group. However, the mRNA expression of smad3 did not differ among the groups.

Western blotting showed higher expression levels of fibronectin, MMP2, and TGF- β 1 in the control group than in the normal group (Fig. 5A and 5B), whereas their expressions were reduced in the doxycycline-treated group than in the normal group. PCR and Western blot also showed lower expression of ECM components inducing fibrosis in the doxycycline-coated stent groups.

DISCUSSION

Tracheal stenosis is an important complication after endotracheal intubation or tracheal tube insertion because it leads to severe airway obstruction. In a prospective study, Stauffer et al. showed a high prevalence of tracheal stenosis following tracheotomy (65%) and endotracheal intubation (19%).¹¹ Tracheal stenosis occurs more often with prolonged rather than with short-term endotracheal intubation and tracheotomy.^{11,12} Apart from duration of intubation, numerous other factors that increase the prevalence of tracheal stenosis include the size of the endotracheal tube, traumatic intubation, presence of an infection while intubated, and gastroesophageal reflux.^{13,14} Many endoscopic methods and surgical procedures can reconstruct the trachea; however, repeated scar formation results in poor surgical outcomes.

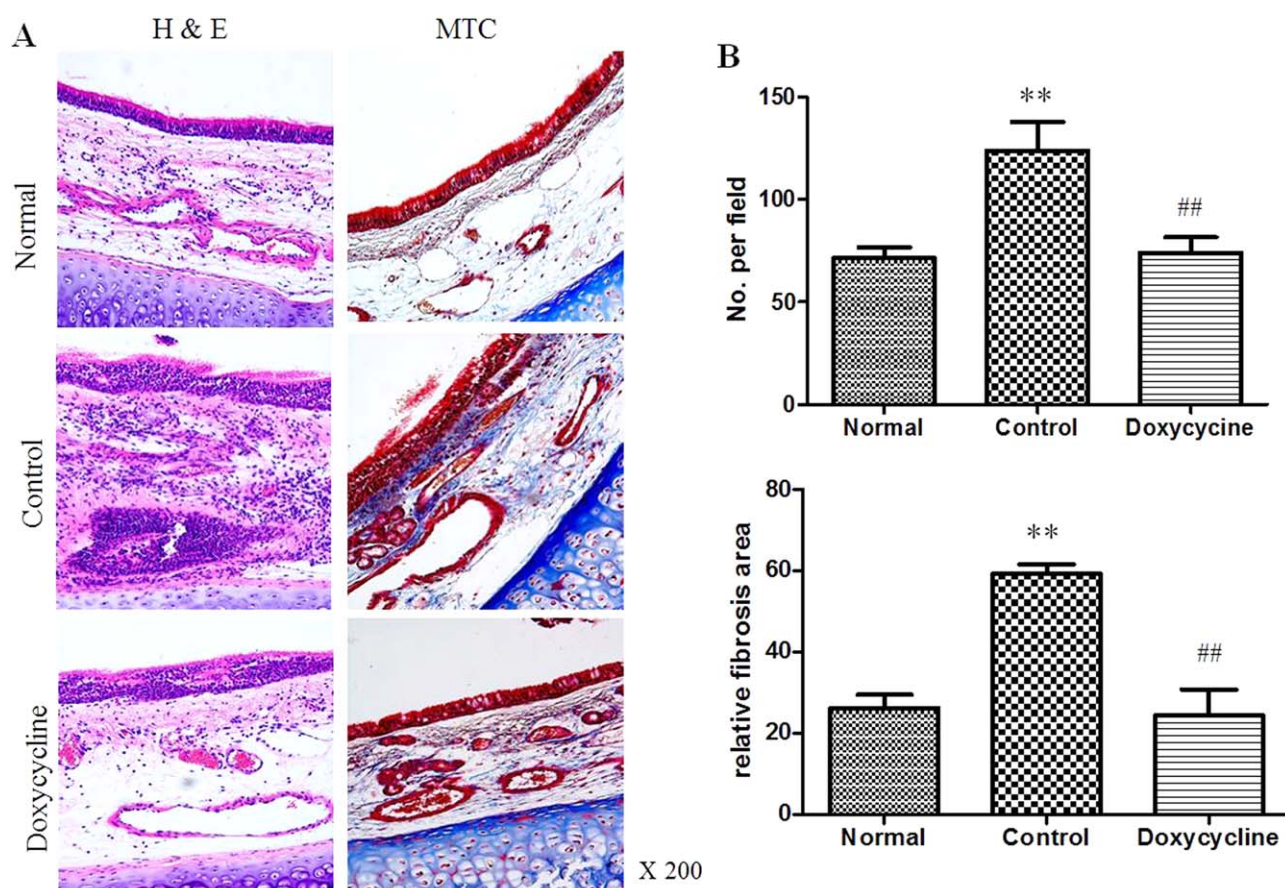


Fig. 3. Histologic findings of tracheal mucosa. (A) H&E and MTC staining ($\times 200$). (B) Differences of inflammatory cells and fibrosis areas in groups. Kruskal-Wallis, Dunn's post hoc test.

*compared to normal group; #compared to control group.

** $P < 0.01$; ## $P < 0.01$ ($n = 36$).

H&E = hematoxylin and eosin; MTC = Masson's Trichrome.

Damaged tissue can be repaired by ECM remodeling and scar formation. Some modulators in the ECM induce a scarless wound: heparin, anti-transforming growth factor beta, mitomycin C, 5-fluorouracil, and triamcinolone have been evaluated for prevention of fibrosis.^{3,4,15,16} Doxycycline has antimicrobial properties and is also effective in ECM modulation, regulation of wound healing, and immunomodulation.^{7,8,17} Hence, we hypothesized that doxycycline may offer a pharmacologic strategy for modulating tracheal stenosis by modification of the tissue repair process.

In this study, we used a nitinol stent coated with doxycycline to regulate the wound-healing process for preventing tracheal inflammation and fibrosis. As compared to the control groups, histologic examination revealed that the doxycycline-coated group showed less flaring and granulation of the tracheal mucosa, fewer inflammatory cells, and decreased area of fibrosis; and PCR and Western blot evaluations showed lower expression of ECM components. This is the first research demonstrating that doxycycline-coated nitinol stent has favorable effects in reducing the tracheal inflammation and fibrosis.

Doxycycline has been extensively studied in human and animal diseases such as osteoarthritis,¹⁸ periodontitis,¹⁹ and alveolar bone loss in rats,²⁰ and in the healing process of ulcers and chronic wounds.²¹ Doxycycline inhibits metalloproteinases and TNF- α -converting enzyme.²² Similar to previous studies, our results showed reduced mRNA expressions of MMP2 and TIMP2, as well as decreased protein expression of MMP2 in the doxycycline-coated group. TIMP2 has dual functions as an MMP inhibitor and an activator.²³

Doxycycline also prevents the release of inflammatory mediators that regulates the activity of neutrophils, eosinophils, and lymphocytes.²² Our histologic examinations showed significantly fewer inflammatory cells in the doxycycline-coated group when compared to the noncoated stent group. These studies suggest that elevated levels of inflammatory cytokines interfere with the healing of the tracheal mucosa by destroying growth factors and essential ECM components required for wound healing. The administration of doxycycline would thereby block the release of inflammatory mediators and have a positive effect on the mucosal healing.^{24,25}

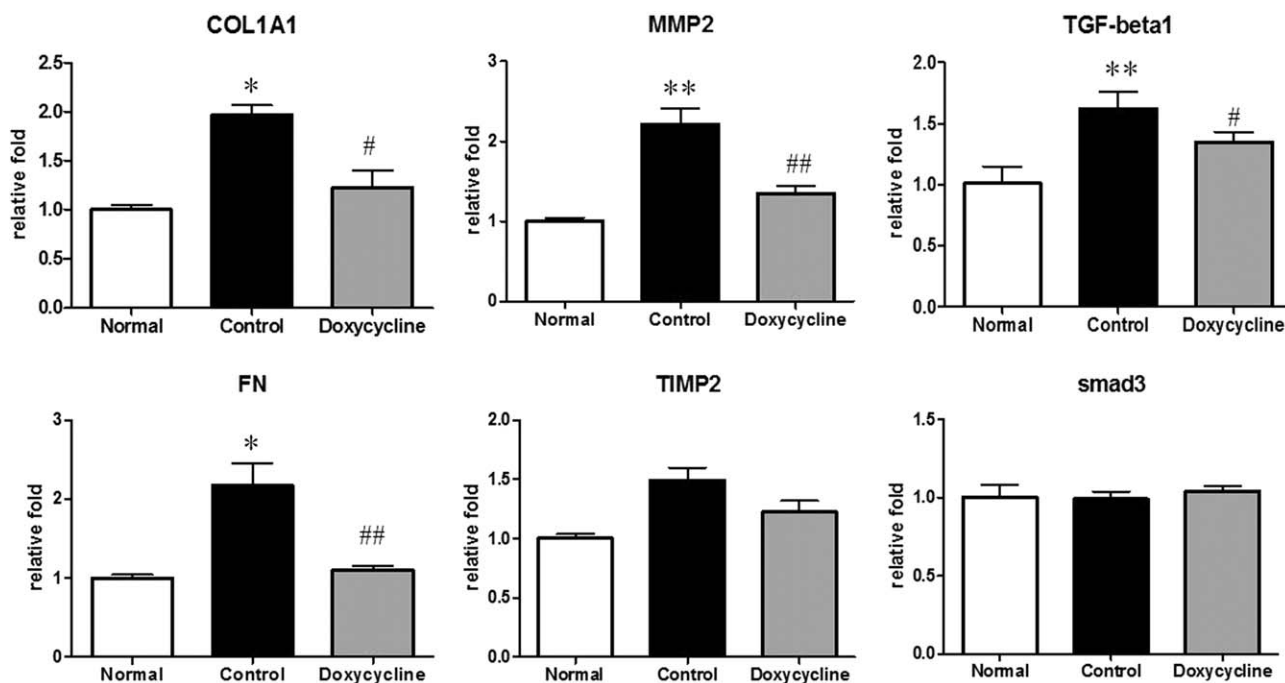


Fig. 4. Effect of doxycycline on messenger RNA of tracheal mucosa. Nitinol stent coated with doxycycline decreases the protein expressions of fibronectin, MMP2, and TGF β 1. Kruskal-Wallis, Dunn's post hoc test.

*compared to normal group; #compared to control group.

* $P < 0.05$; ** $P < 0.01$; # $P < 0.05$; ## $P < 0.01$ ($n = 12$).

MMP = matrix metalloproteinase; TGF =transforming growth factor; TIMP = tissue inhibitor of metalloproteinases.

TGF- β 1 is a multifunctional cytokine having an important role in wound healing and tissue repair.²⁶ In general, the release and activation of TGF- β 1 stimulates the production of extracellular matrix proteins and also inhibits the degradation of these matrix proteins. These mechanisms of TGF- β 1 may involve a component of

tissue fibrosis. In many diseases, excessive TGF- β 1 contributes to a pathologic excess of tissue fibrosis that compromises the normal organ function.²⁶ Histologic evaluations in our study revealed greater fibrosis in the control group, whereas the doxycycline-coated group revealed significantly lesser fibrosis. The expressions of

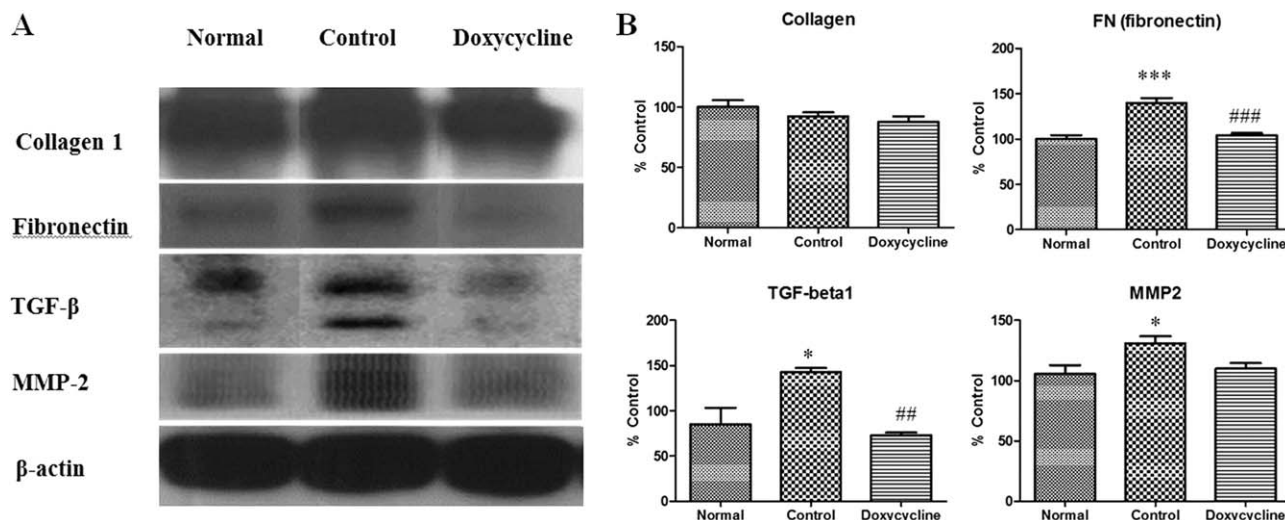


Fig. 5. Effect of doxycycline on protein expressions of tracheal mucosa. (A) Western blot of tracheal mucosa after insertion of nitinol stent coated with doxycycline. (B) Nitinol stent coated with doxycycline decreases the extracellular matrix components (collagen 1, MMP2, fibronectin, TIMP2) and fibrosis signal (TGF- β 1). Kruskal-Wallis, Dunn's post hoc test.

*compared to normal group; #compared to control group.

* $P < 0.05$; *** $P < 0.001$; ## $P < 0.01$; ### $P < 0.001$ ($n = 12$).

MMP = matrix metalloproteinase; TGF =transforming growth factor; TIMP = tissue inhibitor of metalloproteinases.

mRNA and TGF- β 1 protein were increased in the control group and decreased in doxycycline-coating group. These results suggest that the administration of doxycycline may contribute to prevent tracheal fibrosis.

In this study, we fabricated doxycycline-coated nitinol tracheal stents and tried to determine whether they prevented tracheal inflammation and fibrosis in a rabbit model. This coated nitinol tracheal stent is an innovative method for prevention and modulation of the tracheal stenotic section in airway-compromised patients.

Our rabbit animal model is suitable for tracheal stent evaluation, and provides a guide for evaluation of new strategies for treatment of tracheal inflammation and fibrosis. This is because dogs and pigs are expensive and hard to handle, and relevant drugs and antibodies in these animals are not readily available for molecular and immunological studies.

This is a preliminary study that offers well-known agents as a novel alternative for treatment of tracheal inflammation and fibrosis. However, it has some limitations that warrant consideration. First, the number of rabbits used was small. Second, because the observation period was short, further research with longer duration of observation will be necessary to document the changes in tracheal mucosa. Third, our study only shows that doxycycline can help prevent tracheal inflammation and fibrosis after tracheal injury and stent placement. A subsequent study is required to use doxycycline-coated balloons on endotracheal or tracheostomy tubes to show the prevention of tracheal stenosis in animals intubated for long periods of time. Our study supports the hypothesis that doxycycline is clinically useful in prevention of tracheal inflammation and fibrosis.

CONCLUSION

This study showed that a nitinol stent coated with doxycycline may have favorable effects on reducing the tracheal inflammation and fibrosis in a rabbit model. We suggest that local application of doxycycline should be further researched for prevention of tracheal stenosis.

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