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LARYNGOLOGY

Establishment of a novel and effective reflux laryngitis model in rabbits: a preliminary study

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Abstract

Purpose To establish a novel and effective reflux model with a modified nasogastric aspiration tube and to investigate the association between different types of nasogastric aspiration tubes and reflux laryngitis, we conducted this study.

Methods Thirty-eight healthy New Zealand albino rabbits (2.5–3.5 kg) were divided into three groups: control (CTR, $n=6$)—non-intubated; normal nasogastric intubation (NNI, $n=16$)—intubated with 4#, 6#, 8#, and 10# normal nasogastric aspiration tubes; and modified nasogastric intubation (MNI, $n=16$)—intubated with 4#, 6#, 8#, and 10# modified nasogastric aspiration tubes. The laryngoscopy, body weight, and pH values at the esophageal entrance were recorded before and 1, 2, and 4 weeks after intubation. After the final laryngoscopy, the animals in groups with a pH below 4 were sacrificed to obtain histological and gene expression analysis results.

Results The reflux finding score (RFS) after 4 weeks showed that there was a statistically significant difference in the 8# NNI group (7 ± 0.816, $P<0.001$), the 8# MNI group (11.5 ± 2.517, $P<0.001$) and the 10# MNI (12.75 ± 1.893, $P<0.001$) group compared with the control group (1.83 ± 1.602). The pH values of these three groups were lower than 4. However, the weight loss of the rabbits in the 10# NNI and 10# MNI groups was more obvious. Submucous gland hyperplasia and inflammation were significantly increased in the 8# NNI group, 8# MNI group and the 10# MNI group, but in the level of some pro-inflammatory cytokines and COX-2, the MNI group was significantly higher than the NNI group (8# NNI × 8# MNI, $P<0.01$; 8# MNI × 10# MNI, $P<0.01$).

Conclusion This study showed that 8# modified nasogastric intubation (MNI) produces effective reflux laryngitis in the rabbits.

Keywords Laryngopharyngeal reflux · Reflux laryngitis · Reflux finding score (RFS) · pH measurement · mRNA expression · Animal model

Introduction

Recently, the incidence of gastroesophageal reflux disease (GERD) was reported to be increasing in China [1], as well as in western countries. Laryngopharyngeal reflux (LPR) is defined as an extraesophageal manifestation of GERD [2]. LPR has been linked to several disorders, including reflux laryngitis (RL) and reflux cough. Those patients with RL must have one of the following symptoms: hoarseness, excessive phlegm or saliva, throat clearing, throat pain, "lump-in-throat" sensation, or chronic cough [3]. Currently, drug therapy is the main treatment for clinical reflux laryngitis, and proton pump inhibitors (PPIs) are the internationally recognized drugs of choice. A large number of clinical practice and randomized controlled trials have shown that acid-suppressing therapy has achieved good therapeutic results, benefiting the majority of patients [4]. However, there was a meta-analysis found that the efficacy of PPIs in patients with reflux laryngitis is comparable to that of a placebo [5]. In addition, in the past decade, a steadily increasing list of complications following long-term use of PPIs has been reported

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[6]. Thus, an effective and simple experimental reflux laryngitis animal model is needed to verify the efficacy of PPIs, investigate the pathophysiology and explore more safety and efficacious managements for reflux laryngitis.

Nasogastric intubation (NGI) is largely used in clinical practice [7]. However, several complications such as reflux laryngitis [8] and increased mortality because of aspiration pneumonia can be induced by this procedure. Previously, according to this complication, a rat model of reflux laryngitis was established [9]. However, due to the small larynx and the high mortality rate after intubation, the rat model of reflux laryngitis may be not practical. The rabbit is an ideal experiment object because the vocal cord size of rabbits is moderate, and the extracellular matrix (ECM) content is similar to human (Fig. 3a, b). It has been reported in the literature that total or partial cardiomyectomy can induce gastroesophageal reflux in rabbit [10], but this operation is complicated and requires a certain surgical anatomic technique. To establish an effective and simple reflux laryngitis model, we modified the normal nasogastric aspiration tubes. As shown in Fig. 1a, by opening the nasogastric aspiration tube at the esophagus, gastric acid can directly enter the esophagus and then cause reflux without cardiomyectomy. With this modification, we hypothesized that we may establish a more stable reflux laryngitis model, and then we can compare the effects of different types of nasogastric aspiration tubes further.

Materials and methods

Animals

Thirty-eight healthy, male New Zealand albino rabbits with corporal mass between 2.5 and 3.5 kg were used in this study. The animals were randomly assigned into three groups: control (CTR, $n = 6$)—non-intubated; normal nasogastric intubation (NNI, $n = 16$)—intubated with 4#, 6#, 8#, and 10# normal nasogastric aspiration tubes (Jiangyang Special Rubber Products Co., Ltd., Jiangsu, China); and modified nasogastric intubation (MNI, $n = 16$)—intubated with 4#, 6#, 8#, and 10# modified nasogastric aspiration tubes (Fig. 1b).

For the induction of laryngitis, the animals received intramuscular anesthesia with Diazepam (3 mg/kg; Xudong Haipu Pharma Co., Ltd., Shanghai, China), ketamine (40 mg/kg; Gutian Pharma Co., Ltd., Fujian, China) and Su-Mian-Xin (4 mg/kg; Shengda Pharma Co., Jilin, China).

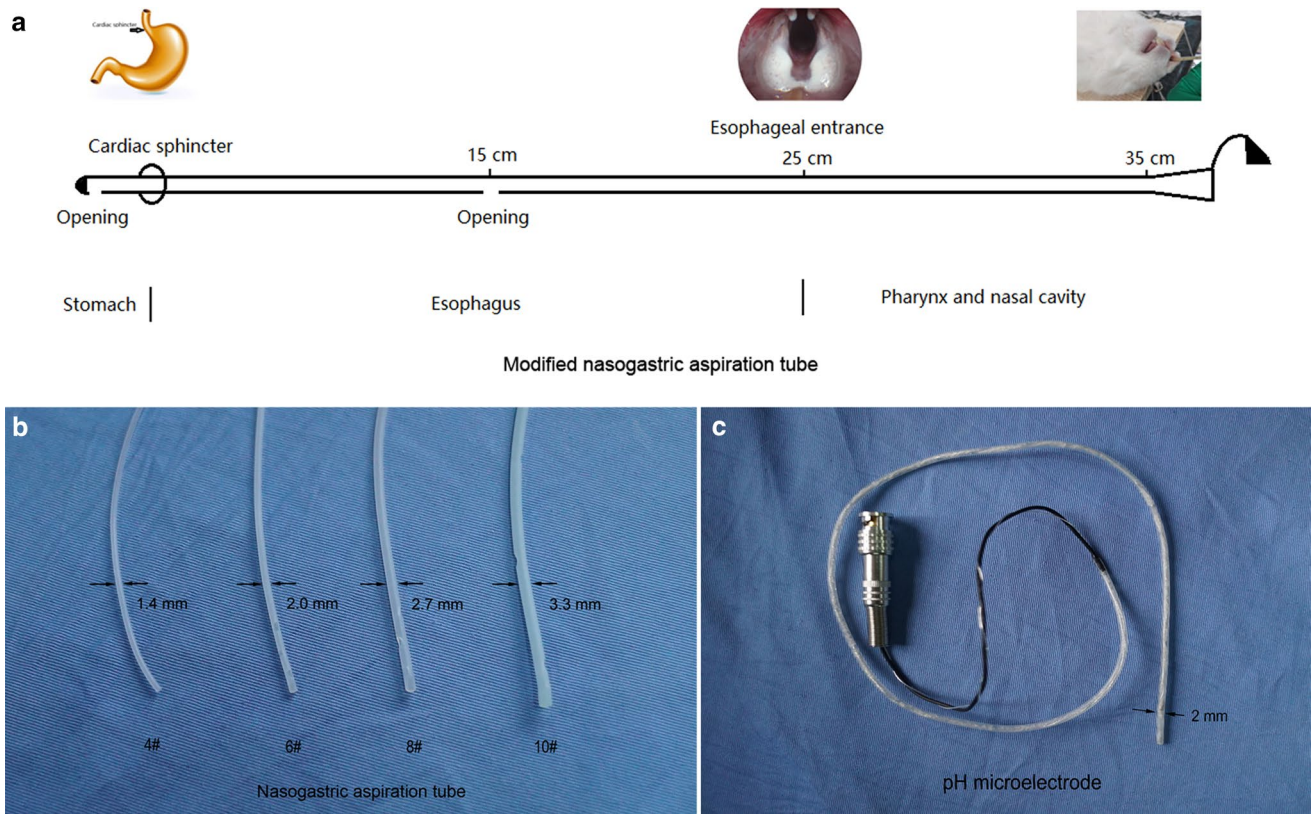

Fig. 1 Nasogastric aspiration tubes and pH microelectrode. **a** Modified nasogastric aspiration tube. **b** Different types of nasogastric aspiration tubes. **c** 2-mm pH microelectrode

A 33–35 cm (Anatomic data before the experiment) narrow-bore nasogastric aspiration tube was inserted through the nasopharynx until it reached the stomach. The external tip was sutured to the nasal lateral cartilages. Subcutaneous Buprenorphine (0.05 mg/kg, 8/8 h, during the first 48 h; BOTaier Biotechnology Co., Ltd., Shenzhen, China) was administrated in the NGI group to reduce the pain. All animals underwent examination with a 3-mm laryngoscope (Tiansong Medical Instrument Co., Ltd., Zhejiang, China) and a pH monitor (Jiaoyuan Analytical Instrument Co., Ltd., Jiangsu, China) with a 2-mm pH microelectrode (Fig. 1c) immediately and 1, 2, and 4 weeks after intubation. At the end of the intubation period (4 weeks), the animals were euthanized with halothane (3%, inhaled) and the larynx was removed to obtain morphological, histological, and gene expression results. The study was approved by the Research Ethics Committee of Eye, Ear, Nose and Throat Hospital of Fudan University.

Morphological evaluation

Laryngoscopy and reflux finding score (RFS)

After intramuscular anesthesia with Diazepam (3 mg/kg; Xudong Haipu Pharma Co., Ltd., Shanghai, China), ketamine (40 mg/kg; Gutian Pharma Co., Ltd., Fujian, China) and Su-Mian-Xin (4 mg/kg; Shengda Pharma Co., Jilin, China), the animals received laryngoscopy. Images were recorded pre and post intubation using a 3-mm endoscope system (Tiansong Medical Instrument Co., Ltd., Zhejiang, China). Because there was no previous assessment of normal values for rabbits, we referenced the method reported by Zhang et al. [10] using a standard RFS system described by Belafsky et al. [11] to analyze images. The RFS was calculated by two independent observers.

Body weight and laryngopharyngeal pH measurement

The changes in body weight reflect the nutritional status of the rabbits, which can be measured by a digital scale (SF-400; Qiantuan Industry and Trade Co., Ltd., Zhejiang, China). Measurements of pH were performed using a 2-mm pH microelectrode attached to a pH meter (Jiaoyuan Analytical Instrument Co., Ltd., Jiangsu, China) (Fig. 1c). We recorded the body weight and pH values at the esophageal entrance (5 s for pH measurement) immediately and at 1, 2, and 4 weeks after intubation. Using the standard marker for reflux, a measured pH below 4 [12], groups with obvious acid reflux underwent histological and gene expression analysis.

Histology

All animals with a measured pH below 4 were sacrificed 4 weeks after intubation. Animals that did not achieve a pH less than 4 were not sacrificed. The 6 non-intubated rabbits were also sacrificed for comparison. Laryngeal tissues were obtained and fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. 4-μm-thick sections were mounted on glass slides and stained with hematoxylin and eosin (H&E). Light microscopy was used to compare the findings among groups in the study. All sections were coded to avoid observers' bias during examination. Histological changes were considered as follows:

Inflammation

At a magnification of 400, the number of lymphocytes present in the submucosa was scored as follows: 0 (20 lymphocytes); 1 (21–50 lymphocytes); 2 (51–80 lymphocytes); 3 (81–120 lymphocytes); 4 (> 120 lymphocytes).

Submucous gland hyperplasia

At a magnification of 200, a score was assigned according to the number of submucous glands visible in section: 0 (less than three glands); 1 (3–10 glands); 2 (11–30 glands); or 3 (> 30 glands).

Gene expression analysis

The real-time polymerase chain reaction (RT-PCR) was performed 4 weeks after intubation. Larynges were excised immediately, macerated, immersed in 0.8 ml of TRIzol (Invitrogen, Carlsbad, CA, USA), and stored at − 80 °C. Total RNA was extracted using Ultrapure RNA Kit (CWBio, Shanghai, China). Quantification of total mRNA was performed by spectrophotometry (NanoDrop Technologies, Inc., Wilmington, NC, USA). Reverse transcription was performed using PrimeScript RT Master Mix (RR036; Takara, Kusatsu, Japan). The cDNA was then subjected to real-time RT-PCR reactions for quantification of mRNA levels of GAPDH, TNF-α, COX-2, IL-6, IL-1β, and IL-10 using the SYBR Premix EX Tap (Takara, Kusatsu, Japan). Primer sequences used to amplify various cDNA are shown in Table 1. A typical real-time RT-PCR protocol was performed under the following conditions: 30 s at 95 °C, followed by 40 cycles (95 °C denaturing for 5 s; 60 °C annealing for 30 s), melting at 60 °C until 95 °C for 90 s, and finally cooling to 35 °C. The specificity of the SYBR Green assays was confirmed by melting point analysis. Gene expression of the housekeeping gene glyceraldehyde-3-phosphate

Table 1 Sequences of primers used for real-time polymerase chain reaction

Target	Oligo	Sequence	Gene Bank ACC
GAPDH	Forward primer	5′-GCTTCTTCTCGTGCAGTGCT-3′	NM_001082253.1
	Reverse primer	5′-GCCGTGGGTGGAATCATACT-3′	
IL-1β	Forward primer	5′-CCAGTGAGATGATGGCTTACC-3′	NM_001082201.1
	Reverse primer	5′-GTTGTAGGGTTGGCAGGAGA-3′	
TNF-α	Forward primer	5′-TAGGAGGGAGAGCAGCAACT-3′	NM_001082263.1
	Reverse primer	5′-GGTGCGTGAGAGAGGAAGAC-3′	
IL-6	Forward primer	5′-TGAACTCCTTCACAAGCGCC-3′	NM_001082064.2
	Reverse primer	5′-TGAAGTGGGGAAAGCGGTAG-3′	
IL-10	Forward primer	5′-TCAGCTCTGCTATGTTGCCC-3′	NM_001082045.1
	Reverse primer	5′-AGTCTTCACCCTGCCAAAGG-3′	
COX-2	Forward primer	5′-AGCCTTCACCAAAGGACTGG-3′	NM_001082388.1
	Reverse primer	5′-AGAGGCGCAGTTTATGCTGT-3′	

dehydrogenase (GAPDH) was used for normalization of the results. The delta–delta cycle threshold (ΔΔCt) was used to calculate the relative fold changes.

Statistical methods

The differences in RFS were evaluated by paired *t* test. Analysis of variance was used to analyze the mRNA levels. Multiple comparisons among groups were made by the Tukey method. The Mann–Whitney *U* test was used to evaluate the histological differences. Data values are shown as mean ± standard deviation. $P < 0.05$ was considered statistically significant. All data were analyzed using SPSS 22 software (IBM, Armonk, NY, USA).

Results

Laryngoscopy and RFS

As shown in Fig. 2, in the groups of 8# NNI, 8# MNI, and 10# MNI, the laryngoscope showed more mucus adhesion in the larynx and edema in the interarytenoid after 4 weeks, but no significant differences were found in the remaining groups. The reflux finding score (RFS) after 4 weeks showed that there was a statistically significant difference in the 8# NNI group (7 ± 0.816, $P < 0.001$), the 8# MNI group (11.5 ± 2.517, $P < 0.001$) and the 10# MNI (12.75 ± 1.893, $P < 0.001$) group compared with the control group (1.83 ± 1.602) (Fig. 3e).

Laryngopharyngeal pH and body weight measurement

Different types of nasogastric aspiration tubes resulted in different changes in the pH recorded at the esophageal entrance. There was no obvious gastric acid reflux (pH > 4) in the groups of 4# NNI, 6# NNI, 10# NNI, 4# MNI, 6# MNI and CTR. 8# MNI group and 10# MNI group showed gastric acid reflux immediately after intubation. Then, the pH was lower than 4 at 1, 2, and 4 weeks after intubation. No obvious gastric acid reflux was observed immediately after intubation in 8# NNI group, but the pH was below 4 at 1, 2, and 4 weeks after intubation (Fig. 3c). In addition, we monitored the weight of the experimental rabbits (representing the nutritional status of the rabbit) and found that the intubation had different degrees of negative effects on the rabbit body weight, and the weight loss of the rabbits in the 10# NNI and 10# MNI groups was more obvious (Fig. 3d).

Histology

Inflammation

There were significant differences between the nasogastric intubation group and the control group in inflammatory cell infiltration (8# NNI, $P < 0.05$; 8# MNI, $P < 0.01$; 10# MNI, $P < 0.01$) (Fig. 4a, b).

Submucous gland hyperplasia

The comparison of submucous gland hyperplasia between the nasogastric intubation group and the control group revealed a significant difference (8# NNI, $P < 0.05$; 8# MNI, $P < 0.01$; 10# MNI, $P < 0.01$) (Fig. 4c, d).

Laryngeal levels of pro-inflammatory and anti-inflammatory cytokines

The 8# NNI, 8# MNI and 10# MNI groups all induced drastic changes of inflammatory mediators. The relative expression of IL-1β mRNA increased significantly in the 8# NNI group ($P < 0.001$), 8# MNI group ($P < 0.001$), and 10# MNI ($P < 0.001$) group compared with the control group. In

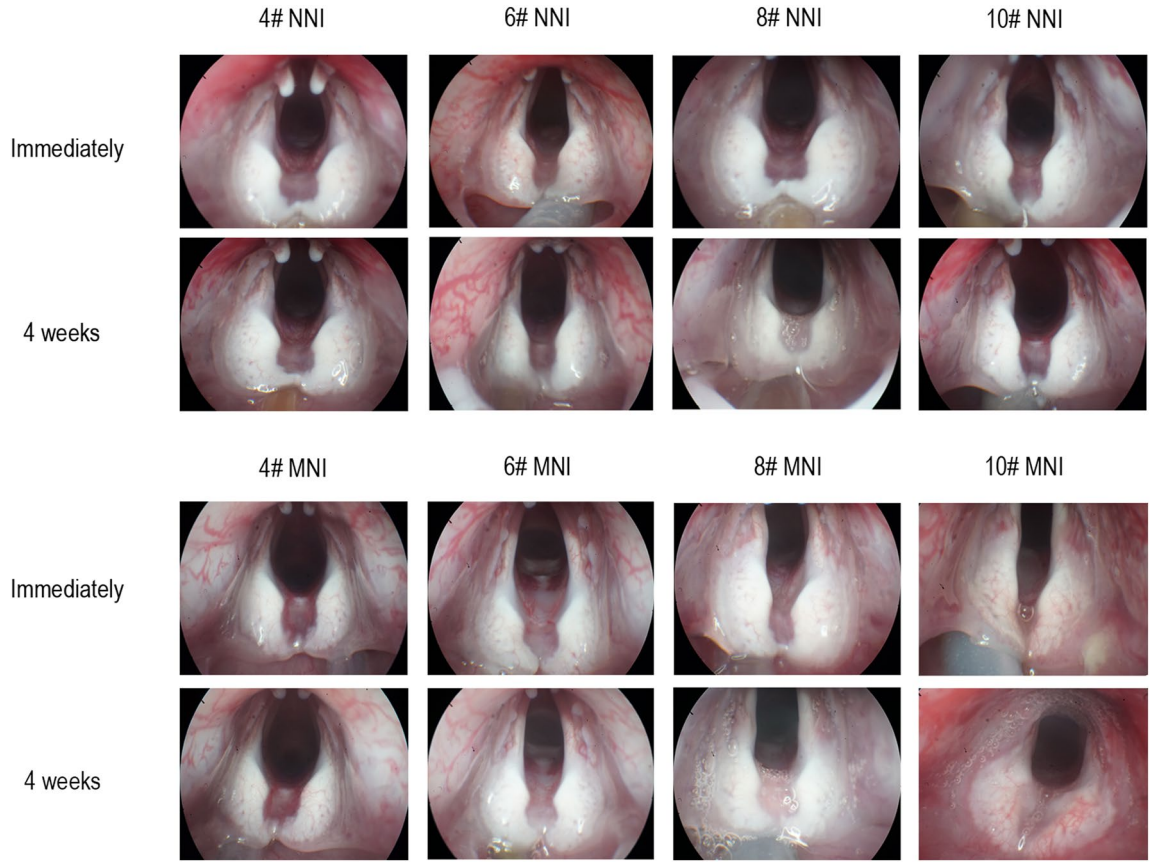

Fig. 2 Laryngoscopy of the rabbits with NNI and MNI. Vocal cord edema and mucus adhesion in the groups of 8# NNI, 8# MNI, and 10# MNI 4 weeks after intubation. No significant differences were found in the remaining groups. NNI: normal nasogastric intubation. MNI: modified nasogastric intubation

addition, the 8# MNI and the 10# MNI groups were significantly higher than the 8# NNI group ($P < 0.05$). There was no statistically significant difference between the 8# MNI group and the 10# MNI group (Fig. 5a). The 8# NNI, 8# MNI, and 10# MNI model also affected the level of expression of IL-6 mRNA, which increased significantly after 4 weeks (8# NNI × CTR, $P < 0.001$; 8# MNI × CTR, $P < 0.001$; 10# MNI × CTR, $P < 0.001$). However, there was no statistically significant difference among them (Fig. 5b). TNF-α mRNA changed significantly in the laryngeal mucosa, showing an increase at 4 weeks after nasogastric intubation (8# NNI × CTR, $P < 0.001$; 8# MNI × CTR, $P < 0.001$; 10# MNI × CTR, $P < 0.001$), but no obvious difference among them (Fig. 5c). Changes in mRNA levels of the anti-inflammatory cytokine IL-10 showed a strong decrease 4 weeks after nasogastric intubation (8# NNI × CTR, $P < 0.01$; 8# MNI × CTR, $P < 0.001$; 10# MNI × CTR, $P < 0.001$). In addition, the mRNA levels in 8# MNI group and 10# MNI group were significantly lower than the one in 8# NNI group ($P < 0.05$), and there was no statistically significant difference between the 8# MNI group and the 10# MNI group (Fig. 5d).

The 8# NNI, 8# MNI, and 10# MNI model also induced changes in the expression of COX-2 mRNA levels in the laryngeal mucosa, with an increase at 4 weeks after nasogastric intubation (8# NNI × CTR, $P < 0.01$; 8# MNI × CTR, $P < 0.001$; 10# MNI × CTR, $P < 0.001$). In addition, the expression in MNI group was significantly higher than the one in NNI group (8# NNI × 8# MNI, $P < 0.01$; 8# MNI × 10# MNI, $P < 0.01$), but there was no statistically significant difference between the 8# MNI group and the 10# MNI group (Fig. 5e).

Discussion

The larynx is a complex organ that is important for airway protection and maintaining safe swallowing and positive pressure in the pulmonary system. Laryngitis, one of the most common diseases of larynx, can impair some or all of these functions. Most investigators agree that the laryngitis is caused by infection (viral, bacterial, or fungal), trauma, alcohol and tobacco consumption, and laryngopharyngeal reflux (LPR) disease. Among them, LPR disease may

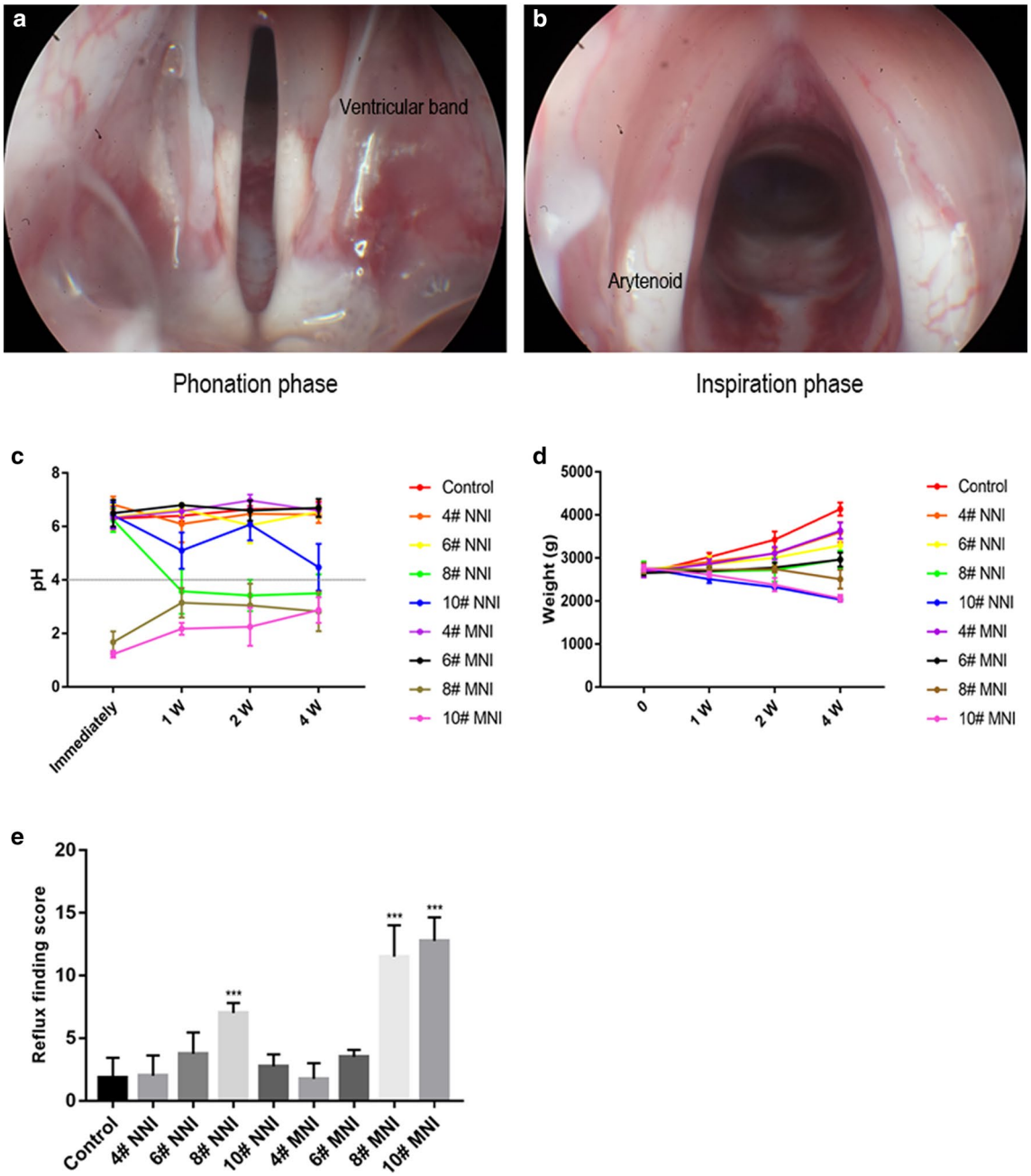

Fig. 3 Normal rabbit vocal cord, **a** phonation phase, **b** inspiration phase. **c** The pH values of three groups at immediately, 1 week, 2 weeks, and 4 weeks after intubation. **d** The body weight of three groups at immediately, 1, 2, and 4 weeks after intubation. **e** The reflux finding score (RFS) of three groups 4 weeks after intubation. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus control

represent the major etiologic factor of laryngitis [13]. Those patients with reflux laryngitis (RL) must have one of the following symptoms: hoarseness, excessive phlegm or saliva, throat clearing, throat pain, "lump-in-throat" sensation, or chronic cough [3]. However, the pathogenesis of laryngopharyngeal reflux is not clear. The lower esophageal sphincter (LES), as the first barrier against reflux, obviously plays an important role in reflux diseases. The myectomy of LES is reported in the literature as a method for making reflux animal models. It has been confirmed that partial and total cardiomyectomy could result in efficacious gastroesophageal and laryngopharyngeal reflux in rabbits [10, 14]. However, this kind of operation is complicated and requires a certain surgical anatomic technique. In this study, we used a modified nasogastric aspiration tube to cross the LES to allow gastric acid to flow directly from the stomach into the

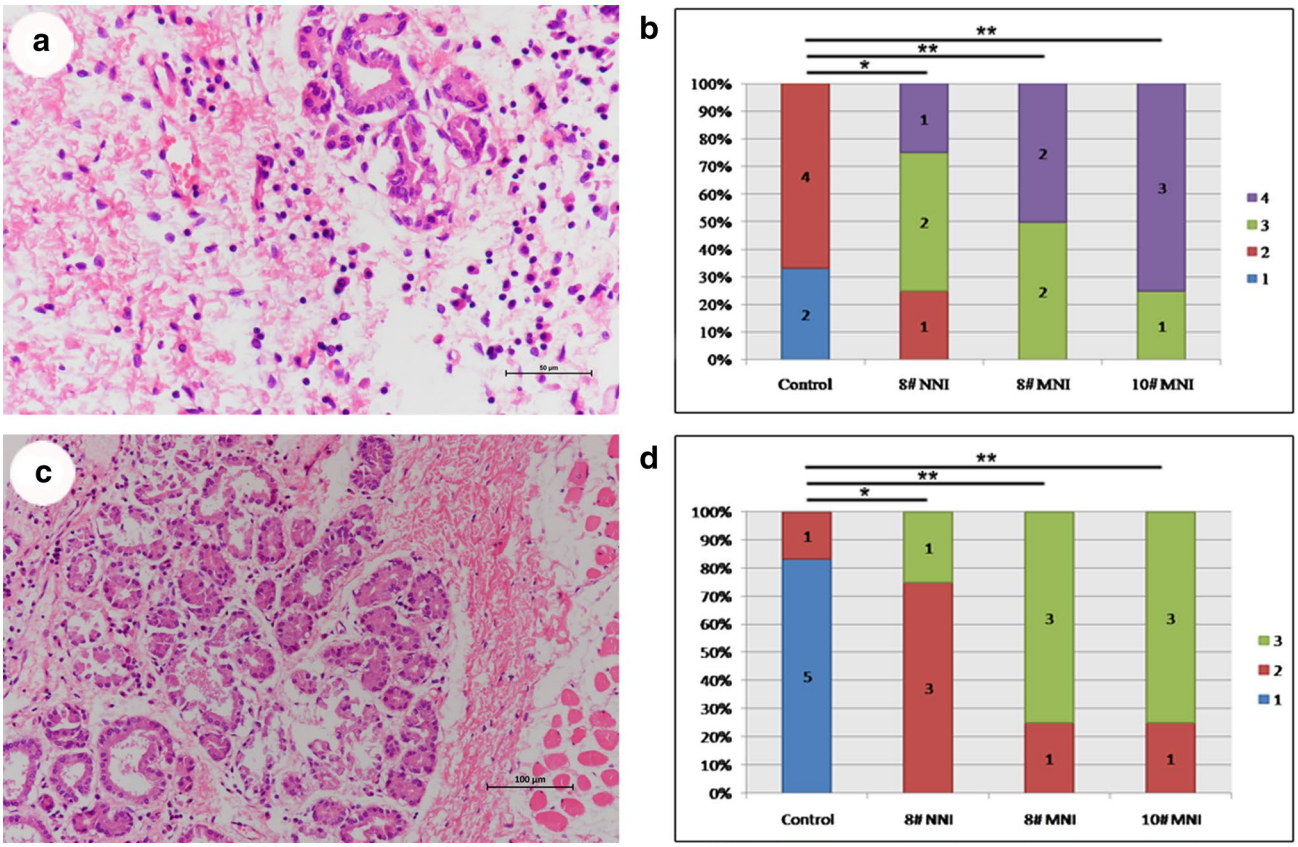

Fig. 4 Histological changes. **a** Inflammatory cell infiltration in laryngeal mucosa. **b** Inflammation distribution of 8# NNI, 8# MNI, 10# MNI and CTR. **c** Submucous gland hyperplasia in laryngeal mucosa. **d** Submucous gland hyperplasia distribution of 8# NNI, 8# MNI, 10# MNI and CTR. CTR: control. *NNI* normal nasogastric intubation, *MNI* modified nasogastric intubation. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus control

esophagus, thereby establishing a simple and effective gastroesophageal and laryngopharyngeal reflux model without cardiomyectomy.

Ambulatory 24-h dual probe pH-metry was once considered the best method for gastroesophageal reflux testing [15]. However, for the diagnosis of laryngopharyngeal reflux, several authors hold the view that a hypopharyngeal sensor is required due to the lack of direct evidence for reflux in the laryngopharynx [16]. In our study, we put the pH sensor at the esophageal entrance to detect whether the gastric contents had reached the laryngopharynx. Currently, a criterion for the diagnosis of laryngopharyngeal reflux is pH level < 4, because the laryngopharyngeal mucosa is easier to be damaged in acid than the esophageal mucosa [12].

In our study, we found that the 8# NNI group, 8# MNI group and 10# MNI group each had laryngopharyngeal reflux after intubation. In NNI group, a nasogastric tube that is too thin is not enough to open the cardia, while a too thick nasogastric tube can open the cardia but will affect the reflux of gastric acid. The lessened reflux seen with the larger tubes may be due to the lessened space between the esophageal wall and the wall of the tube. This would limit the volume of gastric contents that are refluxed. Thus, the 8# nasogastric tube is the ideal size for this group. In the MNI group, we found that the 8# and 10# nasogastric aspiration tubes could cause more obvious laryngopharyngeal reflux. It may be because the nasogastric aspiration tubes (4# and 6#) are too thin to pass the gastric acid, and the thin tubes are easily blocked by the gastric contents which affect the reflux effect. In addition, we also found that the weight loss of rabbits in the 10# nasogastric intubation group was more obvious, which may be caused by the nasogastric aspiration tube being too thick thus hindering food swallowing.

LPR is a chronic process. Most patients of LPR have symptoms and signs for months, even years. In this study, we only performed histological analysis at 4 weeks after intubation and found significant histological changes of the larynx. Shimazu et al. [17] used a rat model with induced chronic acid reflux esophagitis to observe histological changes of the pharynx and the larynx. There were no pathological changes in the mucosa of the pharynx and the larynx 2 weeks after surgery; however, there

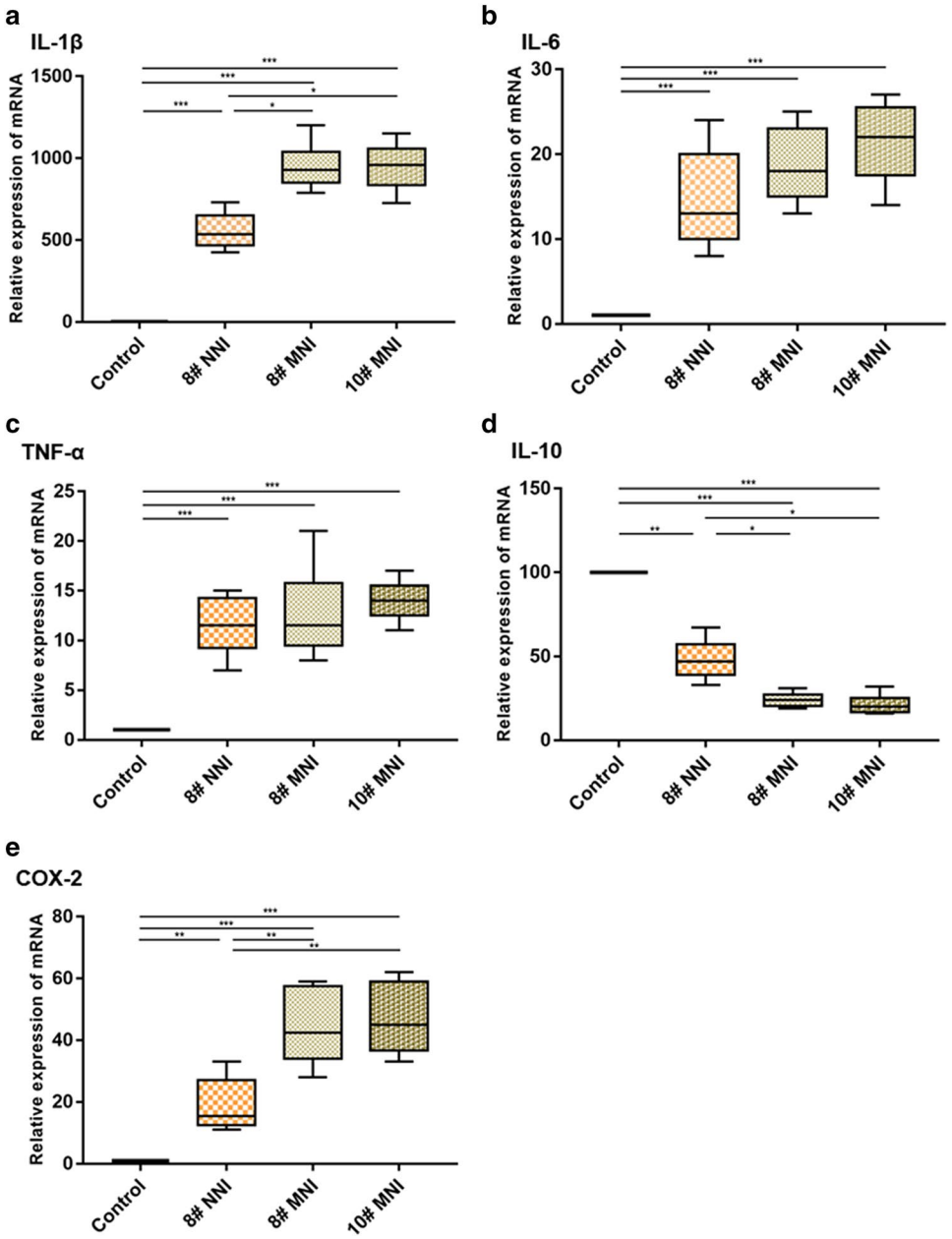

Fig. 5 Relative expression of mRNA levels obtained for pro-inflammatory and anti-inflammatory cytokines in the laryngeal mucosa of animals with 8# NNI, 8# MNI and 10# MNI. Note the different levels of expression of interleukin (IL)-1β (**a**), IL-6 (**b**), tumor necrosis factor (TNF)-α (**c**), IL-10 (**d**) and COX-2 (**e**). NNI: normal nasogastric intubation. MNI: modified nasogastric intubation. $*P<0.05$; $**P<0.01$; $***P<0.001$

were pathological changes such as thickening of mucosa as well as inflammatory infiltration in the pharynx and larynx 8 and 12 weeks after surgery, respectively. In the study of Zhang et al. [10], they used a rabbit model with total cardiomyectomy, and found significant pathological changes 8 weeks after surgery. In the current study, obvious histological changes were observed in 8# NNI group, 8# MNI group and 10# MNI group 4 weeks after intubation, which indicates that appropriate NNI and MNI all can cause reflux laryngitis, and the reflux effect is more obvious than the cardiomyectomy. Of course, further experiments are required to compare these two kinds of methods directly.

Interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α are pro-inflammatory cytokines which are produced after damage induces COX-2 synthesis and cells release prostaglandins (PGs) [18]. IL-10 is an anti-inflammatory cytokine. In this study, we found 8# NNI, 8# MNI, and 10# MNI groups have different degrees of increase in the level of some pro-inflammatory cytokines and COX-2, but the MNI group was significantly higher than the NNI group. Of course, there are some limitations in this study. For instance, we only analyzed the histological and gene expression results of each group at the forth week and lacked a dynamic study. We also did not directly compare the effects of the modified nasogastric intubation and cardiomyectomy.

In follow-up studies, we will use the model to investigate the pathophysiological mechanisms of reflux laryngitis and evaluate the effectiveness of existing acid-suppressing drugs, such as PPI, which was previously documented as having the same efficacy as placebo for the symptoms of laryngopharyngeal reflux [19].

Conclusion

This study showed that 8# modified nasogastric intubation (MNI) may produces significantly effective reflux laryngitis in the rabbit, and thus provides an experimental model (1) to test the effectiveness of anti-reflux procedures, (2) to investigate the pathophysiological mechanisms of reflux laryngitis, (3) to evaluate the effect of new therapeutic approaches for clinical treatment of reflux laryngitis.

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Compliance with ethical standards

Conflict of interest No conflict of interest exists in the submission of this manuscript.

Ethical approval Ethical approval was obtained from the ethics committee of Eye, Ear, Nose and Throat Hospital of Fudan University (2017061).

Informed consent Informed consent was obtained from all individual participants included in the study.

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