Original Article

Strongly induced hypertrophic scar in a rat model of tracheal stenosis

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Abstract: Objective: Previous rat models of tracheal stenosis have demonstrated a high mortality rate with little scar formation as well as interference by chemical factors. This study aimed to develop an improved rat model for tracheal stenosis. Methods: Forty-five Wistar albino rats were randomly divided into two control groups and one experimental group with 15 rats in each group. Electrical cauterization was performed anterior of the 1-2 and 4-6 trachea rings through the tracheostoma. About 0.5 cm of skin on both sides of the incision in the experimental group was removed and fixed to keep the tracheostoma open. In control group 1, the incisions were closed after electrical cauterization, whereas rats in control group 2 had an open tracheostoma but no electrocautery injury. Results: Fourteen (93.3%) of 15 animals in the experimental group survived. All rats survived in control group 2, but a high rate of mortality (100%) was observed in control group 1. Compared with control group 2, the experimental group showed a significant increase in lamina propria thickness at days 7, 14, and 21, resulting in luminal narrowing with the maximal stenosis degree at day 21 (67.8%). Conclusion: We developed a rat model of tracheal stenosis, resulting from mucosal injury administered by electrocauterization. The advantages of this model are ease of handling, a high survival rate, and remarkable scar hyperplasia.

Keywords: Animal model, tracheostomy, electrocauterization, tracheal mucosa, upper airway obstruction

Introduction

Postoperative recurrence of cicatricial tracheal stenosis (TS) is still a difficult problem to overcome. Current strategies, such as end-to-end anastomosis, CO₂ laser incision, balloon dilation, and stent replacement cannot guarantee favorable results and may lead to complications [1-3]. Therefore, various animal models have been developed to study the mechanism of TS and to explore effective treatment methods. Previously, large and medium sized animals such as pigs, dogs, and rabbits were used to demonstrate effects related to airway injury [4-6]. TS is easy to induce in these animal models because of their large airway lumen. However, because relevant drugs and antibodies are not readily available, the use of these animal models precludes molecular biology or immunology studies [7]. Although mouse TS models have been commonly used for genetic and molecular investigation, they are difficult to use because their airway lumen diameter is too small to provide sufficient scar tissue for further study [8, 9].

Recently, rats have been used as an animal model to study airway stenosis. However, these models do not develop sufficient scar hyperplasia in contrast to that seen in the clinic. This is because mild damage to the tracheal mucosa only induces a small quantity of scar tissue, whereas a deep and severe injury induces a large amount of scarring but might lead to a high rate of mortality in rats. To resolve this issue, we developed an improved rat model that produces an adequate cicatrix for the study of TS. This model demonstrated a high probability of survival and might be useful for the evaluation of therapies to prevent or reverse TS.
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Material and methods

Experimental animals

This study was approved by the institutional Animal Studies Committee of Sun Yat-sen University (Permit Number: 2012-349), and was conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. All efforts were made to ensure minimal animal suffering during the experimental procedures. Forty-five healthy Wistar Albino female rats were purchased from Sun Yat-sen University Laboratory Animal Center. Prior to experiments, rats weighed 220-250 g. The rats were randomly divided into one experimental group and two control groups of 15 rats per group. All animals were kept at a room temperature of 22°C and were adapted to diet for one week according to the feeding requirements of specific pathogen free rats.

Surgical procedure

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 ml/kg). Rats were placed in a supine position, the anterior or cervical area was shaved, and then prepped with povidone-iodine solution. A 1.5-cm vertical incision was made in the midline of the neck between the level of the hyoid bone and suprasternal fossa. The underlying strap muscles were dissected bluntly to expose the trachea below the level of the cricoid cartilage. After making a transverse incision between the second and third tracheal cartilage, a tracheostoma (2×1.5 mm) was made by partially excising the anterior wall of the third tracheal ring and intervals above and below it. Monopolar electrocauterization (Spring Medical, Wuhan, Hubei, China) was set at 10 W, and the anterior part of the electrosurgical cautery unit was bent at a right angle. Wounds were made on the anterior of the 1-2 and 4-6 tracheal rings by intratrachea-
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al electric coagulation. The mucosa and the underlying perichondrium and cartilage were injured at a width of 1.5 mm, using 6-second exposures. When the trachea wall became white, the intended depth of injury in the lateral surface of cartilage was reached. In the experimental group, about 0.5 cm of skin on both sides of the incision was removed, then the strap muscles were separated and fixed with 4-0 silk sutures in each side of the skin flap to keep the tracheostoma open (Figure 1A-D). In control group 1, rats received incisions that were closed after electrical cauterization, whereas rats in control group 2 had open tracheostoma but no electrocautery injury. To minimize variations in the intervention, all operations were performed by a single surgeon, who used the instruments in a uniform fashion.

Post-operative care

After surgery, the rats were returned to their cages and supplied with food and water. They were monitored at 20-min intervals until they recovered from consciousness and were able to lift their heads. Airways and wounds were checked frequently for the first 48 hours. During airway checks, rats were evaluated for respiratory distress and obstructing sputum and blood clots were suctioned away. The rats were also observed for level of mental state, activity and appetite. The presence or absence of symptoms as well as the weight of the rats were recorded.

Tissue collection and histopathological analysis

Animals were euthanized painlessly at days 7, 14, and 21 after surgery for histologic analysis (n=5 for each group at each time point). Tracheal specimens were fixed in 10% buffered formalin solution for 24 hours, followed by dehydration in ethanol and embedding in paraffin. Cross sections of 0.4-µm thickness were obtained from these samples and histochemical hematoxylin-eosin and Masson’s trichrome stains were applied. Specimens were assessed to determine the lamina propria (LP) thickness, and identify angiogenesis, fibroblasts, and collagen production. Measurements were taken from the medial aspect of the tracheal cartilage to the epithelial basement membrane at ×40 magnification.

Statistical analysis

To determine differences in the degree of stenosis between groups, the Mann-Whitney U-test was performed using SPSS 17.0 software for Windows (Chicago, IL, USA). All values were expressed as the mean ± the standard error of the mean. Statistical significance was assumed at P<0.05.

Results

High survival rate of TS in experimental rats

In the experimental group, 14 (93.3%) of 15 animals survived until sacrifice. Only one rat

Figure 2. Airway histologic characteristics. (A) Control airway displaying normal epithelium, lamina propria, and cartilage layers (H&E, original magnification ×40). (B-D) Anterior injury model (B, H&E, original magnification ×40; C, Masson trichrome stain, original magnification ×100; D, H&E, original magnification ×40) showing thickened lamina propria (LP), narrowed tracheal lumen (TL), collagen bundle (CB), new hypervascularity (HV), fibrosis of lamina propria (FB), and re-epithelialization (RE).
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(6.7%) died of an intestinal obstruction at day 11 postoperatively as determined by autopsy. All rats survived in control group 2; however, in control group 1, 4 of 5 rats died within 24 hours after surgery and the others died at postoperative day 5. Autopsy showed that the airway obstruction that caused death was due to excretions, accompanied by pulmonary edema and bleeding. Because of the high mortality rate in control group 1, the subsequent study of this group was terminated. Animals in control group 2 and the experimental group developed a reduced mental state, decreased activity, dietary reduction after the first day, and an unobstructed stoma. On the second day, there was decreased activity but an improved mental state as the wounds began to scab and the stoma was partially obstructed. On the third day, the rats were livelier with increased activity and normal appetite, and the stoma was completely closed by normal wound healing processes and the sutures were removed. Thereafter, the condition of the rats improved. Our study showed the mean body weights were not significantly changed in both groups during the experimental period.

Establishment of a rat model of hypertrophic scarring for TS

Airways were sectioned and stained with H&E to observe the general histologic and morphologic characteristics and Masson trichrome staining was performed to identify the collagen.
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Figure 2A-D shows representative images from a control rat (Figure 2A) and a rat with trachea injured by electrocauterization (Figure 2B-D). Lower magnification images demonstrated (1) anterior narrowing of the tracheal lumen, and (2) an increased thickness in the LP (Figure 2B). Higher magnification images demonstrated (1) the destruction of normal epithelium and re-epithelialization at the anterior trachea, (2) fibrosis, infiltration of inflammatory cells, and proliferation of fibroblasts in the LP driven by an increase in collagen contents as indicated by trichrome staining, and (3) an increase in blood vessel size and number (Figure 2C and 2D).

Marked increase in LP thickness following electrical cauterization

Histologic examination showed submucosal hypertrophy at the cauterized site resulting in a luminal narrowing of the trachea. Significant differences in thickness of the LP and the cross-sectional luminal area of the trachea were observed between the injury group and the control group. Compared with control group 2, the experimental group showed and maintained a significant increase in LP thickness at days 7, 14, and 21 (635.2 ± 87.4 µm vs. 28.0 ± 11.2 µm, 670.8 ± 98.2 µm vs. 30.2 ± 10.5 µm, and 700.6 ± 100.4 µm vs. 30.4 ± 11.5 µm, respectively; P<0.0001). Model specimens at days 14 and 21 demonstrated a thickened LP compared with day 7, but without significant difference (Figure 3A and 3B). The cross-sectional area of the model group was narrower than that of control group 2 (Figure 4A and 4B), with the greatest difference at day 21 (Figure 4C).

Discussion

Currently, several methods are used to develop TS rat models. The endotracheal tube method [10] uses a 10-mm-long stent made from an 8 F feeding tube inserted into the trachea after an incision, and then the wounds are sutured. After 10 days, the stent is removed under general anesthesia. In that study, the thickness of the LP was increased 3-fold and the mortality rate was 15% [10]. The tracheostomy cannula method [11] uses a 6 F feeding tube as a tracheostomy cannula that is fixed to peripheral tissues and skin. After 10 days, the tracheal cannula is removed. A previous study showed that the LP thickness increased 4-fold by fibrosis and the mortality rate was 20.83% [11]. Electrocauterization [7] is performed on the anterior 180° of the tracheal mucosa and then incisions are closed. It was reported that after 4 weeks, the LP thickness was increased by 100 µm with a mortality rate of 13.33%. In the scratching method [12], the mucosa of the posterior half of the trachea is damaged until the perichondrium is reached by biomicroscopic visualization. Using this method, it was reported that the LP thickness increased 3-fold, equivalent to around 60 µm, without related
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deads [12]. Although these models successfully modeled TS, they had low levels of tissue injury, low levels of scar tissue, and no significant effect on tracheal ventilation functions, all of which are different from that observed in clinical practice. Recently, the nylon brush method [13] was described, where the mucosa is scraped with a nylon brush through the tracheostoma. The degree of stenosis was 26.8% at 9 days after scraping, with a low mortality rate of 5.25%. However, it was hard to control the definite extent and depth of the injury using this method, and it produced limited scar tissues.

Compared with the above methods, our method has the following advantages: 1) low mortality rate. There were no postoperative deaths in 15 rats of the experimental group, although one died of an intestinal obstruction as determined by autopsy; thus, its death was related to intraperitoneal anesthesia; 2) marked scar formation. The LP was thickened by the hyperplasia of fibroblasts, collagen, and granulation tissue. The LP thickness of 608.14 ± 269.24 µm at 3 weeks after surgery, which reached a maximum of 991.02 µm, was much thicker than that reported for other methods; and 3) the operation is simple, and postoperative special care is not required.

Two key points contributed to the success of our model. First, tracheostomy and the stoma closed on its own 2-3 days after surgery. Swollen airways caused by injury or exudates can result in death by obstructing the trachea, which can be prevented by tracheostomy. The most critical period was at 24 hours after surgery. If rats survive to this time point, they generally do not suffocate at later stages; however, persistence of the stoma might cause infection of the lower respiratory tract and even death. Therefore, opening the stoma at a certain time-point and the ability to self-heal and close after the critical stages are important factors for the success of our model. Second, the controllability of the depth and extent of injury. The use of biomicroscopy and transparency of the rat trachea allowed the depth of the injury to be evaluated in real time to avoid over or insufficient damage. The extent of injury was managed by reshaping electrodes. In addition, it is important to avoid repeated suction in the trachea by intraoperative control of hemorrhages and to keep the stoma open on postoperative day 1 by suturing the strap muscles to the skin flap to an appropriate degree of tightness.

Conclusion

This study reports an improved rat model for the study of TS. Ease in animal handling, low material costs, flexibility in experimental design, and availability of molecular reagents are the benefits of this model. Its limitation was the short follow-up period. Therefore, further research with a longer follow-up period will be necessary to document the changes in tissue hyperplasia over time.

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Disclosure of conflict of interest

None.

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