Serial Histological Changes in the Cartilaginous Eustachian Tube in the Rat Following Balloon Dilation

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Abstract

Although balloon dilation has shown promising results in the treatment of dilatory Eustachian tube (ET) dysfunction, the histological effects of ET balloon dilation (ETBD) remain unclear. We evaluated this in a rat model. The left ET of 20 Wistar rats was dilated with a balloon catheter and the right ET was used as a control. Five rats were sacrificed immediately after ETBD, at 1, 4 and 12 weeks after the procedure for histological examination. The epithelial cells, presence of squamous metaplasia, and the proportion of the goblet cells in the epithelium; the vascular structures and dimensions of the submucosa; and presence of cartilage fracture and the area of the ET lumen were evaluated and compared between the groups. Desquamation of nearly all epithelial cells and the fracture of tubal cartilages were observed immediately after ETBD. At 1-week post-ETBD, the ciliated epithelial cells started to recover with squamous metaplasia and epithelial hyperplasia. The goblet cells recovered by 4 weeks post-ETBD and epithelial hyperplasia decreased but was still present at 12 weeks post-ETBD. The depth of the submucosa increased and neovascularization in this region was observed at 1-week post-ETBD and persisted up to 12 weeks post-ETBD. The lumen of the cartilaginous ET increased immediately after ETBD but decreased at 1-week post-ETBD. The cartilaginous ET lumen recovered to the normal value at 4 weeks post-ETBD. This study is the first to describe the serial histological changes to the cartilaginous ET after ETBD and helps our understanding of the histological changes that occur after an ETBD intervention for intractable ET dysfunction.

Introduction

As the sole connector of the middle ear to the nasopharynx, a functioning eustachian tube (ET) is important for maintaining a healthy well-aerated middle ear¹. The functions of the ET include middle ear ventilation, as well as the transport and secretion of pathogens and the nasopharyngeal reflux^{2,3}. A dysfunctional ET can lead to acute and chronic otitis media, one of the most common disease entities encountered in otolaryngology⁴. Conditions that interrupt the proper opening of the ET, such as an inflammatory response within its lumen caused by irritants and infectious reactions, can result in ET dysfunction (ETD). The inability of the ET to open also results in a gas equilibrium failure between the middle ear and nasopharynx. Atmospheric gases diffuse across the venous capillary cell membranes in the middle ear. So dilatory dysfunction of the ET results in a net negative pressure within the middle ear, leading to symptoms such as aural fullness or 'popping sounds', reduced hearing, tinnitus, autophony, otalgia, and imbalance⁵.

Until recent years, the understanding of ETD among otolaryngologists was limited, and few treatment options were available⁶⁻¹¹. Surgical management is now available after the introduction of the ET balloon dilation (ETBD) procedure¹². Since Ockermann et al. reported their first experience with ETBD in 2010¹³, many studies have since reported this procedure to be feasible and safe for the treatment of ETD¹⁴⁻²¹. The reported success rates of ETBD range from 36–80%^{16,22-25}.

Although ETBD is reported to be superior to the conventional medical management of ETD, there is still a population of affected patients who do not respond to this intervention. No consensus has yet been reached regarding the further management of ETBD failure cases. This is mainly due to the limited understanding of the mechanisms underlying ETBD due to a lack of histologic studies of the ET after this surgery. A proposed underlying mechanism of ETBD is microtears in the cartilaginous part of the ET¹² and a resulting decrease in mucosal inflammation and reduced biofilm load²⁶. A previous histological study by Kivekas et al. reported that thinning of the mucosa, shearing of the epithelium, and a crush injury to the submucosa, particularly involving lymphocytic infiltrates, were the immediate responses to balloon dilation, and that a healthy pseudocolumnar epithelium and replacement of lymphocytic infiltrates with a thinner layer of fibrous tissue could be observed postoperatively²⁶. One drawback of that prior report however was that the ET mucosal specimens were taken from the nasopharyngeal opening of the tube which does not include the true cartilaginous portion.

Histological examination of the whole human cartilaginous ET after ETBD is impossible. Therefore, an animal study is warranted to verify the histological changes of the ET after ETBD. In this study, we aimed to evaluate the serial histological changes of the ET after balloon dilation in a rat model

Results

Normal Histological Findings for the Rat Eustachian Tube

All 20 animals completed the whole study. The technical success rate of ETBD was 100%. The epithelium of the rat ET is a ciliated respiratory epithelium. This ET epithelium was divided into cartilaginous and nasopharyngeal ends (Fig. 1). The epithelial cells at the nasopharyngeal end were columnar and contained goblet cells (secretory mucous cells). The epithelial cells at the cartilaginous end were more cuboidal in shape and contained few secretory mucous cells. The rat ET was surrounded by cartilage in the posterosuperior direction. A thin submucosa was found between the epithelium and the cartilage. The submucosa consisted of fibroblasts, collagen fibers and a few vessels. There was no lymphocytic involvement in the rat ET. In addition, as the rat ET extends toward the nasopharynx, the cartilage disappears and submucous glands appear, most notably in the medial portion.

Changes to the Rat Eustachian Tube Epithelium after Balloon Dilation

The epithelial cells at the nasopharyngeal end of the rat ET were desquamated immediately after ETBD. At 1-week post-ETBD, the morphology of these epithelial cells had recovered but goblet cells were not seen. At 4 weeks post-ETBD, goblet cells could be identified but in a less ordered manner. At 12 weeks after the BD procedure, the columnar epithelium and goblet cells had fully recovered (Fig. 2). At the cartilaginous end of the rat ET, the epithelial cells were desquamated immediately after ETBD. At 1-week post-ETBD, the epithelial cells showed a morphological change to squamous metaplasia. At 4 weeks

post-procedure, the epithelial hyperplasia decreased, and the original morphology was finally recovered at 12 weeks post-ETBD (Fig. 3).

Figure 4A shows the changes in the portion of the epithelium with goblet cells in proportion to the whole perimeter of the ET lumen. The goblet cell proportion in the epithelium decreased to zero immediately after ETBD and remained at zero until 1 week post-ETBD. At 4 weeks post-surgery however, the proportion of goblet cells increased significantly ($28.7 \pm 16.5\%$) to a level that was not statistically different to the normal group ($32.0 \pm 3.4\%$). At 12 weeks post-ETBD, the proportion of goblet cells remained at a similar level to the normal group ($31.9 \pm 6.8\%$).

Figure 4B shows the changes in the portion of the epithelium showing squamous metaplasia in proportion to the whole perimeter of the ET lumen. This level of squamous metaplasia significantly increased at 1-week post-dilation ($33.7 \pm 6.4\%$), then decreased significantly to $10.1 \pm 7.7\%$ at 4-weeks post-ETBD. At 12 weeks after ETBD, the level of squamous metaplasia decreased to $4.2 \pm 3.7\%$, which was not statistically different to the normal control. Figure 4C shows the changes of the maximum cell layer count of the epithelium. Immediately after ETBD, all of the epithelia had fallen off and the maximum cell layer count was zero. A significant increase in the maximum cell layer count was evident at 1-week post-dilation (5.0 ± 0.9), which decreased to 2.8 ± 0.4 and 2.4 ± 0.5 at 4 and 12 weeks after the procedure, respectively. The maximum cell layer count was significantly increased at 12 weeks compared to the normal control ($p < 0.05$).

Serial changes in the submucosa after ETBD

Figure 5A shows the changes observed in the vascular structures in the submucosa. Immediately after ETBD, no vessels could be identified. The average number of vessels per slide was 2.4 ± 1.4 , 2.2 ± 1.3 and 2.4 ± 2.0 at 1-, 4-, and 12-weeks post-ETBD, respectively and was greater at 12 weeks compared to the normal control ($p < 0.05$). Figure 5B shows the observed changes in the depth of the submucosa. No significant alterations in this depth were evident immediately after ETBD, but it was found to be increased to $157 \pm 54.3 \mu\text{m}$ at 1 week post-ETBD. The depth of the submucosa at 12 weeks was $105.2 \pm 20.7 \mu\text{m}$ which was significantly thicker than the normal group ($58.5 \pm 21.3 \mu\text{m}$, $p = 0.03$). The submucosa was compared in the different experimental groups by the proportion of the area calculated in each histology section. This value was increased to $23.2 \pm 1.3 \%$ at 1 week, $24.4 \pm 2.6 \%$ at 4 weeks, and $25.9 \pm 1.8 \%$ at 12 weeks after the ETBD procedure and was significantly greater at 12 weeks compared to the normal group ($18.7 \pm 2.4 \%$, $p = 0.03$, Fig. 5C).

The proportional area of the ET lumen was compared in each of the histology sections and was found to change throughout the 12-week time period but to reach and maintain a normal level at 12 weeks after the ETBD procedure (Fig. 5D).

Serial changes in the tubal cartilage after ETBD

Out of the 20 rat ETs that were analyzed, 6 showed no frank fracture lines whereas a total of 21 fracture lines were identified in the remaining 14 ETs. These fracture lines were found at 3 distinctive locations: at

the midpoint between the medial and lateral lamina of the tubal cartilage, and at the lateral 2 points that divide the lateral lamina into thirds (Fig. 6). The frequency at which the fracture lines were identified were 48%, 19%, and 33% respectively, from the midpoint toward the far lateral. All of the cartilage fracture lines were observed immediately after ETBD. Figure 7 summarizes all of the changes identified in this study in the epithelium, submucosa and lumen.

Discussion

With the introduction of the ETBD procedure, chronic ETD could be surgically managed with a reported success rate of 36–80%^{12,16,22,23}. However, 20–64% of ETD patients do not respond to this ETBD treatment modality, thus necessitating further management options. There is no consensus at present about the optimal balloon catheter sizes or lengths to use in patients with ET disorders²⁷. The difficulties in developing further management approaches for ETD may lie in the lack of histological studies. Hence, we speculated that a thorough investigation of the histologic changes that occur in the ET after dilation with balloons of different diameters and lengths might provide new insights into treating and managing ETD.

Kivekas et al. have previously obtained pre- and postoperative biopsy specimens in 13 patients who underwent ETBD²⁶. For practical reasons however, histopathologic studies of the human ET could only be carried out at the nasopharyngeal orifice. The authors suggested in their analyses that the crushing effect of the balloon on lymphocytes and lymphocytic follicles that were later replaced with a thinner fibrous scar is a possible mechanism underlying the therapeutic effects of ETBD²⁶. However, the whole length of the ET needed to be investigated to properly evaluate the effects of ETBD, particularly as the cartilaginous ET is considered a crucial structure in maintaining normal ET function. Hence, a proper histopathological study of the whole ET using appropriate animal models became necessary. Generally, sheep and pigs are considered to be good model systems for the human middle ear²⁸. However, a smaller animal model enables larger scale and higher throughput studies, making it more cost-efficient, and more suited to observing the tissue reactions after ETBD which was an important consideration²⁹. In our current study, we were able to dilate the ET in the rat using commercially available micro balloon catheters.

We here investigated the serial histological changes in the rat ET after ETBD for up to 12 weeks. Immediately after ETBD, we observed desquamation of nearly all epithelial cells and fracture of the tubal cartilage. At 1-week post-ETBD, the ciliated epithelia cells started to recover via squamous metaplasia and epithelial hyperplasia but goblet cells were still missing. The goblet cells recovered by 4 weeks post-ETBD, but epithelial hyperplasia (maximum cell layer count) was still decreased at 12 weeks post-ETBD. The depth of the submucosa increased and neovascularization in the submucosa was observed at 1-week post-ETBD, which persisted at 12 weeks post-ETBD. The lumen of the cartilaginous ET increased immediately after ETBD but decreased at 1-week post-ETBD due to the increased depth of the submucosa and onset of epithelial hyperplasia. The cartilaginous ET lumen recovered to normal at by 4 weeks post-ETBD.

Increased submucosal fibrosis after ETBD was the significant finding of our current study. The effect of ETBD seems to be related to the fibrosis of the submucosal connective tissue. In terms of prior histopathologic exams of similar tissues, Modi et al. performed ETBD of the rabbit subglottis³⁰. The subglottis and ET both have a cartilaginous framework lined by a respiratory epithelium and mucosa. Thirty days after dilation, these authors observed regeneration of the normal epithelial lining and submucosal fibrosis, particularly when larger balloon sizes had been used. We speculated that submucosal fibrosis after ETBD may stiffen and keep the ET patent.

In the urethra, another non-vascular structure formed by the urothelium, submucosal connective tissue, and smooth muscle, the formation of strictures has been with associated collagen-rich connective tissue with few fibroblasts or smooth muscle fibers³¹. Excess type 3 collagen in comparison with type 1 collagen has been detected in cases of ureteral strictures and is considered to be the cause of ureteral fibrosis and stenosis of the lumen^{32–35}.

In our present study of the rat ET, epithelial damage was observed immediately after ETBD. However, the mucosal lining regenerated at 1 week post-ETBD, the goblet cells had recovered by 4 weeks post-ETBD and the squamous metaplasia and epithelial hyperplasia decreased to nearly normal levels at 12 weeks after the procedure. The epithelium thus seems to recover within 12 weeks. It can be argued that a regenerated healthy mucosa will be thinner, thus facilitating ET opening, and the possibly recovered function of the cilia and of mucus production may aid the clearance of secretions from the middle ear³⁶. In addition, the ET mucosa is known to generate a surfactant that reduces surface tension, which would be reduced in otitis media with effusion^{37,38}, and a restored healthy mucosa may secrete surfactant, thus aiding ET opening.

The dimensions of the tubal cartilage in the rat ET did not change throughout our current study period. The cartilage healing process after fracture in general involves an equilibrium between the deposition of type I collagen (scar tissue) and expression of type II collagen (repair). Small full-thickness cartilage defects are mainly replaced by fibrocartilage, whereas partial-thickness defects heal through the deposition of fibrous scar tissue³⁹. Full-thickness cartilage fractures were induced in our current rat model. Furthermore, cartilaginous structures tend to return to their original shape unless the deformation is maintained for several months⁴⁰. These factors could account for the lack of changes to the cartilage framework following ETBD and this may suggest that this is a safe procedure. If the thickness of the cartilage did change, we would not be able to exclude the possibility of cartilage contracture leading to a worsening of the luminal stricture.

The main limitation of our current study was that no functional assessment of the ET could be done. The ET is a dynamic organ that remains closed at rest and opens during certain movements such as swallowing or yawning. The compliance of the ET is a known characteristic of its proper function⁴¹. Hence, we cannot yet ascertain how the submucosal fibrosis induced by ETBD affects the ET function. Another limitation was that our analyses were carried out on rats with a normal ET and we could not therefore analyze lymphocytic involvement. Moreover, ETBD was found to induce a thicker submucosa

but no obvious changes in the lumen. In a chronically inflamed ET, the lumen would be narrower, and further studies in a rat model of otitis media and ETD might therefore provide better insights into the histologic changes caused by ETBD.

Conclusion

Increased submucosal fibrosis and maintenance of the cartilage framework despite cartilage fractures after ETBD in the rat were the significant findings of our current study. At 12-weeks post-dilation, the observed findings were increased epithelial hyperplasia and increased depth of submucosa accompanied by neovascularization. This study is the first to describe serial histological changes after ETBD and these observations will assist with the planning of future histological studies of the middle ear in animal models after the placement of various types of balloons and stents that are typically used to treat intractable ET dysfunction in humans.

Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee at Asan Institute for Life Science and conformed to US National Institutes of Health guidelines for humane handling of laboratory animals and is reported in accordance with ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). Twenty male Wistar rats, 10 weeks of age (300–350 g; Orient Bio, Seongnam, Korea)), were included in our study. The left ET was dilated with a balloon catheter and the right ET was used as a normal control in all rats. The rats were randomly assigned to four groups of each five for serial histological examination. All rats were sacrificed immediately ($n = 5$), 1 week ($n = 5$), 4 weeks ($n = 5$), and 12 weeks ($n = 5$). All animals were sacrificed by administering inhalable pure carbon dioxide. All animals were housed at one per cage in a room with a 12-hour contrast cycle at $24 \pm 1^\circ\text{C}$ with a relative humidity of $55 \pm 10\%$. Standard rodent chow and water were provided ad libitum. All animals were acclimated for at least 1 week prior to conducting the experiments.

Fluoroscopic eustachian tube balloon dilation

All ETBD procedures were performed under fluoroscopic guidance by an interventional radiologist and otologist. Anesthesia was induced by means of an intramuscular injection of 50 mg/kg zolazepam and tiletamine (Zoletil 50; Virbac, Carros, France) and 10 mg/kg xylazine (Rompun; Bayer Healthcare, Leverkusen, Germany). After disinfection of the left external auditory canal with 0.05% chlorhexidine hydrochloride, the left tympanic membrane was punctured using a micro puncture needle (Cook, Bloomington, IN). The needle was directed 45 degrees anteriorly and in a horizontal plane under fluoroscopic guidance. The tip of the needle was located inside the tympanic cavity and about 0.2 ml of contrast media was injected into this cavity to visualize the tympanic orifice of the ET. The tip of the needle was then relocated close to the tympanic orifice. A 0.014-inch micro guidewire (Transend; Boston Scientific, Fremont, CA) was next gently advanced through the ET and eventually out of the nasal cavity.

After removal of the micro puncture needle, a micro balloon catheter (Genoss, Suwon, Korea) was introduced over the guidewire from the nasal side and located at the middle portion of the ET to cover the whole tube structure. The balloon catheter was then inflated at a pressure of 10 atmospheres for 1 minute. During balloon inflation, waist formation and subsequent disappearance of the waist was observed. The micro balloon catheter and micro guidewire were removed after deflation of the balloon.

Histopathologic examinations

Surgical exploration was performed immediately after sacrifice. The mandible and anatomical structures anterior to the soft palate were then resected and the nasopharyngeal opening of the ET was identified. The soft tissue structures around the ET were dissected away whilst taking care not to disrupt the anatomy of the tube. Head samples were fixed in 10% neutral buffered formalin for 48 hours, followed by decalcification for 2 weeks (Osteosoft, Merck, Germany). The samples were then coronally sectioned from the nasopharynx to posterior wall of the tympanic cavity at intervals of 200 μm . Paraffin blocks were prepared from the sliced head samples from which 5 μm slices were obtained with an MR2258 microtome (Histoline, Pantigliate, Italy) and placed on slides. The slides were then stained with hematoxylin-eosin (H&E).

Whole slides of the ET for each rat were reviewed. The cartilage surrounding the ET was first identified. The section in which the sectioned ET cartilage was in the form of a 'comma' shape, showing both cartilaginous and nasopharyngeal epithelia, was selected for analysis (Supplementary Fig. 1). Evaluations included an assessment of changes in the epithelial cells, in the dimensions of the submucosa (degree of fibrotic changes), or in the lumen. In the epithelium, the presence of goblet cells, squamous metaplasia and epithelial hyperplasia was assessed. In order to quantify changes in any of these aspects of the epithelium, the length of the epithelium exhibiting goblet cells, and the level of squamous metaplasia or epithelial hyperplasia were measured and divided by the whole perimeter of the ET lumen in the corresponding slide. These measurements were then compared among our 5 study groups: normal (before) group, immediate, and 1, 4, and 12 weeks after ETBD.

In the submucosa, vessels were identified and those under the tubal cartilage were counted and analyzed as numbers per slide. To evaluate the extent of fibrosis caused by ETBD, the depth of the submucosa was measured. The depth of the submucosa of the left ET (balloon) was measured at 9 different points in total in the medial, middle and lateral areas of the surrounding cartilage. The same was done for sections that were 200 μm proximal and distal to the reference slide. The 9 measurements were averaged to obtain the representative depth of the submucosa. The depth of the submucosa of the normal right ET was also measured as a control using the same points measured on the left side.

Due to the variation of the angles at which the ETs were sectioned among the slides, linear measurements alone may not accurately represent the thickness of the submucosa. The area of the submucosa was also therefore calculated. Histomorphometric analysis was conducted using CaseViewer, version 2.4 (3DHISTECH, Budapest, Hungary). The area of the lumen was also recorded. The relative proportions of the observed absolute values were compared.

Statistical analysis

Difference between the groups were analyzed using the Mann Whitney U test. A p-value of < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS software (version 24.0; SPSS, IBM, Chicago, IL).

Declarations

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Author contribution statement

Y.K., and H.J.P. designed the study and analyzed data. Y.K., J.M.K., and D.S.R. carried out the experiment, and collected data. J-H.P, W.S.K. contributed to the interpretation of the results. Y.K. wrote the paper. H.J.P. supervised the project. All authors read and approved the final manuscript.

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Figures

Figure 1

(A) Normal histology of the rat Eustachian tube (ET) (15x power field view; hematoxylin and eosin (H&E) stain; scale bar represents 100 μ m). The ET consists of the cartilaginous end (*) and the nasopharyngeal end (**). (B) High-power field view of the epithelium at the cartilaginous ET (50x power field view; H&E stain; scale bar represents 50 μ m). The epithelial cells are cuboidal and contains no goblet cells. (C) High-power field view of the epithelium at the nasopharyngeal end (50x power field view; H&E stain; scale bar represents 50 μ m). The epithelial cells are columnar and include goblet cells.

Figure 2

Serial epithelial changes at the nasopharyngeal end of the rat ET after ETBD. A 15x power field view and 50x power field sections are shown in the left and right columns, respectively. The epithelial cells were desquamated immediately after ETBD. At 1-week post-dilation, the morphology of the epithelial cells had recovered but goblet cells were not seen. At 4- and 12-weeks post-dilation, the columnar epithelium and goblet cells had fully recovered. Hematoxylin and eosin stain, scale bar represents 50µm.

Figure 3

Serial epithelial changes at the cartilaginous end of the rat ET after ETBD. A low- and high-power field of view are shown in the left and right columns, respectively. The epithelial cells were desquamated immediately after ETBD. At 1-week post-dilation, the epithelial cell morphology had changed to squamous metaplasia. At 4 weeks after the procedure, the epithelial hyperplasia had decreased, and recovery to the original morphology was finally observed at 12 weeks post-ETBD. Hematoxylin and eosin stain, scale bar represents 50µm.

Figure 4

Serial changes in the proportion of the goblet cells in the epithelium of the nasopharyngeal end of the rat ET (A). Serial changes of the proportion of squamous metaplasia (B), and the maximum cell layer counts (C) present in the epithelium of the cartilaginous end of the ET are also shown; *p<0.05.

Figure 5

Serial changes at the cartilaginous end of the rat ET including (A) vascular structures; (B) submucosal depth; (C) the area of the submucosa (%); and (D) the area of the ET lumen (%); *p<0.05.

Figure 6

Sites of cartilage fracture observed in 14 of the experimental rats. The cartilage tended to fracture at three distinctive locations i.e. at the midpoint between the medial and lateral lamina of the tubal cartilage (arrow), and at the lateral two points that divide the lateral lamina into thirds (arrowhead). Hematoxylin and eosin stain, scale bar represents 100μm.

Figure 7

Histologic changes at the epithelium, submucosa and lumen of the rat ET. The asterisk denotes a significant difference between the period before surgery and at 12 weeks post-ETBD ($p < 0.05$).

Supplementary Files

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- [Supplementaryfigure1.tif](#)