Development of a Survival Animal Model for Subglottic Stenosis

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Objective: To develop a reproducible survival animal model for subglottic stenosis. Study Design: Prospective study. Methods: We evaluated five methods of inducing airway injury in 30 New Zealand white rabbits to produce a subglottic stenosis model. Experimental groups comprised: group 1 (n = 5), which underwent 4-hour intubation; group 2 (n = 5), which underwent induced subglottic injury with a nylon brush; group 3 (n = 10), which underwent subglottic injury with a nylon brush, followed by 4-hour intubation; group 4 (n = 5), which underwent subglottic injury with Bugbee cautery in 50% of the subglottic circumference, followed by 4-hour intubation; and group 5 (n = 5), which underwent subglottic injury with Bugbee cautery in 75% of the subglottic circumference, followed by 4-hour intubation. Five animals were used as controls. Endoscopy of the airway and sacrifice of animals were planned at an interval of 14 days postinjury. Histologic measurements were analyzed. Results: No animals in groups 1 or 2 developed stenosis. In group 3, 50% of animals developed symptomatic grade 3 subglottic and tracheal stenosis, necessitating early endoscopy and sacrifice in three animals. Four animals in group 4 developed grade 1 subglottic stenosis, and four in group 5 developed grade 2 subglottic stenosis. Histologic measurements of lumen areas within each of these two groups were similar; all animals survived the follow-up period. Conclusion: We successfully developed a reproducible survival model for induced subglottic stenosis using a combination of cautery-induced subglottic injury followed by 4-hour intubation. This model lays the foundation for future studies that evaluate endoscopic interventions for the management of subglottic stenosis. Key Words: Subglottic stenosis, rabbit, animal model. Level of Evidence: NA

INTRODUCTION

Over the past several decades, endoscopic techniques such as balloon dilation have become an invaluable tool for the management of acquired subglottic stenosis (SGS). Despite widespread use of these techniques, effective evidence-based protocols for the endoscopic management of SGS are lacking and require the development of animal models of subglottic injury. A wide array of methods for inducing airway stenosis in animal models has been described, including intubation only, brushing, application of silver nitrate or hydrochloric acid, use of electrocautery, and use of the carbon-dioxide laser. Previous authors have not, however, consistently assessed the postinjury endoscopic appearance of the airway, postinjury respiratory symptoms, and histologic findings. In addition, most previous animal models with induced SGS have involved open approaches. These approaches disrupt the cricoid cartilage, making it difficult to further assess the outcomes of balloon dilation—a management approach that has steadily gained favor in many pediatric institutions. Models in which endoscopic injury is used are rare and have been associated with limitations such as high mortality, early sacrifice, and reporting of histologic findings alone.

With these collective issues in mind, the aim of our study was to create a reproducible survival animal model for acute airway stenosis using an endoscopic approach and to document this model symptomatically, endoscopically, and histologically. Given the previous research demonstrating that New Zealand white rabbits provide a reliable model for studying airway stenosis, we used these animals to develop our model.

MATERIALS AND METHODS

Experimental Study Groups

Our study included 35 New Zealand white rabbits with an average weight of 3.66 kg (range 3.2–4.2 kg). All animals were used for 129:989–994, 2019
given at least 3 days to acclimate to our animal facility prior to airway intervention. Over the course of the study, we sequentially evaluated five distinct methods of inducing airway injury to produce a survival SGS model. Experimental groups were characterized as follows: group 1 (n = 5) underwent a 4-hour period of intubation; group 2 (n = 5) received induced subglottic injury with a nylon brush; group 3 (n = 10) received induced subglottic injury with a nylon brush, followed immediately by a 4-hour period of intubation; group 4 (n = 5) received induced subglottic injury with monopolar Bugbee cautery in 50% of the circumference of the subglottis, followed immediately by a 4-hour period of intubation; and group 5 (n = 5) received induced subglottic injury with monopolar Bugbee cautery in 75% of the circumference of the subglottis, followed immediately by a 4-hour period of intubation. Five additional rabbits were used as controls. These animals received no induced airway injury; however, they underwent airway endoscopy under general anesthesia after being acclimated and were sacrificed 14 days after this procedure.

**Anesthesia Induction and Endoscopy**

General anesthesia induction for all groups was carried out using intramuscular ketamine (35 mg/kg) and xylazine (5 mg/kg) and was maintained with inhaled 2% isoflurane. Isoflurane was continued during the 4-hour period of intubation.

A Miller size 1 laryngoscope and a 2.7-mm Hopkins rod telescope (Karl Storz, Tuttingen, Germany) were used to visualize the larynx. The vocal folds were anesthetized with 0.5 mL of atomized lidocaine (1 mg/mL). The telescope was advanced into the airway to assess laryngeal and tracheal anatomy. During this procedure, video and photo-documentation were carried out. Sizing of the airway was also performed.

**Inducing Airway Injury**

Intubation (used in groups 1, 3, 4, and 5) was carried out with a 3.5-mm cuffed Portex endotracheal tube (Smiths Medical, Dublin, OH) under direct vision. The nylon brush used in groups 2 and 3 was approximately 5 mm in diameter and 10 mm in length. The brush was inserted into the subglottis and rotated 40 times to induce mucosal injury. Bugbee monopolar cautery used in groups 4 and 5 was inserted under endoscopic visualization and used to injure either 50% (group 4) or 75% (group 5) of the circumference of the posterior subglottic area with 7 watts of power for approximately 5 seconds (Fig. 1).

**Posttreatment Protocol**

After the 4-hour period of intubation, rabbits were extubated and monitored in a temperature-controlled chamber for 1 to 2 hours. They were then transferred to their respective cages, where they were monitored for the 14-day follow-up period. Buprenorphine (0.03 mg/kg) was given for analgesia every 6 to 12 hours postoperatively. At day 14, we again performed endoscopy and sizing of the airway using the same anesthesia induction method described above. Stenoses were graded according to the Myer-Cotton classification system. Images from airway endoscopies were recorded. The rabbits were then euthanized with sodium pentobarbital (100 mg/kg), which was administered directly into the heart while the animals remained under general anesthesia.

The larynx and trachea from the level of the hyoid bone to 2 cm below the cricoid were then harvested. Each specimen was sectioned and fixed in 10% buffered formalin, embedded in paraffin, and sectioned into 5 μm sections. A hematoxylin and eosin stain was used for histological analysis. Pathology slides were analyzed under an optical microscope (Carl Zeiss Microscopy, Thornwood, NY), and two different areas of the airway were measured using AxiosVision software SE64 Rel. 4.9.1 (Carl Zeiss, Thornwood, NY). These areas comprised the narrowest part of the cricoid only and the narrowest part of the cricoid at the level of the trapped tracheal rings. The circumference of the lumen was manually demarcated with an AxiosVision software tool (Carl Zeiss), and the inner area was then calculated by this software.

All procedures were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center, and all procedures were conducted at an approved animal surgical facility. The care and handling of the animals were done in accordance with guidelines specified by the National Institutes of Health.

**Data Analysis**

The following parameters were documented in each of our five experimental groups and in controls: respiratory symptoms, endoscopic airway sizing, grade of stenosis, and histologic findings. Percentage of airway obstruction for histologic measurements was calculated using the following formula:

\[
\text{Airway obstruction (\%)} = \left[1 - \frac{\text{(experimental group area / control area)}}{100}ight]
\]

We examined the distribution of the data using scatterplots and descriptive statistics, including medians and ranges. The Wilcoxon rank sum test was used to compare intraluminal area (mm²) in the trapped ring and cricoid between each experimental group and the control group. In addition, a comparison between group 4 and group 5 was tested. For the purposes of analyses, group 3 was split into two groups based on stenosis. Raw \( P \) values were calculated, and a Bonferroni-Holm adjustment for multiple comparisons was also considered. \( P \) values < 0.05 were considered significant. All statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC).

**RESULTS**

All rabbits recovered and showed no evidence of respiratory difficulties in the immediate postoperative period. However, two animals from group 4 (cautery in

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50% of the circumference of the subglottis and intubation) and three from group 5 (cauter in 75% of the circumference of the subglottis and intubation) showed poor oral intake in the first few postoperative days but recovered completely in 5 days. These animals received extra doses of buprenorphine for pain control, which improved their oral intake.

No animals in the control group or in groups 1 (intubation only) and 2 (brush-induced injury) developed any stenosis. All animals in the control group sized with a 3.5 endotracheal tube (ETT) with a free leak.

Of the 10 animals in group 3 (brush-induced injury and intubation), five (50%) developed symptomatic grade 3 subglottic (Figs. 2 and 3) and tracheal stenosis; in three of these five, disease severity and concomitant respiratory symptoms required early endoscopy and sacrifice at days 9 to 11 following injury. These rabbits developed severe stridor, chest retractions, nasal flaring, and inability to feed. Sizing was not attempted because these animals showed a pinhole stenosis. The five other animals in group 3 developed only mild scarring of the subglottic mucosa and showed no respiratory symptoms. They sized with a 3.5 ETT with a free leak.

Four (80%) animals in group 4 (cauter in 50% of the circumference of the subglottis and intubation) developed asymptomatic grade 1 SGS, sizing with a 3.0 ETT with a free leak and a 3.5 ETT with no leak at the day of sacrifice; one animal developed grade 2 SGS, sizing with a 2.5 ETT with no leak and stridor when agitated. Four (80%) animals in group 5 (cauter in 75% of the circumference of the subglottis and intubation) developed grade 2 SGS (Figs. 4 and 5), sizing with a 2.5 ETT with no leak. These rabbits demonstrated stridor when agitated. One animal in this group developed asymptomatic grade 1 SGS, sizing with a 3.0 ETT with a free leak.

Except for three rabbits in group 3 that underwent early sacrifice, all other animals survived until the planned sacrifice at postoperative day 14.

Microscopic measures of the cricoid and of the trapped rings are shown in Figures 6 and 7. Endoscopic and histologic findings are summarized in Table I. The median cricoid area of the controls was 25.24 mm². The median cricoid area in group 4 was 16.23 mm², corresponding to an obstruction of 32.71% of the lumen.
compared to controls; and 9 mm² in group 5, corresponding to an obstruction of 62.44% of the lumen. Both group 4 and group 5 were significantly different from controls ($P = 0.01$) ($P = 0.08$ with adjustment for multiple comparisons). The median cricoid area at the level of the trapped rings of the five animals in group 3 that developed SGS was 3.3 mm², corresponding to an airway obstruction of more than 80% compared to control measurements at the same level of the airway. This was also significantly different from controls ($P = 0.01$) ($P = 0.08$ with adjustment for multiple comparisons).

**DISCUSSION**

After sequentially employing a number of previously described techniques to induce airway stenosis, we successfully developed a reproducible survival endoscopic animal model of SGS using a combination of cautery-induced subglottic injury and 4-hour intubation in New Zealand white rabbits. This technique consistently induced stenosis limited to the subglottis because it allowed us to apply spot burns to this specific anatomic area under direct visualization. In contrast to previous studies, we were able to show the results of induced SGS symptomatically, endoscopically (sizing with endotracheal tubes), and histologically (measuring areas of the subglottic lumen). Moreover, we found that all animals exhibited consistency across these three measures, as determined by percentages of airway obstruction. No less important, all animals ($n = 5$) in our combined cautery and intubation model survived the planned follow-up period, which is essential for future studies on the development of treatment protocols. Also noteworthy, at the time of necropsy and histologic evaluation we observed a consistent finding of 1 to 2 trapped rings within the lower edge of the cricoid. This is important to note because it represents a difference between rabbits and humans—with trapped rings being relatively uncommon in humans. The significance of this difference is that it is difficult to induce a "pure" subglottic stenosis model because these rings are frequently injured by the induction of stenosis, regardless of the technique used.

Most previous studies that have developed animal models of airway stenosis have used an open approach. Authors have induced airway injury with a wide variety of methods, including brushing of the trachea, the use of the carbon-dioxide laser, the application of silver nitrate or hydrochloric acid to the airway mucosa, and cautery. Balloon dilation is frequently used for the management of SGS; however, this modality disrupts scar tissue through radial high-pressure force and may fracture the cricoid cartilage, depending on balloon size and pressure. Given that all these open approaches result in cricoid disruption, which does not allow for balloon dilation until several weeks after the procedure, developing an animal model with open approaches is indubitably not ideal.
Previous models in which an endoscopic injury technique was used have been associated with problems such as reporting limited histologic findings consisting only of measures of thickness of the lamina propria and submucosa; high morbidity and mortality rates; the need to euthanize some animals as soon as 3 days after injury, making it impossible to determine the likelihood of survival beyond this brief period; or determination of the grade of stenosis based solely on subjective measurement.

Two studies reported the use of intubation alone to induce injury. Kelly et al. created SGS in eight rabbits using a 3-cm length of an endotracheal tube one or two sizes above the appropriate tube size for the animal. It was held in situ with a suture placed through the trachea via an external approach and secured over a button in the neck for 7 days. All animals developed SGS 1 week after tube removal; however, one animal died and one was excluded from the study due to tube obstruction and the need for tube removal. Kumar et al. induced SGS by intubation alone in 12 rabbits: four were intubated for 2 hours, four were intubated for 4 hours, and four were intubated for 6 hours. Authors reported that 1) 2-hour intubation did not induce subglottic injury; 2) all four rabbits that underwent 6-hour intubation died from airway obstruction; and 3) all animals that underwent 4-hour intubation developed some degree of SGS. In an effort to replicate the technique of inducing injury reported by these authors, we initially attempted to introduce a 4.0 Portex endotracheal tube (Smiths Medical) (our experimental group 1). In most animals, however, we were unable to comfortably pass this tube beyond the glottis. Although Kumar et al. reported the same difficulty, they stated that the resistance they felt when the tube was introduced was overcome with some force. It is possible that they could have caused additional mucosal injury during the intubation process itself and that SGS was actually induced by a combination of traumatic intubation followed by 4-hour intubation with a large tube. This could explain the lack of reproducibility using even a smaller tube, as we used in group 1 (intubation only).

Two models of stenosis have involved the brushing of the airway mucosa. Using an open approach, Nakagishi et al. brushed the trachea in eight rabbits. Authors reported high variability in the grades of stenosis produced, ranging from 20% to 70% obstruction. Steehler et al. used a nylon brush to induce tracheal stenosis. No stenosis was observed in rabbits that received injury through an open approach, whereas the eight rabbits that underwent an endoscopic approach developed a wide range of tracheal stenosis (10%–80% obstruction). Such a range limits the use of this particular model for our stated goals. Nevertheless, in group 2, we evaluated this model, endeavoring to develop SGS in our rabbits using the same approach. Our inability to do so may be due to a difference in brushing technique or the type of brush used.

In group 3 (brushing and intubation), we modified the endoscopic model of Steehler et al. with brushing, adding intubation for 4 hours immediately after brushing. Our outcomes were inconsistent. Five of our rabbits presented with nonobstructive subglottic scar bands, stridor, chest retractions, nasal flaring, and inability to feed. The other five rabbits developed severe stenosis (grade 3) and severe symptoms, requiring early sacrifice. In addition, the location of the stenosis was difficult to control, with some rabbits developing combined subglottic and tracheal stenosis. The animals in which stenosis was successfully created developed narrowing at the level of the first trapped rings, and a long segment of tracheal stenosis as well. The variation in the extent and location of the injury using the nylon brush was likely due to not being able to induce the injury under direct visualization because the brush was too large to pass through a bronchoscope.

Although two previously published studies developed models of airway stenosis using electrocautery, their technique involved an open approach. One of these studies reported a high mortality rate (50%) when the full thickness of the subglottic mucosa was burned. The authors therefore recommended performing only partial-thickness electrocauterization of the mucosa to achieve a survival model of SGS in the rabbit. This model cannot, however, be reproduced with an endoscopic approach because we cannot visualize the full thickness of the mucosa without a criciodotomy and cannot be certain.

<table>
<thead>
<tr>
<th>Group Number (type of injury)</th>
<th>Number of Animals</th>
<th>Endoscopic Outcomes at Time of Sacrifice (region and grade of stenosis)</th>
<th>Histologic Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (endoscopy alone)</td>
<td>5</td>
<td>No stenosis</td>
<td>Normal</td>
</tr>
<tr>
<td>1 (4-hour intubation)</td>
<td>5</td>
<td>No stenosis</td>
<td>7.1% 0%</td>
</tr>
<tr>
<td>2 (brushing)</td>
<td>5</td>
<td>No stenosis</td>
<td>5.94% 0%</td>
</tr>
<tr>
<td>3 (brushing + 4-hour intubation)</td>
<td>10</td>
<td>5 animals: grade 3 SGS + tracheal stenosis</td>
<td>23.81% 83.89%</td>
</tr>
<tr>
<td>4 (cautery in 50% of the subglottic circumference + 4-hour intubation)</td>
<td>5</td>
<td>Grade 1 SGS</td>
<td>32.71% 17.99%</td>
</tr>
<tr>
<td>5 (cautery in 75% of the subglottic circumference + 4-hour intubation)</td>
<td>5</td>
<td>Grade 2 SGS</td>
<td>62.44% 41.65%</td>
</tr>
</tbody>
</table>

SGS = subglottic stenosis.
about the depth of the mucosa that we are burning.\textsuperscript{7} The other study\textsuperscript{9} induced tracheal stenosis rather than SGS.

In sum, our study successfully resulted in the development of a reproducible survival animal model using endoscopic cautery-induced injury to the subglottis and intubation. This model paves the way for further translational research that assesses various surgical interventions for symptomatic SGS.

CONCLUSION

In our study, we found that previous animal models for SGS were not reproducible. We were, however, able to create a reproducible model of induced subglottic injury with a small number (n = 10) of animals. This model lays the foundation for future studies that evaluate endoscopic interventions for the management of SGS.

BIBLIOGRAPHY


