

A Bama Minipig Model of Laryngopharyngeal Reflux and the Change of Laryngopharyngeal Mucosal Ultrastructure

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Background/Aims

To establish an animal model of laryngopharyngeal reflux (LPR) and study the effect of LPR on the laryngopharyngeal mucosal ultrastructure.

Methods

Ten Bama minipigs were randomly divided into control group and stent group. Every pig underwent endoscopy, and baseline pH was monitored for 4 hours at laryngopharynx and distal esophagus, then specimens from laryngopharyngeal mucosa were biopsied. For the control group, these procedures were repeated on the 14th day. In the stent group, a custom-designed esophageal stent suit was implanted into esophagus, laryngopharyngeal and distal esophageal pH monitoring lasted for 2 hours, then stent suit was removed 3 days later. At last, the same procedures were done as the control group on the 14th day. Specimens were observed under transmission electron microscope to measure the intercellular space and desmosome number.

Results

In the control group, there was no laryngopharyngeal reflux on the first day and 14th day. Before the stent was implanted, there was also no laryngopharyngeal reflux in the stent group. In both 2 hours and 14 days after stent implantation, the number of reflux, reflux time, and percentage time of pH < 4.0 were significantly increased ($P < 0.05$) in the stent group. There was no difference in intercellular space and desmosomes in the control group between baseline and 14th day. In the stent group, intercellular space of laryngopharyngeal mucosa was significantly increased (0.37 μm vs 0.96 μm , $P = 0.008$), and the number of desmosomes was significantly decreased (20.25 vs 9.5, $P = 0.003$).

Conclusions

A Bama minipig model of LPR was established by implanting a custom-designed stent suit. LPR might destroy the laryngophar

Received: September 30, 2014 Revised: December 23, 2014 Accepted: December 25, 2014

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Financial support: This study was supported by National Natural Science Foundation of China (project No. 812245).

Conflicts of interest: None.

Author contributions: Guijian Feng, planning and conducting the study, collecting and interpreting data, and drafting the manuscript; Zhenyu Zhang, stent implanting; Chunyan Diao, stent implanting; Jun Jiang, minipig breeding; Shuying Zheng, Electron Microscope; and Yulan Liu, planning and conducting the study.

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yngeal mucosal barrier.

(*J Neurogastroenterol Motil* 2015;21:182-188)

Key Words

Extracellular space; Laryngopharyngeal reflux; Models, animal

Introduction

Laryngopharyngeal reflux (LPR) was first defined by the American Academy of Otolaryngology-Head and Neck Surgery in 2002.¹ LPR disease (LPRD) refers to the backflow of stomach contents (eg, acid, bile, pepsinogen, etc) into the laryngopharynx through the upper esophageal sphincter, resulting in injuries to the laryngopharynx, oral cavity, and respiratory tract. It was reported that 4-10% of patients referred to ear, nose, and throat (ENT) physicians were diagnosed with LPR,^{2,3} and a number of patients with gastroesophageal reflux disease (GERD) in the department of gastroenterology were also identified to be suffering from LPR.⁴ LPRD is usually complicated with chronic pharyngitis, laryngitis, laryngeal carcinoma, bronchial asthma, and sleep apnea syndrome, which can impact the quality of life.⁵⁻⁸ Compared with GERD, patients with LPRD had a longer treatment course and higher cost of treatment. Moreover, the incidence of LPR has been increasing over the past few years. Therefore, it would be very important to establish LPR animal model in order to study the mechanism and treatment to LPR.

In order to establish the exact model of LPR, it should be kept in mind that the abnormal dynamic barrier is the initiator of LPR. The gastroesophageal junction constitutes the first barrier against LPR, including lower esophageal sphincter (LES) and phrenic angle. Normal motility and clearing function of esophageal body are the second barriers against LPR. In GERD patients, especially in some patients with reflux esophagitis, esophageal motility was found to be ineffective.^{9,10} Knight et al¹¹ also found 73% patients with LPR symptoms to have esophageal motility problems. Tsutsui et al¹² reported that 47.9% had abnormal esophageal motility among patients with proton pump inhibitor (PPI)-resistant LPRD. The last barrier against LPR is upper esophageal sphincter (UES) in which tonic cricopharyngeal muscle contraction maintains the high pressure of UES. One recent study observed that abnormal UES and esophageal motility associated with impaired bolus clearance in patients with chronic cough were mostly marked in patients with a positive reflux-

cough symptom association.¹³

If there is a way to make esophagus lose these barriers, then a new laryngopharyngeal reflux model will be established. Esophageal stent implant is an important method to treat esophageal stenosis with many kinds of stents showing anti reflux function.¹⁴⁻¹⁶ The stent without anti reflux function is likely to damage barrier function of esophagus and cardia. When a stent is placed under the UES or LES, the function of esophageal body is completely shielded, leading to the induction of LPR. In the present study, we for the first time used a specialized custom stent suit to destroy LES and the barrier of esophageal body, and successfully established a Bama minipig model of LPR. Furthermore, the changes of laryngopharyngeal mucous ultrastructure have not been largely studied. In GERD, intracellular space of esophageal cells is increased,¹⁷⁻¹⁹ and in LPR with GERD, Park et al²⁰ reported increased intracellular space also in esophagus. We have also studied the ultrastructure of laryngopharyngeal mucosa in this model to clarify the pathological mechanism of LPR.

Materials and Methods

Animals

Ten male Bama minipigs were purchased from Beijing chuang shi century minipig breeding base. The Bama minipigs were 4-6 months old, the mean weight was 10 kg, and the mean length was 71 cm. Minipigs were maintained in a pathogen-free environment with a 12:12-hour light-dark cycle, temperature (20-22°C) and humidity (50-60%) for 1 week prior to experiment in order to adapt to environment. Ten Bama minipigs were randomly divided into control group and stent group. All animal care and procedures were approved by the Animal Care Committee of Beijing University People's Hospital and complied with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication 80-23, revised 1996).

pH Monitoring

Anesthesia was induced with intramuscular injection of a mixture of 1 mg ketamine and Sumianxin II for each pig. Anesthesia was maintained with Phenobarbital when needed by 1 mg prn. The dose was adjusted according to the depth of anesthesia. Trachea cannula was inserted when the eyelash reflex ceased.

The minipigs were examined using endoscopy (GIF-2T200 and GIF-XP-260N; Olympus Medical Systems Corp, Tokyo, Japan) to determine the distance from esophageal orifice to the incisor tooth and the distance from the cardia dentate line to the incisor tooth. Oropharyngeal electrode was implanted in the esophageal orifice to monitor pharynx pH using the Restech Dx-pH system (Restech Company, San Diego, CA, USA). The distal esophagus electrode was implanted 5 cm above the cardia dentate line to monitor esophagus pH using the Sandhill system (Sandhill Z07-2000B-B monitor; Sandhill Scientific, Highlands Ranch, CO, USA). Endoscope was used to determine the success of implanting electrode. After baseline pH was monitored for 4 hours, the laryngohypopharynx mucosa specimens were immediately excised and fixed in 4% formaldehyde solution for electron microscope examination. All the pH monitoring data were analyzed by the manufacturer's software.

Stent Implantation

According to the literature, esophageal length of the Bama minipig was about 25 cm. The manufacturer can only provide stent less than 18 cm. Esophageal stent suit was custom-designed by Micro-Tech Co, Ltd (Nanjing, China). The stent suit included 2 stents made from nickel-titanium alloy. The length of each stent was 14 cm, and the diameter was 2.2 cm and 2.0 cm. The basilar stent had an umbrella in order to be fixed in the gastric cardia and the upper stent had a recycling line at the upper part to be fixed through the nasal cavity, while the body of stents was covered by membrane (Fig. 1). The location of the cardia was determined by endoscope, and the head of the guidewire was put in the antrum of the stomach, then we draw back the endoscope, the stent towveyor entered the stomach and the baseline stent was released. We repeated this procedure to implant another stent. Then the pH of pharyngolarynx and distal esophagus were monitored for approximately 2 hours. On the third day after surgery, the stent was removed. On the 14th day after surgery, the pH of pharyngolarynx and distal esophagus was monitored again, and the laryngopharynx mucosa specimens were excised by biopsy forceps simultaneously.



Figure 1. Images of the custom-designed stent suits. (A) The upper stent had a recycling line to be fixed through the nasal cavity and to be removed more easily. (B) The basilar stent had an umbrella in order to be fixed in the gastric cardia.

Transmission Electron Microscope Examination

Tissue preparation for electron microscopy was according to the previous study. Ten pictures were obtained from each case, one entire cell was selected in each picture, and intercellular space was measured from 10 random directions (vertical to the neighboring cell membrane, and no less than 1 μm between each direction) using a transmission electron microscope (Tecani spirit; FEI, Hillsboro, Oregon, USA). The average width of 100 intercellular spaces was considered as the intercellular spaces of one case. The average desmosome number of ten intercellular spaces from 10 pictures was considered as the desmosome number of one case.

Statistical Methods

Data were expressed as mean \pm standard deviation. Chi-square test, paired samples *t* test, independent samples *t* test and one-way ANOVA with repeated measures were used for statistical analysis where appropriate. Analyses were performed by the SPSS software (Statistical Package for the Social Sciences version 18.0; SPSS Inc, Chicago, IL, USA). *P* < 0.05 was considered statistically significant.

Results

There were 10 Bama minipigs enrolled in this procedure, one

Table 1. Data From Laryngopharynx

	Baseline		2 hr		14 days		P-value
	Control group (n = 4)	Stent group (n = 4)	Control group (n = 4)	Stent group (n = 4)	Control group (n = 4)	Stent group (n = 4)	
Reflux time (min)	0	0	ND	6.08 ± 4.67	0	2.35 ± 1.82	$P_{14dS-C} = 0.021$ $P_{2hs-S} = 0.040$
Reflux times	0	0	ND	5.00 ± 2.83	0	4.50 ± 1.73	$P_{14dS-S} = 0.041$ $P_{14dS-C} = 0.001$
Reflux percent (%)	0	0	ND	5.10 ± 4.31	0	0.97 ± 0.74	$P_{2hs-S} = 0.019$ $P_{14dS-S} = 0.011$ $P_{14dS-C} = 0.019$
Intercellular space (um)	0.418 ± 0.041	0.367 ± 0.066	ND	ND	0.429 ± 0.261	0.960 ± 0.183	$P_{2hs-S} = 0.049$ $P_{14dS-S} = 0.049$
Number of desmosomes	18.0 ± 0.8	20.3 ± 1.5	ND	ND	17.8 ± 1.7	9.5 ± 2.08	$P_{14dS-C} = 0.001$ $P_{14dS-S} = 0.008$ $P_{14dS-C} = 0.001$ $P_{14dS-S} = 0.003$

ND, no data; P_{14dS-C} indicates the P-value of stent group comparing to control group at the 14th day; P_{2hs-S} indicates the P-value of 2 hours data comparing to baseline data from stent group; P_{14dS-S} indicates the P-value 14th day data comparing to baseline data from the stent group.

from each of two group died of undetermined choke during the procedure. Because one pig died from each group, the success rates for both the acute and benign LPR model were 80%.

Evaluation of the Laryngopharyngeal Reflux Model

In the control group, no reflux was observed, as assessed by laryngopharyngeal pH monitoring on the first and 14th day after surgery (Table 1). Before the stent implantation, there was also no reflux in the stent group. Therefore we concluded that the model of LPR could be considered successful if reflux occurred at least once after stent implantation.

Two hours after stent implantation, the number of reflux events, reflux time, and percentage were significantly higher ($P < 0.05$) in the stent group compared with baseline (Fig. 2). Eleven days after removing the stent (ie, 14 days after stent implantation), there was still reflux. One or more reflux events occurred in every pig, not only 2 hours after stent implantation but also on the 14th day.

Changes in Gastroesophageal Reflux

In this LPR model, gastroesophageal reflux (GER) was also induced. In the control group, there was no change in GER between the first and 14th days after the procedure (Table 2). However, 2 hours after stent implantation the number of reflux events, reflux time, and percentage were significantly higher ($P < 0.05$) in the stent group compared with the control group.

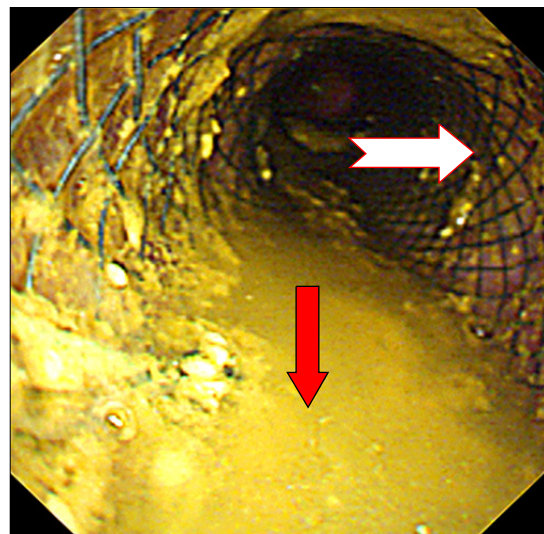


Figure 2. Endoscope examination of the stent in esophagus and reflux. There are stents in esophagus (white arrow), and much of reflux content (red arrow).

Eleven days after removing the stent (14 days after stent implantation), there was still reflux increasing, and the differences between the control and stent groups were significant ($P < 0.05$) except for acid reflux.

Changes in Intercellular Space and Number of Desmosomes

In the control group, there were no changes in intercellular space or number of desmosomes between the first day and 14th day after the procedure (Table 1). However, in the stent group the intercellular space was significantly higher on the 14th day (0.96 μm) compared with the first day (0.37 μm , $P = 0.008$), and the number of desmosomes were significantly lower (20.25 on the first day, compared with 9.5 on the 14th; $P = 0.003$; Fig. 3).

Discussion

In the present study, a Bama minipig model of LPR was established by implanting a custom-designed stent suit, which was assessed by pH monitoring at the sites of laryngopharynx and distal esophagus. Intercellular space was significantly increased and the number of desmosomes was significantly decreased in the laryngopharyngeal mucus of LPR model, suggesting that LPR might destroy the laryngopharyngeal mucosal barrier by change the mucosal ultrastructure.

Gill et al²¹ studied human and non-human laryngeal tissues (mouse, rat, guinea pig, porcine, and rabbit) and found that porcine tissue revealed both respiratory-type and stratified squamous epithelium, as seen in the human larynx. The authors indicated that the porcine larynx was a superior model for research of epithelial damage occurring during LPR.²¹ In the present study, Bama minipigs were used. The miniature pig has been widely accepted as the experimental animal.²²⁻²⁵ With its body size being only one-fourth to one-third of an ordinary pig,²⁶ it is easy to be operated and thus suitable to establish a LPR model.

Although the pathological mechanisms of LPR and GERD are similar, LPR model was rarely reported, compared to GERD model. There were 2 previous methods to establish GERD animal model: one was endogenous GERD induced by its own gastrointestinal reflux and the other was an exogenous GERD induced by exposure to acidic solutions. In endogenous GERD model, surgical operation was used to change normal gastrointestinal tract anatomy resulting in reflux of gastric juice, duodenal juice, or the mixture. The operation methods included ligation of pylorus and proximal small bowel, destruction of cardia, and dif-

Table 2. Data From Distal Esophagus

	Baseline		2 hr		14 days		P-value
	Control group (n = 4)	Stent group (n = 4)	Control group (n = 4)	Stent group (n = 4)	Control group (n = 4)	Stent group (n = 4)	
Reflux time (min)	0.1 ± 0.2	0.15 ± 0.3	ND	40.36 ± 60.05	0.2 ± 0.4	18.88 ± 12.53	$P_{1,44\text{S-C}} = 0.013$ $P_{2,1\text{BS-BS}} = 0.034$
Reflux Times	0.5 ± 1.0	0.25 ± 0.5	ND	3.50 ± 1.29	0.5 ± 1.0	19.0 ± 10.0	$P_{1,44\text{S-BS}} = 0.034$ $P_{1,44\text{S-C}} = 0.001$ $P_{2,1\text{BS-BS}} = 0.033$
Reflux percent (%)	0.04 ± 0.08	0.05 ± 0.10	ND	31.38 ± 45.40	0.08 ± 0.15	7.85 ± 5.16	$P_{1,44\text{S-BS}} = 0.034$ $P_{1,44\text{S-C}} = 0.019$ $P_{2,1\text{BS-BS}} = 0.034$
No acid reflux	0.25 ± 0.50	1.0 ± 1.1	ND	6.5 ± 3.1	4.25 ± 7.23	6.25 ± 2.23	$P_{1,44\text{S-C}} = 0.308$ $P_{2,1\text{BS-BS}} = 0.051$ $P_{1,44\text{S-BS}} = 0.034$

ND, no data; $P_{1,44\text{S-C}}$ indicates the P-value of stent group comparing to control group at the 14th day; $P_{2,1\text{BS-BS}}$ indicates the P-value of 2 hours data comparing to baseline data from stent group; $P_{1,44\text{S-BS}}$ indicates the P-value 14th day data comparing to baseline data from the stent group.

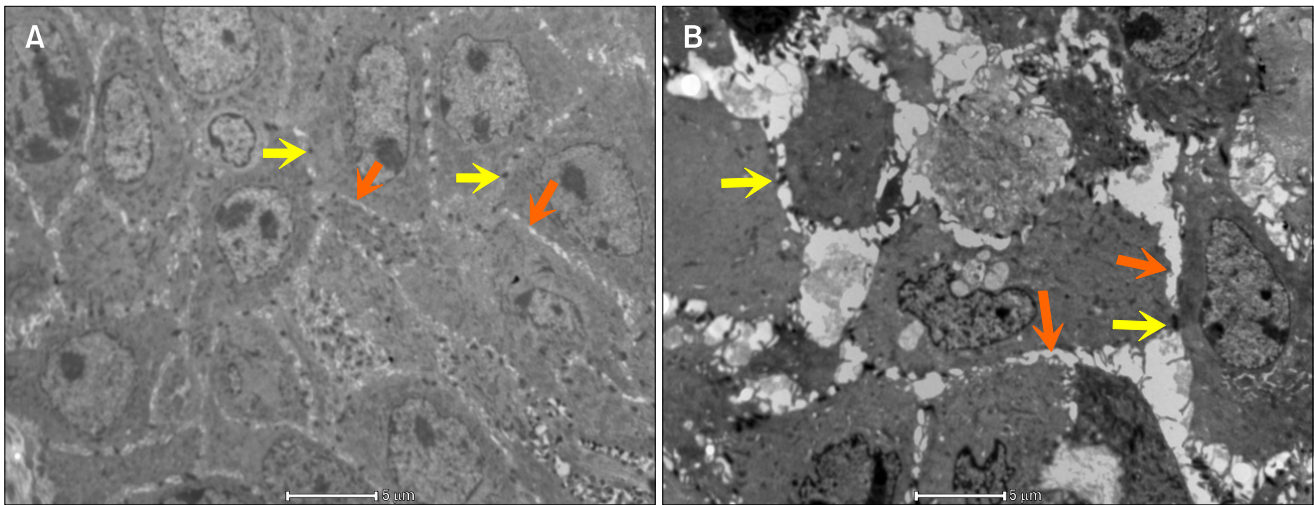


Figure 3. The change of laryngopharyngeal mucosal ultrastructure after stent implant. (A) Intercellular space and desmosomes of laryngopharynx before stent implant: orange arrows indicate intercellular space and yellow arrows indicate desmosomes. (B) Intercellular space and desmosomes of laryngopharynx after stent implant: orange arrows indicate increased intercellular space and yellow arrows indicate decreased desmosomes.

ferent kinds of esophageal gastrointestinal anastomosis. Endogenous GERD model had some advantages for studying the acute pathophysiology of GERD, but it also had key limitations such as the animals needing to have a strong tolerance for the operation and that operators should have a high level of technical skill. In addition, the model animal often died due to various complications as they struggled to survive for a long time, thereby precluding the potential to fully understand the entire development of GERD which is a chronic disease. The advantages of exogenous model included simple operation, high survival rate, and long-term observation. However, there are 2 major limitations of exogenous model: (1) chemicals perfused in esophagus were very different to the complex reflux content in vivo and (2) could not reflect the pathogenesis of reflux disease. Exogenous models are used to study esophageal mucosal damage and defense mechanism. However, the LPR animal model has been reported limitedly. Hu et al²⁷ induced LPR by total cardiomyectomy on albino rabbits in New Zealand to study the association between LPR and GERD. But they did not monitor the objective evidence of reflux, for example the pH value.

In our study, a custom-designed stent was implanted to destroy kinetic barrier against LPR resulting in stomach contents backflow into oropharynx. We also assessed the reflux by pH monitoring at the sites of laryngopharynx and distal esophagus and found that this Bama minipig model of LPR was successful. Moreover, there was still reflux 14 days after stent implantation. There is no report about stent implantation to establish LPR

model. Our method had advantages including simple operation, high survival rate, less damage, and applicability to study both acute and chronic reflux. So it provided a new method to understand the whole development and pathogenesis of LPR.

In addition, studying mucosal ultrastructure changes may clarify pathogenesis of LPR.^{27,28} Compared with esophageal squamous mucosa, hypopharynx mucosa is easily injured by reflux contents.²⁹ In the esophagus, upper limit of tolerance to GER is 50 times per day in the normal physiological condition, but in the hypopharynx, upper limit is generally below 7 times per day.³⁰ Many studies found that esophageal intercellular space was increased in GERD and decreased after treatment.^{19,31-33} Significant changes in the intercellular space were also found in the esophageal and laryngeal samples of a rabbit reflux model induced by total cardiomyectomy.²⁷ Park et al. found that both LPR patients with and without GERD had a dilated intercellular space in esophageal tissues ($0.95 \pm 0.44 \mu\text{m}$ and $0.61 \pm 0.47 \mu\text{m}$, respectively), compared to the control group ($0.35 \pm 0.27 \mu\text{m}$).²⁰ In the present study, we studied the mucosal ultrastructure changes in this LPR model. In the stent group, intercellular space was significantly increased, and the number of desmosomes was significantly decreased, which suggested that LPR damages the laryngopharyngeal mucus.

A Bama minipig model of LPR was established by implanting a custom-designed stent suit. Our model indicates that LPR may damage the laryngopharyngeal mucosal barrier.

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