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NOVEL RAT MODEL OF TYMPANOSTOMY TUBE OTORRHEA

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Abstract

OBJECTIVE—Tympanostomy tube otorrhea (TTO), caused by the presence of pathogenic bacteria in the middle ear, is the most common complication of TT insertion. No studies have described a reproducible animal model of TTO. We aimed to develop a rat model of TTO which, in turn, could be used to assay the levels of TNF- α and IL-1 β through the course of the infection.

METHODS—The left Eustachian tubes of 55 male Sprague-Dawley albino rats were occluded with gutta-percha (ETO=Eustachian Tube Occlusion). Middle ear (ME) effusion was ascertained by weekly otomicroscopy. At 3 weeks tympanostomy tubes were placed bilaterally and the MEs were inoculated bilaterally with *Streptococcus pneumoniae* through the tubes. The rats were randomly assigned to one of two daily ototopical treatments: ciprofloxacin/dexamethasone (CDX) or placebo. The animals in each of the two treatment groups were further divided to receive 1, 2, 5 or 7 days of treatment. The rats were sacrificed after treatment was finished. The rates of otorrhea, positive middle ear (ME) cultures, and levels of TNF- α and IL-1 β in the ME fluid were measured.

RESULTS—Left ETO followed by ME inoculation with *S. pneumoniae* and treatment with placebo resulted in persistent infection (100% culture-positive ME fluid at 10 days) and otorrhea (85.7%). Persistent infection of the left ear was accompanied by significantly elevated the levels of IL-1 β and TNF- α . Ears treated with **CDX** had lower rates of otorrhea at all time points and lower levels of IL-1 β and TNF- α .

CONCLUSIONS—This study is the first to describe a reproducible animal model of acute TTO. Surgical obstruction of the ET, followed by TT placement and ME inoculation with *S. pneumoniae* induced persistent otorrhea and infection. Both IL-1 β and TNF- α appear to be potential markers of persistent middle ear infection. This novel model may be used in future studies of the pathogenesis and therapy of TTO.

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Keywords

tympanostomy tube otorrhea; rat model; cytokines

INTRODUCTION

Tympanostomy tube (TT) insertion is the most common surgery performed in children in the United States of America under general anesthesia.¹ Up to 83% of patients suffer from post-tympanostomy tube otorrhea (TTO), which represents the most frequent complication of the procedure.² The etiology of TTO is distinct in that it parallels that of acute otitis media (AOM) with an intact tympanic membrane during the respiratory season with similar microbiology^{3–5} yet, also has a second epidemiological peak in the swimming season with a completely different set of pathogens typically isolated. Further distinguishing TTO are the route of therapy, the influence of potential pathogens in the external auditory canal (EAC), the changes in the microbial flora produced by the use of broad-spectrum antibiotics and the presence of the tube itself. These differences clearly mandate that TTO be considered as a distinct middle ear infection that must be studied as such. Furthermore, the differences significantly impact on the treatment of TTO. An animal model of the condition is paramount to the investigation of TTO's pathogenesis, as well as safety and efficacy of therapies.

Researchers have described various methods to induce middle ear inflammation in chinchillas, cats, gerbils, guinea pigs, rats and mice. The techniques include functional or mechanical obstruction of the Eustachian tube (ET), and inoculation with human pathogens, purified bacterial products or inflammatory mediators through different routes.⁶ The rat was chosen for this experiment because it is widely available at reasonable cost, it can readily be infected with human pathogens, surgical techniques have been described to occlude its ET, and reagents are commercially available to measure inflammatory cytokines. Also, the size of the external auditory canal and tympanic membrane allows the placement of a tympanostomy tube.

The inducement of persistent middle ear (ME) inflammation and infection in the rat has been achieved consistently through obstruction of the ET with or without bacterial inoculation of the ME cavity.^{7–10} Although previous models have contributed to a better understanding of the pathogenesis, prevention and treatment of otitis media with an intact tympanic membrane, their findings cannot be automatically extrapolated to TTO. To the best of our knowledge, no published animal model currently exists specifically designed to induce TTO. Therefore, we sought to develop reproducible TTO in an animal which modeled the human condition in its course, including the improvement with topical antimicrobial therapy.

MATERIALS AND METHODS**Animals and Experimental Model**

All animal experiments were conducted under an approved protocol compliant with the Children's Hospital of Pittsburgh animal care and use committee regulations. Pathogen-free male Sprague-Dawley albino rats (Hilltop Laboratory Animals, Inc, Scottsdale, PA) weighing between 250g and 350g were used. The rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (1.0 mg/kg), administered intramuscularly. All animals were examined by otomicroscopy to document normal middle ear (ME) status and then underwent surgical occlusion of the left Eustachian tube (ET) as previously described.^{9–10} In summary, the procedure consisted of occlusion of the bony ET with gutta-percha (American

Dental Cooperative, St Louis, MO). The ears were examined 48 hours later, and successful left ET occlusion was confirmed by tympanic membrane retraction, and the presence of middle ear effusion.

Three weeks after left ET occlusion, Umbrella Ventilation Tubes (Medtronic Xomed, Jacksonville, FL) were placed bilaterally, through wide posterior myringotomy incisions. Immediately following the placement of the TTs, both middle ears of each animal were inoculated with 15 µl of phosphate-buffered saline (PBS) containing 1×10^8 colony-forming units (CFU) of a penicillin-sensitive strain of *Streptococcus pneumoniae*, type 6A. The inoculum was applied directly through the lumen of the TT with a micropipette with microscopic guidance.

An initial culture specimen was obtained 24 hours after inoculation, by swabbing the outer flange of the TT or any visible otorrhea. The animals were then randomly assigned to one of two treatments: ciprofloxacin 0.3% and dexamethasone 0.1% (CDX); or placebo (ototopical drop vehicle solution). The animals were anesthetized for the daily treatments with a mixture of ketamine (50 mg/kg) and xylazine (0.5 mg/kg). The ears were examined under the microscope and the presence of otorrhea assessed. The ear canals were suctioned if the otorrhea occluded the view of the TT, and one drop of the treatment solution was applied into each ear.

The animals in each of the two treatment groups were further assigned to receive 1, 2, 5 or 7 days of treatment. All were euthanized 24 hours after the last dose, except the rats in the 7-day treatment group, which were sacrificed 3 days after the last treatment. Euthanasia consisted of an intracardiac injection of 0.5 ml of Euthasol (Virbac AH, Inc., Fort Worth, TX) under anesthesia, followed by decapitation. The middle ear bullae were dissected out of the whole heads immediately after euthanasia, and opened aseptically. Culture swabs were obtained directly from the middle ear mucosa, and transferred onto chocolate agar plates. The culture plates were incubated at 37°C for 48 hours in a 5% CO₂ environment, and then qualitatively examined for recovery of *S. pneumoniae*. The middle ear cavities were flushed with 25 µl of PBS. The effusion+wash was stored frozen for later measurements of IL-1β and TNF-α, using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

Data Analysis

Statistical analysis was performed using SAS (PC-version 9.2, SAS Institute, Cary, NC). Fisher's Exact test was used for comparisons among the study groups for categorical variables. Kruskal-Wallis test with group as the covariant was used for comparisons for numerical variables, followed by the Tukey's Studentized Range test if the overall test was significant. The confidence level was set to 95% for all tests.

RESULTS

A total of 55 rats were used. Eight died through the course of the experiment at various time points, and could not be included in the analysis. The final sample consisted of 47 animals. Of these 47 rats, six failed to develop signs of left ET occlusion, but were still included. A flow diagram of the study design is presented in Figure 1.

Following bilateral insertion of TTs and bilateral inoculation with *S. pneumoniae*, the left ears had higher rates of otorrhea compared to the right ears at all time points (Figure 2). There was persistent discharge in the left ears in the placebo group in 86% of the animals at day 8 after bacterial inoculation. The left ears treated with CDX had significantly less

discharge compared to left ears treated with placebo starting at day 2 until the latest time point – 0/6 compared to 6/7 at day 8 after inoculation ($p=0.004$).

Culture results were available in 36 of the 47 rats (Table 1). The tympanic bullae of the remaining 11 rats were stored frozen for future histopathology analysis. Overall, right and left ears treated with placebo had identical rates of positive cultures (96%). All ears from the placebo group had positive cultures at the latest time point, despite the absence of visible discharge in five of 14 ears. As expected, ears treated with placebo had a higher rate of positive cultures compared to CDX (34/36 vs. 9/36, $p<0.001$).

In regards to the levels of TNF- α in the ME effusions, there were significant differences among the treatment groups using the Kruskal-Wallis test ($p=0.009$). Combined left ears treated with placebo had higher levels of TNF- α compared to all other groups. Stratifying by time point, there were significant differences on day 2 only. For IL-1 β , there were significant differences among the treatment groups using the Kruskal-Wallis test ($p = 0.02$). Subsequent Tukey's Studentized Range Test demonstrated that left ears treated with placebo showed significantly higher levels of IL-1 β compared to all other groups. After stratifying by time point, one subgroup experienced significant differences: left ears treated with placebo at 5 days ($p = 0.03$).

COMMENTS

To our knowledge, this is the first report describing a reproducible animal model of acute TTO. We showed that surgical occlusion of the left Eustachian tube, followed by tympanostomy tube insertion and direct bacterial inoculation of the tympanic bulla resulted in persistent infection and otorrhea in this rat model. Previous work by Söderberg *et al* has described the use of tympanostomy tubes in the rat. These authors performed a midline palatal division to induce spontaneous purulent otitis media, and immediately placed a tympanostomy tube unilaterally. They reported a decreased likelihood of purulent otitis media on the side with the TT, thereby providing support to the use of TTs in the prevention of infection.¹¹ The fundamental difference between that report and our study is that our aim was to induce TTO, not prevent it with TTs. Also, we used a ventilation tube designed for humans, which may have improved the delivery of ototopical medications.

Secondarily, we found that the levels of IL-1 β and TNF- α were up-regulated throughout the course of untreated infection in the left ear, which is consistent with previous studies measuring these early inflammatory mediators in otitis media with an intact tympanic membrane in both animals and humans.^{10, 12–15} It is likely that early molecular patterns of host inflammatory middle ear response are similar in both AOM and TTO. Interestingly, the levels of the two cytokines rose continuously up until the latest measurement at day 10 of infection. Since acute otorrhea precedes chronic suppurative otitis media (CSOM), we hypothesize that persistently elevated levels of IL-1 β and TNF- α may play a role in that transition.¹⁶ Further research is needed to systematically study this question and the viability of cytokine levels as surrogate markers of infection in animal models or humans.

The choice of our treatment drug (CDX) stemmed from current best practices that recommend the use of ototopical therapy as first line of treatment of TTO.¹⁷ Ciprofloxacin is one of two available fluoroquinolones approved by the Food and Drug Administration (FDA) for topical use in patients with non-intact tympanic membranes. The combinatorial agent ciprofloxacin/dexamethasone is more effective than otic ofloxacin or oral amoxicillin/clavulanic acid and induces a more rapid resolution of otorrhea than otic ciprofloxacin alone in the treatment of TTO.^{18–20} Although ciprofloxacin administered systemically is not

highly active against *S. pneumoniae*, the topical concentration of ciprofloxacin exceeds the MICs and MBCs of even multi-drug resistant *S. pneumonia* (i.e. serotype 19F).

We clearly observed that the treatment solution penetrated through the tubes during the applications, and verified the presence of the typical CDX crystalline precipitate over the promontory when the bullae were opened. We therefore assume that the effects of treatment occurred due to direct middle ear activity rather than changes in the external auditory canal. However, based on our data, it is not possible to determine the individual contributions of the antibiotic and steroid components of the suspension. This question may be studied in the future using the same animal model.

In our model we have demonstrated that in the absence of ETO the ME infection begins to clear during the first week, as shown by the course of infection and inflammation in the right ears of the placebo-treated group. Hence, obstruction of the ET is a key component for establishing TTO in this model. Similarly, our group has found the same to be true in the rat model of OM without TT placement.¹⁶

Animal models to simulate otitis media have inherent limitations. In our study, the mechanism of infection – a direct injection of bacterial concentrate through the TT – does not reflect the most likely conduit for infection in humans. However, other validated models of acute otitis media have used transbullar and transtympanic injections, directly introducing bacteria or bacterial toxins into the middle ear cleft, suggesting that such methodology has become a reasonable and accepted standard experimental practice.⁶ A second limitation of our study was the relatively small number of animals. In retrospect, the use of fewer time points could have increased group sizes and increased the power of the statistical analysis. Also, the daily treatments with placebo and CDX required anesthesia with half the dose required for the initial left ET occlusion procedure. The repeated use of anesthetics might have contributed to the rate of mortality (8/55) and further decreased our numbers. Thirdly, the examiner evaluating the presence of otorrhea was not blinded to treatment groups, which could have added bias. However, the culture results mirrored the clinical examination and actually identified additional cases of persistent infection. Furthermore, we focused on TTO induced by a common bacterial nasopharyngeal (NP) pathogen. However it is known that otorrhea may be caused by viruses as well as other bacteria present in the external auditory canal.^{21,22} We believe the same animal model may be adapted to investigate these other etiologies.

In summary, we demonstrated a novel rat model of TTO, which induces persistent infection, otorrhea and elevation of pro-inflammatory cytokines. This model may become an important tool to further investigate the pathogenesis and therapy of this common complication of TT placement. We also identified TNF- α and IL-1 β as viable putative surrogate markers of infection and may serve as objective secondary outcome measures for clinical trials.

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Figure 1.
Flowchart of the study design

Figure 2.
Proportion of ears with otorrhea by day of treatment

Table 1

Rates of positive and negative middle ear cultures according to treatment duration with either placebo or CDX*

Group	Placebo (n=18)		CDX (n=18)	
	Left ear	Right ear	Left ear	Right ear
1-day	4	4	5	5
Negative	1 (25%)	1 (25%)	3 (60%)	4 (80%)
Positive	3 (75%)	3 (75%)	2 (40%)	1 (20%)
2-day	5	5	5	5
Negative	0 (0%)	0 (0%)	5 (100%)	3 (60%)
Positive	5 (100%)	5 (100%)	0 (0%)	2 (40%)
5-day	5	5	4	4
Negative	0 (0%)	0 (0%)	3 (75%)	2 (50%)
Positive	5 (100%)	5 (100%)	1 (25%)	2 (50%)
7-day	4	4	4	4
Negative	0 (0%)	0 (0%)	3 (75%)	4 (100%)
Positive	4 (100%)	4 (100%)	1 (25%)	0 (0%)
Combined	18	18	18	18
Negative	1 (6%)	1 (6%)	14 (78%)	13 (72%)
Positive	17 (96%)	17 (96%)	4 (22%)	5 (38%)

* CDX, ciprofloxacin/dexamethasone.