The efficacy of betahistine as treatment for eustachian tube dysfunction in an allergic rat model

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THE EFFICACY OF BETAHISTINE AS TREATMENT FOR EUSTACHIAN TUBE DYSFUNCTION IN AN ALLERGIC RAT MODEL

by

JAMES DAVID WILSON

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Approved by

First Reader

________________________________________

Barbara A. Seaton, Ph.D.
Professor of Physiology and Biophysics

Second Reader

________________________________________

Douglas C. Fitzpatrick, Ph.D.
Research Associate Professor
University of North Carolina at Chapel Hill
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ABSTRACT

Otitis media is a quite common disease, especially in children due largely to their underdeveloped Eustachian tubes. One potential factor, thought to be a large contributor to the disease, is an allergic reaction causing congestion and blockage of the Eustachian tube, leaving the middle ear prone to bacterial infection and effusions. The H₃ receptor has recently been discovered in the nasal mucosa of humans and rodents and is linked to the immune response. Excess histamine released in an allergic response causes nasal vascular constriction and congestion. By blocking the H₃ receptor, the local vasculature may be allowed to dilate, resulting in decongestion. This could play a large role in the treatment of otitis media with effusion.

The effectiveness of betahistine dihydrochloride, an H₃ receptor blocker, in providing possible relief from middle ear congestion was tested using a rat model. An allergic response was induced in rats followed by one of two betahistine dihydrochloride treatment regimens: drug delivery via transtympanic or intranasal route. Changes in Eustachian tube function were monitored during this process. Four measurements were used to measure the function of the Eustachian tube: passive opening pressure, passive closing pressure, active clearance of negative pressure, and Mucociliary transit time. Lower opening
pressure and closing pressure, higher clearance of negative pressure, and shorter Mucociliary transit time were indications of better Eustachian tube function.

Regardless of delivery method, no significant results were found among the experimental groups to suggest improved Eustachian tube function after drug treatment. Although the middle dose of betahistine dihydrochloride (50 mg/mL) delivered transtympanically followed the expected response outcome, the trend did not achieve statistical significance. Overall, the results of this study are inconclusive for measuring the beneficial effects of betahistine dihydrochloride on Eustachian tube function. Further investigations are being conducted to measure the magnitude and duration of the effects of allergic responses on Eustachian tube anatomy and physiology.
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LIST OF ABBREVIATIONS

ACNP .......................................................... Active Clearance of Negative Pressure
AR ................................................................. Allergic Rhinitis
AOM ............................................................. Acute Otitis Media
EPR ............................................................... Early Phase Response
ET ................................................................. Eustachian Tube
IACUC ......................................................... Institutional Animal Care and Use Committee
IN ................................................................. Intranasal
LPR ............................................................... Late Phase Response
MCTT ............................................................ Mucociliary Transit Time
OME ............................................................. Otitis Media with Effusion
OVA ............................................................... Ovalbumin
PBS ............................................................... Phosphate-Buffered Saline
PCP ............................................................... Passive Closing Pressure
POP ............................................................... Passive Opening Pressure
TM ............................................................... Tympanic Membrane
TT ............................................................... Transtympanic
INTRODUCTION

1. Otitis Media

Otitis media encompasses a variety of disease states that fall across a spectrum from acute to chronic and as symptomatic or asymptomatic (Stool, 1998). Otitis media is a large concern for public health as $4 billion is spent annually for its evaluation and treatment (Durland et al., 2000). It is divided into two main forms that have differing symptoms and effects: acute otitis media (AOM) and otitis media with effusion (OME), the latter being of greater relevance to this study. Half of all children will have at least one occurrence of AOM in their first year of life and greater than 60% of children are affected by age 2 (Casselbrant & Mandel, 2003), while the incidence of OME is impossible to determine due to its general lack of symptoms.

AOM is diagnosed clinically based on three components: 1) visible infection of middle ear, 2) moderate to severe bulging of the middle ear, and 3) redness of the manubrium and tympanic membrane (TM) (Siddiq, Grainger, & Prentice, 2014). These factors typically are accompanied by a rapid onset of discomfort in the affected ear suggestive of a viral infection (AAPS, 2004). Other factors that could also indicate the presence of AOM include milder bulging of TM and tugging/holding of the ear. When a patient does present with AOM, antibiotics are the primary modality of treatment. A risk-benefit analysis should be determined before beginning an antibiotic regimen. With the use of antibiotics come certain risks, including nausea and allergic reaction which must be taken
into account along with the possibility that a “watch and see” observational approach may allow AOM to run its course without requiring therapeutic intervention. If AOM does not resolve upon initial observation or application of topical decongestant to the nasopharynx through the nose, delayed treatment does not have a significant effect on the efficacy of therapeutic treatments (AAPS, 2004). Figure 1 below shows the standard of care for a patient following the initial decision to treat with antibiotic or observation.

Figure 1. Standard of care for treatment of patient following initial decision of antibiotic treatment versus observation (AAPS, 2004).

OME can occur as a sequela to AOM, or without history of prior infection, and differs from AOM in its clinical presentation. Also known as “glue ear,” OME is characterized by a buildup of fluid in the middle ear cavity behind the tympanic
membrane (TM) that is unable to be cleared through the Eustachian tube (Gates et al., 2002; van Zon et al., 2012). Though commonly lacking symptoms, OME is a concern nonetheless. When OME of unknown duration is found upon exam, spontaneous resolution is much less common than when the onset of disease is known (Casselbrant & Mandel, 2003). By allowing a buildup of fluid in the middle ear, there is a short-term conductive hearing loss. If the effusions persist, especially in the case of bilateral OME, more significant hearing loss can occur accompanied with impaired development of speech and normal behavior (Shekelle et al., 2002; Gouma et al., 2011).

Furthermore, it is important for providers to better distinguish between AOM and OME in the future. Deferring antibiotic use in OME may allow the process to resolve on its own and could lead to 8 million fewer antibiotic prescriptions annually (Dowell et al., 1998). Bacterial resistance is currently of great concern when it comes to the use of antibiotics in the community as well as the individual. With antimicrobial use there is an increased likelihood of resistant bacteria settling in the body that will not respond to subsequent therapy (Dowell et al., 1998).

2. Eustachian Tube

The Eustachian tube (ET), which provides information between the middle ear and nasopharynx, is an important component of normal middle ear function. Some of its important functional roles include regulation of middle ear air
pressure, transportation of secretions away from the middle ear by ciliary movement, and a barrier to entry of sound pressure and fluids present in the nasopharynx (Takahashi et al., 1989; Fireman, 1997). These functions are illustrated below in Figure 2. Anatomically there are several differences between child and adult ET that can help explain the increased incidence of OME in the young. These differences include the diameter, angle, and musculature of the ET (Holborow, 1970, 1975). With a smaller diameter, more horizontal orientation, and lower frequency of opening due to immature tubal muscles, the young ET is physiologically less suitable for clearance of foreign material from the middle ear (Mann et al., 1979). If an obstruction occurs in the ET, which for the aforementioned reasons is much more likely in a child, negative middle ear pressures can occur as the trapped air of the middle ear is taken up by surrounding mucosa. As this negative pressure is created by the absorbance of air, effusions from the tissues of the middle ear often present themselves as a consequence (J. M. Bernstein, 1996).
Figure 2. Functions of Eustachian Tube in Regulation of Middle Ear. A) Allows equalization of middle ear pressure with atmosphere. B) Allows drainage of fluids from middle ear to nasopharynx with aid of ciliary action. C) Protects middle ear from sound pressure and nasopharyngeal secretions (Fireman, 1997).

3. Pathogenesis of OME

Historically the prevalence of OME in young children has been attributed to an immature ET as discussed previously, which leads to improper drainage of the middle ear. Though this is one factor, allergy has been found to be another important factor. In fact, children with allergic rhinitis (AR) are twice as likely to
develop OME as children with no reported allergies (Draper, 1967). The middle ear is a common locus for allergy and effusion causing agents, resulting in the swelling and obstruction of the ET that can lead to OME (Bernstein, 1975; Labadie et al., 1999; Sobol et al., 2002). The allergic response can be generalized as follows: an antigen presenting cell, such as a dendritic cell, carries a particular allergen (i.e. pollen, pet dander, dust mite fecal protein, etc.) to a CD4+ T-lymphocyte which in turn releases a variety of interleukins and other TH2 cytokines into the blood stream (Oates et al., 1991). These cytokines then interact with plasma cells, mast cells, and eosinophils, which ultimately produce the immunoglobulins that respond to repeated exposures of the allergens. This process is known as sensitization and is visualized below in Figure 3. Furthermore, the responses to repeated allergen exposures can be divided into an early phase response (EPR) followed by a late phase response (LPR).
Figure 3. Sensitization to Allergen. An allergen is presented to a CD4+ T cell by the antigen-presenting cell (dendritic cell) where it is processed and ultimately allergen-specific IgE antibodies are created to respond to subsequent exposure to the same allergen (Oates et al., 1991).

A. Early Phase Response

The EPR occurs shortly after repeat exposure to an allergen and leads to symptoms including itching, sneezing, and runny nose (Skoner, 2001). This rapid response is mediated by the presence of immunoglobulin E (IgE) coated mast cells being present in the surface epithelium of the exposed area. Once there is an interaction between IgE and allergen, the mast cells degranulate causing the symptoms related to EPR (Oates et al., 1991). Degranulation products causing EPR include histamine, kininogenase, and heparin. Other products released by
the mast cells, but not stored in the granules, include prostaglandin D2 and a variety of leukotrienes that cause swelling and leaking of blood vessels, which results in the fluid present in a runny nose. Local glands are also stimulated during this process to release mucoglycoconjugates and antimicrobial materials that cause sinus filling via local blood vessel dilation as well as afferent nerve stimulation, resulting in the sensation of itching and action of sneezing (Mygind & Naclerio, 1993). A schematic for the EPR along with the LPR can be seen below in figure 4.

**B. Late Phase Response**

Clinically, the LPR is similar to that of the EPR with added congestion, fatigue, irritability, and even a potential decrease of cognitive function (Pollock et al., 2002). LPR is characterized by goblet cell hyperplasia causing an increase in mucous production and secretion, recruitment of inflammatory cells including eosinophils, and tissue swelling. These effects have been shown to last 1-2 days following allergen exposure and leave the tissue in a highly sensitive state for repeat activation of the EPR for several subsequent weeks (Fadal, 1993). A previous study in our lab has supported the presence of LPR for at least 36 hours following exposure of allergen to the middle ear confirmed by the presence of Eustachian tube dysfunction and middle ear effusions (Hardy et al., 2001). During the time period leading up to the LPR, chemoattractant cytokines, including some of the interleukins, cause protective granular cells to accumulate
around the area of allergen exposure (Naclerio et al., 1985). These protective cells, once activated, promote reactivation of the EPR pathway as well as leading to longer-lasting changes in the makeup of nasal tissue. The more a subject is exposed to an allergen the less of the allergen is needed to evoke the allergic response. This phenomenon is known as “priming” and is of interest because it can leave the body more prone to respond to other allergens (Connell, 1969). Figure 4 below gives a visual representation of how the body responds to allergens following sensitization.

Figure 4. Early and Late Phase Response to Repeated Allergen Exposure. Once the body has been sensitized to a particular allergen, repeated exposure to that allergen sets in motion the simplified pathway above. The EPR is illustrated to the left and LPR occurs as the diagram progresses to the right (Oates et al., 1991).
4. Histamine

Mast cells (and other granular cells) release histamine during an allergic response, which has been shown to be responsible for eliciting symptoms of AR. It is a neuromodulatory compound capable of causing sneezing, overactive glandular release, and vasodilation leading to congestion along with its other functions throughout the body (Monroe et al., 1997). Several investigations have shown that symptoms of AR can be evoked in non-allergic subjects simply by giving them an application of histamine, thus demonstrating its important role in the allergic process (Doyle et al., 1990; Rajakulasingam et al., 1993; Howarth et al., 2000).

Histamine causes its effects on the nasal mucosa largely through its interaction with the to the H₁ receptor (Hilberg et al., 1995). H₂ receptor mediated effects of histamine may contribute to the mucosal response, but the evidence of its role in the process is not nearly as strong as that of the H₁ receptors (Wood-Baker et al., 1996). H₁ receptor blockers are commonly used for treatment of AR alleviating sneezing and hypersecretion but have not been effective in relieving blockage (Nakaya et al., 2005).

A third histamine receptor, H₃ has been found to have a role as a presynaptic autoreceptor, essentially monitoring the level of histamine released by histaminergic neurons (Arrang et al., 1983). Originally thought to only be present in the central nervous system, the H₃ receptor has recently been shown to be present in the nasal mucosa of both humans and rodents (Nakaya et al.,
2005; Suzuki et al., 2008) and has a complicated pharmacology consisting of around 20 isoforms (Hancock et al., 2003). Furthermore, these receptors have been demonstrated in association with both sympathetic nerve endings and submucosal glands, suggesting a role in the allergic pathway (Taylor-Clark et al., 2005; Suzuki et al., 2008). By blocking $H_3$ receptors, histaminergic neurons continue to release histamine as well as acetylcholine, norepinephrine, serotonin and other neurotransmitters (West et al., 1990). It has been suggested that activation of the sympathetic pathway via this inhibition of the $H_3$ receptor could serve a role in decongestion by causing vasoconstriction of local vasculature (Taylor-Clark et al., 2005). Despite this further release of histamine, the sympathetic effects appear to have a greater impact on ET function (Franz et al., 2011). A fourth recently discovered histamine receptor (Nguyen et al., 2001), $H_4$, is located in the nasopharynx along with $H_3$ receptors, but as yet little is known about its function or if it has a role in the nasal allergic response (Nakaya et al., 2005). Table 1 summarizes the location, function, and nasal symptoms produced by the four known histamine receptors.
Table 1. Histamine Receptors and Their Functions (Taken from Lieberman, 2011).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Location</th>
<th>Activities</th>
<th>Nasal Symptoms</th>
</tr>
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<tbody>
<tr>
<td>H₁</td>
<td>Blood vessels, sensory nerves (smooth muscle bronchi, GI tract, cardiac tissue, endothelium, CNS)</td>
<td>Increases vascular permeability, stimulation sensory nerves of airways, eosinophil chemotaxis, smooth muscle contraction in bronchi and GI tract, stimulation of vagal nerve receptors producing reflex smooth muscle contraction in airways, decreased AV node conduction time, enhancement of release of histamine and arachidonic acid derivatives, nitric oxide formation</td>
<td>Sneezing, itching, rhinorrhea, and perhaps some degree of nasal congestion via increased vascular permeability with leakage of fluid into the tissues and vasodilatation</td>
</tr>
<tr>
<td>H₂</td>
<td>Vascular bed, epithelium of mucosa of nose, submucosal glands in nose, mucosa of stomach, CNS, cardiac tissue, uterus, smooth muscle</td>
<td>Stimulate mucous glands in airways, increases vascular permeability, direct chronotropic effect on atrium and inotropic action on ventricle, relaxation of esophageal sphincter, stimulation of suppressor T cells, decrease in neutrophil and basophil chemotaxis and activation, proliferation of lymphocytes, activity of NK cells</td>
<td>Potentially increase nasal airway swelling, producing nasal decongestion and perhaps increasing rhinorrhea</td>
</tr>
<tr>
<td>H₃</td>
<td>Presynaptic nerves in the peripheral sympathetic adrenergic system, nasal submucosal glands, CNS (histaminergic nerves), airways, GI tract</td>
<td>Suppression of norepinephrine release at presynaptic nerve endings, stimulates nasal submucosal gland secretion, opposes bronchoconstriction and gastric acid</td>
<td>Can produce nasal congestion by prevention of norepinephrine after synaptic release</td>
</tr>
<tr>
<td>H₄</td>
<td>Eosinophils, mast cells, basophils neutrophils, nasal turbinates (nerves), lung colon, epicanthus, bone marrow, spleen, liver</td>
<td>Chemotaxis and chemokinesis of mast cells and eosinophils, enhancement of the activity of other chemoattractants (eg, chemokines) on eosinophils, upregulation of adhesion molecules</td>
<td>Could enhance the inflammatory response to nasal allergen exposure</td>
</tr>
</tbody>
</table>

Abbreviations: AV, atrioventricular; CNS, central nervous system; GI, gastrointestinal; NK, natural killer.
5. Betahistine as Therapeutic

The compound betahistine is known for its antagonistic effects on the H\textsubscript{3} receptor, while also showing mild agonistic effects on the H\textsubscript{1} receptor (Arrang et al., 1985), though more recently it has been reclassified as an inverse agonist to the H\textsubscript{3} receptor (Hancock, 2006). Betahistine has been in clinical use for quite some time primarily to treat balance disorders, including Meniere’s disease (Rascol et al., 1995). The more recent finding of H\textsubscript{3} receptors located in the nasal mucosa has opened the possibility for additional therapeutic use of the drug, including treatment of AR and OME.

As mentioned previously, H\textsubscript{1} receptor blockers typically are able to alleviate the symptoms of sneezing and hypersecretion in AR, but not blockage. Recent evidence has shown support for the use of betahistine in improving ET function in non-allergic Sprague-Dawley rats with the use of a topical intranasal application suggesting that the beneficial effects are primarily due to H\textsubscript{3} receptor specificity, rather than H\textsubscript{1} (Franz et al., 2011). Further support for the H\textsubscript{3} receptor hypothesis comes from the observation that ciproxifan, a pure H\textsubscript{3} receptor antagonist, shows similar beneficial effects on ET function and that intranasal drug delivery is preferable to systemic administration (Franz et al., 2011).

Hydrostatic properties such as surface tension and adhesion can affect the pressure needed to open the ET (Hills, 2002). Therefore changes in ET function are likely due, in part, to changes in the makeup and amount of secretions in the ET lumen (Franz & Anderson, 2007). Currently, it is thought that
the nasopharyngeal side of the ET controls tubal opening via musculature and secretary cell presence. Franz (2011) investigated the question of whether or not intranasal application of betahistine had strong enough local effects on the nasopharyngeal side of the ET to alter adhesive forces holding the tube closed, finding that intranasal topical application had the same effect as when the drug was applied transtympanically into the middle ear cavity and forced through the ET with use of positive pressure (Franz et al., 2011).

6. Specific Aims/Objectives

The objective of the study is the test whether betahistine can improve ET function in an allergic rat model in hope of providing a viable clinical alternative to use of antibiotics in OME patients. Specifically, the study will attempt to answer the following questions:

1) Does the use of betahistine improve ET function in an allergic rat model?
2) Is one of the drug delivery methods, transtympanic or intranasal, more effective than the other?
3) What dosage of betahistine is most efficacious as treatment?

Bacterial resistance as a result of antibiotic usage is a large concern to the world population. By finding an alternative to antibiotic treatment for a common disease process like OME that leads to an abundance of yearly antibiotic prescriptions (especially in children), it is hoped the progression of bacterial
resistance can be hampered. Treatment of middle ear inflammation should always be closely monitored due to the potential lasting effects of persistent effusion presence.
METHODS

1. Laboratory Animals

All animals were handled in compliance to an Institutional Animal Care and Use Committee (IACUC) approved protocol (University of North Carolina at Chapel Hill). Brown Norway rats between 150-300 grams were used as the experimental subjects. Initially the rats were divided into two groups representing two unique drug delivery methods: a transtympanic approach (TT) and an intranasal approach (IN). These delivery classes were then further subdivided into five or six treatment groups for the transtympanic and intranasal class, respectively, as shown in Table 2 below. Each delivery approach had a phosphate buffered saline (PBS) control group and an allergic control group. Rats were randomly assigned to their treatment groups.

Table 2. Drug delivery methods and treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Transtympanic Approach (TT)</th>
<th>Intranasal Approach (IN)</th>
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<tbody>
<tr>
<td>I</td>
<td>PBS Control</td>
<td>PBS Control</td>
</tr>
<tr>
<td>II</td>
<td>Allergic Control</td>
<td>Allergic Control</td>
</tr>
<tr>
<td>III</td>
<td>10 mg/mL Treatment</td>
<td>10 mg/mL Treatment</td>
</tr>
<tr>
<td>IV</td>
<td>50 mg/mL Treatment</td>
<td>50 mg/mL Treatment</td>
</tr>
<tr>
<td>V</td>
<td>200 mg/mL Treatment</td>
<td>100 mg/mL Treatment</td>
</tr>
<tr>
<td>VI</td>
<td>---</td>
<td>200 mg/mL Treatment</td>
</tr>
</tbody>
</table>

The TT group was studied prior to the IN group and followed a timeline displayed below in Figure 5. Small modifications to the timeline were made before continuing with the IN groups including adding an extra treatment dose and removing the final challenge day (see Figure 6). These changes were
applied as a result of the findings from the TT group and will be further discussed below.

**Figure 5. Timeline for Transtympanic Group.** For these animals, ovalbumin (OVA), challenges were delivered transtympanically as were betaistine dihydrochloride treatments. Treatment box: 10 mg/ml = TT Group III; 50 mg/ml = TT Group IV; 200 mg/ml = TT Group V. SQ, subcutaneous injection.
Figure 6. Timeline for Intranasal Group. For these animals, ovalbumin (OVA) challenges and beta histine dihydrochloride treatments were delivered via an intranasal delivery method. Treatment box: 10 mg/ml = IN Group III; 50 mg/ml = IN Group IV; 100 mg/ml = IN Group V; 200 mg/ml = IN Group VI. SQ, subcutaneous injection.

2. Sensitization and Challenges

*PBS Control Group.* Rats in the Phosphate buffered saline (PBS) sensitized control group (Group I, Table 2) received sterile PBS solution, pH 7.4 (1 X PBS-Cell Culture Grade; Lineberger Tissue Culture Facility, Chapel Hill, NC) on all sensitization and challenge days indicated in Figures 5 and 6. Challenges administered through the tympanic membrane consisted of 35 microliters of PBS delivered via syringe directly to the middle ear. Intranasal challenges consisted of
50 microliters of PBS sprayed directly into the nasopharynx via the right nostril by a Paasch airbrush (Paasch Airbrush VL231135, Chicago, IL).

**Allergic control and treatment groups.** Rats in the ovalbumin (OVA) control group (Group II, Table 2) and all treatment group (Group II - VI, Table 2) were sensitized to OVA (A5503; Ovalbumin, Sigma-Aldrich Co., St. Louis, MO) via subcutaneous injections of 1.2 mg OVA in 0.6 mL PBS and 5.14 mg aluminum hydroxide (21645-51-2; Aluminum Hydroxide Gel Dried USP, EMD Chemicals Inc., Gibbstown, NJ) in 0.6 mL PBS as a local adjuvant on days 0 and 7. Rats in the TT delivery class received 0.1 mg of OVA in 35 microliters of PBS transtympanically on challenge days and those in the IN delivery class received 0.1 mg of OVA in 50 microliters of PBS via an intranasal route.

3. **Treatment**

A formulation of betahistine dihydrochloride was supplied by Otifex Therapeutics Pty Ltd (Melbourne, Australia) as a stock solution with concentration of 200 mg/mL. This formulation was used as treatment for the subjects following sensitizations and challenges at the original concentration (200 mg/mL) or diluted to 10 mg/mL, 50 mg/mL, or 100 mg/mL. All dilutions were made with the same clinical grade vehicle (proprietary) in which the drug is carried.
4. Assessment of Eustachian Tube Function

The procedures used in this experiment are adapted from those described previously (Hardy et al., 2001; Ebert et al., 2002, 2006). To begin, the subjects were anesthetized with a mixture containing 91 mg/mL of ketamine and 9 mg/mL of xylazine. After being weighed, the animals were anesthetized and then placed in a lateral recumbent position on a thermistor controlled heating pad. The heating pad was used to keep the subject’s body temperature at a constant level throughout each evaluation. The TM of the subject was then visualized though an operating microscope. Once visualized, a myringotomy was performed with a 27-gauge needle in the anterior-inferior portion of the tympanic membrane to serve as a connection between the middle ear and infusion/withdrawal pump system (BS-8000 Multi-Phaser Programmable Syringe Pump, Braintree Scientific Inc., Braintree, MA). See Figure 7 for a summary diagram of the experimental procedures and apparatus.
Figure 7. Pressure Measurement Apparatus Setup. After a myringotomy is performed to create a hole in the TM (4), the rubber cuff (3) of the Foley catheter when inflated creates a closed system between the middle ear (5), infusion/withdrawal pump (2), and water monometer (1). As pressure is added or removed from the system, the water level of the monometer changes to serve as a gauge that measures the pressure level of the system in cm H$_2$O (Ebert et al., 2006).

Using a technique originally described by Flisberg et al. (1963), the passive opening and closing pressures of the ET were made able to be quantified as follows. Firstly, a 6 French latex pediatric Foley catheter with a 3 mL balloon (6 French latex; Medline; Mundelein, IL) was inserted in the ear canal of the subject and the balloon was inflated to create an airtight seal following a myringotomy. The opposite end of the Foley catheter was attached to a T-
connector and coupled to a water monometer with pressure measurements labeled in cm H$_2$O and a Braintree infusion pump to either introduce or remove pressure from the system at a uniform rate of 16 mL/min.

As pressure was slowly introduced into the system, the water monometer reflected the growing pressure in the system. Once the pressure built to a level at which the Eustachian tube was forced open, the meniscus of water in the monometer reversed from convex to concave representing the *passive opening pressure* (POP). At this point, the infusion/withdrawal pump was turned off and pressure was released through the Eustachian tube. Once the monometer settled at a certain value, it was determined that the Eustachian tube was closed and the value was recorded as the *passive closing pressure* (PCP).

In order to quantify the ability of the Eustachian tube to actively clear middle ear pressure, subjects were put in a supine position. A nasal speculum was used to visualize the back of the mouth. The infusion/withdrawal pump was then used to create a negative pressure of -10 cm H$_2$O in the middle ear. Once this negative pressure system was created, the rat hypopharynx was stimulated with a piece of 0.047” outer diameter silicone tubing attached to the end of polyethylene tubing to induce repeated swallowing. The tubing caused the tensor veli palatini muscle to contract, which in turn caused a temporary active opening of the Eustachian tube. Thus, each time the rat swallowed, a small amount of pressure was able to escape from the system and be visualized as the water monometer level gradually returned back toward 0 with subsequent swallows.
Once the monometer level stabilized, and the animal was stimulated to induce 10 additional swallows with no further pressure release, it was deemed the rat had cleared the negative pressure to its greatest possible extent. The difference in pressures between the start point and end point of swallow stimulation was divided by 10 cm H$_2$O to obtain a percentage *active clearance of negative pressure* (ACNP). A diagram of the three pressure measurements across time can be seen below in Figure 8.

**Figure 8. Distinguishing the Pressure Measurements.** At point a, pressure is introduced to the system by the infusion/withdrawal pump until it reaches the POP. This pressure, point b, occurs when the Eustachian tube opens and the shape of the meniscus in the water monometer changes from concave to convex. When the POP is reached, the infusion/withdrawal pump is turned off and pressure is allowed to exit the system through the Eustachian tube. Once pressure is no longer exiting the system indicating the Eustachian tube is closed, the PCP has been reached (point c). The infusion/withdrawal pump is then used again to withdrawal pressure to -10 cm H$_2$O and swallows are induced until pressure no longer exits the system indicating the ACNP. POP- Passive Opening Pressure, PCP- Passive Closing Pressure, ACNP- Active Clearance of Negative Pressure.
During the final evaluation of Eustachian tube function, a measure of ciliary function was included called the mucociliary transit time (MCTT). To measure the MCTT it was necessary for the subject to be deeply anesthetized due to the invasive nature of the procedure needed to split the soft palate. The subject was placed again in the supine position and the soft palate visualized with the aid of a nasal speculum and operating microscope. A midline incision was then made just posterior to the hard palate transecting the entire soft palate. Hemostasis was maintained with small balls of cotton being held with pressure by forceps. Once the soft palate had been opened, a miniature hand mirror could be positioned within the incision and used to visualize the opening of the Eustachian tube into the nasopharynx. The subject was then moved to the lateral recumbent position and the tympanic membrane was once again visualized. A syringe with a 27-gauge needle filled with blue dye (Coomassie Brilliant Blue, B-0149; Sigma Chemical Co., St. Louis, MO) was then used to inject a few drops into the bulla transtympanically. Once injected, a timer was used and the animal was returned to the supine position in order to visualize the opening of the ET into the nasopharynx.

Once blue dye was visualized escaping through the ET, the timer was stopped, indicating the MCTT. The maximum time waited for visualization of the blue dye was 15 minutes, as our preliminary observations suggested that if the dye had not escaped within 15 minutes, it never would. Once a MCTT was recorded, 1 mL of potassium chloride was injected via an intracardiac route as
euthanasia. Once euthanized, the subjects were decapitated as a second method of sacrifice.

5. Changes to the Transtymppanic Versus Intranasal Timeline

Modifications were made to the experimental timeline prior to continuing with the IN group of which three can be noted in Figures 5 and 6 above. First, the final challenge following treatment was eliminated. By eliminating the final challenge, the overall timeline shortened from 44 to 43 days. Also, the affected ear/Eustachian tube was switched from left to right in the IN group due to handedness of the experimenter.

6. Statistical Analysis

All statistical analyses for this study were performed with the use of IBM SPSS Statistics 22. For the POP the Wilcoxon signed-rank test was used. This non-parametric test was used due to the fact that during several evaluations the Eustachian tube did not open, so an arbitrary value of 130 cm H$_2$O representing the highest point of the monometer was used. For the PCP and ACNP a mixed ANOVA was used. The ACNP was made into a percentage of clearance prior to analysis. Finally for the MCTT, a one-was ANOVA was used. Graphs were created using the mean values of the measurements with standard error of measurement included.
RESULTS

1. Transtympanic Approach

   A. Passive Opening Pressure

   The POP for the five transtympanic approach subgroups can be seen below in Figure 9. Values displayed are an average of the pressures experienced by the subjects of each experimental group. Blue columns represent measured values after sensitization and repeated challenges. Yellow columns represent values attained following treatment. No changes were found to be significant (p > 0.05 using Wilcoxon signed-rank test). Starting from the left in the figure is Group I which experienced a rise in POP from 62.0 (+/- 4.0) to 74.5 (+/- 5.9). Group II experienced a rise in POP from 79.0 (+/- 11.4) to 83.6 (+/- 7.9) following treatment and final challenge. Groups III, IV, and V are arranged by increasing treatment dose. Group III experienced a rise from 64.6 (+/- 8.9) to 83.4 (+/- 8.8) after treatment. Group IV had the largest decrease in POP following treatment from 83.4 (+/- 10.8) to 63.8 (+/- 3.5). Though the average drop was large, it was not statistically significant likely due to large variation within the group. Lastly, group V had a minimal drop from 89.0 (+/- 10.7) to 87.6 (+/- 9.6). Despite the lack of significance for this measure, the 50 mg/mL (Group IV) treatment dose administered transtympanically did, by Day 44, reduce the opening pressure to near those of the PBS controls.
Figure 9. The Passive Opening Pressures (POP) of the Transtympanic Approach Groups. The POP of the Eustachian tube refers to the pressure at which the Eustachian tube opens during infusion of pressure into the middle ear. The measurements are organized by experimental subgroup including the two evaluation points for the subgroups. The blue bars refer to the measurement taken on day 29 following sensitization and challenges, and the yellow bars refer to a final measurement taken on day 44 following treatment dates and a final TT challenge.

B. Passive Closing Pressure

The passive closing pressure from the transtympanic approach group can be seen below in Figure 10. The values displayed are the average pressure values of the different experimental groups at the two evaluation dates. All PCP changes were non-significant (p > 0.05 using mixed ANOVA). Starting from the left on the x-axis, Group I experienced a rise in PCP from 42.8 (+/- 6.0) to
46.3(+/- 4.6). Group II PCP rose from 34.1 (+/- 1.4) to 41.5(+/- 3.9). Group III PCP average rose from 32.7 (+/- 2.9) to 41.8(+/- 3.4) while Group IV value fell from 39.1 (+/- 5.9) to 37.2(+/- 1.8). Lastly, Group V rose from 35.6 (+/- 8.0) to 40.6(+/- 3.1).

**Figure 10.** The Passive Closing Pressures (PCP) of the Transtympanic Approach Groups. The PCP of the Eustachian tube refers to the pressure at which the Eustachian tube closes, visualized by cessation of monometer level change following opening. The measurements are organized by experimental subgroup including the two evaluation points for the subgroups. The blue bars refer to the measurement taken on day 29 following sensitization and challenges and the yellow bars refer to the second measurement taken on day 44 following treatment dates and a final TT challenge.
C. Active Clearance of Negative Pressure

The percentage of negative pressure reduced by active clearance can be seen below in Figure 11. No recorded changes in ACNP were found to be significant (p>0.05 using mixed ANOVA). Starting from the left on the figure, Group I demonstrated less clearance at the final post-treatment evaluation with the percentage of clearance dropping from 28.8 (+/- 6.9) to 25.9 (+/- 9.8). Group II experienced an increase in clearance from 27.9 (+/- 7.6) to 30.5 (+/- 11.8). Group III percentage dropped from 19.3 (+/- 8.7) to 9.3 (+/- 4.1) following treatment. Group IV rose in clearance from 18.9 (+/- 9.5) to 31.6 (+/- 12.0). Group V displayed a drop in clearance following treatment from 17.8 (+/- 5.9) to 12.8 (+/- 5.5). Of the three treatment groups only Group IV (50 mg/mL) had a large increase in clearance following treatment.
Figure 11. The Active Clearance of Negative Pressure (ACNP) of the Transtympanic Approach Groups. The ACNP refers to the percentage of \(-10\) cm H\(_2\)O released by stimulated swallowing during light anesthesia with 100% being the maximum. The measurements are organized by experimental subgroup including the two evaluation points for the subgroups. The blue bars refer to the measurement taken on day 29 following sensitization and challenges and the yellow bars refer to a final measurement taken on day 44 following treatment dates and a final TT challenge.

D. Mucociliary Transit Time

For this measure, two types of outliers were removed from MCTT analysis. One type was animals where the dye never appeared after 15 minutes, and the other was where the dye appeared in less than 30 seconds (dye presented itself by means determined not to be due to ciliary action). The resultant means with outliers removed can be seen below in Figure 12. No
statistically significant differences were found between the TT approach groups, F (4,28)=1.783, P=0.160. From left to right, Group I had a value of 204.5 seconds (+/- 78.4). Group II had an average clearance time of 92.4 seconds (+/- 28.0). The low treatment group, Group III, had the fastest clearance of 61.0 seconds (+/- 10.5). Group IV cleared the blue dye at an average of 125.7 seconds (+/- 21.1). Group V had an average clearance of 114.8 seconds (+/- 36.1).

**Figure 12. The Mucociliary Transit Time (MCTT) of the Transtympanic Approach Groups.** The MCTT refers to the amount of time for blue dye injected transtympanically to travel from the middle ear to the opening of the ET in the nasopharynx via ciliary action. The MCTT measurement was taken on the final, post-treatment evaluation (day 44). The means of the five subgroups are displayed with standard error bars included.
2. Intranasal Approach

A. Passive Opening Pressure

The POP for the six intranasal approach subgroups can be seen below in Figure 13. Blue columns represent measured values after sensitization and repeated challenges. Yellow columns represent values attained following treatment. No challenge following treatment was performed in the IN approach group. Starting from the left of the figure is Group I, which experienced a statistically significant (p=0.028) drop in POP from 72.9 (+/- 3.2) to 64.1 (+/- 3.1). Group II experienced a fall in POP from 71.5 (+/- 3.3) to 67.5 (+/- 2.1) following treatment. Next, the four treatment groups, Groups III-VI, are arranged by increasing dose of treatment. The low dosage group, Group III, experienced a drop from 65.2 (+/- 1.2) to 61.9 (+/- 2.7) after treatment. Group IV experienced a slight fall in POP following treatment from 63.9 (+/- 5.0) to 63.0 (+/- 3.0). Group V fell from 69.4 (+/- 3.0) to 64.7 (+/- 2.9). Lastly, Group VI rose slightly from 65.2 (+/- 3.1) to 66.3 (+/- 3.3).
Figure 13. The Passive Opening Pressures (POP) of the Intranasal Approach Groups. The POP of the Eustachian tube refers to the pressure at which the Eustachian tube opens during infusion of pressure into the middle ear. The measurements are organized by experimental subgroup including the two evaluation points for the subgroups. The blue bars refer to the measurement taken on day 29 following sensitization and challenges, and the yellow bars refer to a final measurement taken on day 44 following treatment dates and a final TT challenge. Star denotes statistical significance.

B. Passive Closing Pressure

The PCP averages for the IN approach group can be found below in Figure 14. All subgroups experienced a rise in PCP following treatment. Group I demonstrated a rise of 32.7 (+/- 2.2) to 35.0 (+/- 1.0). Group II rose from 33.5 (+/- 2.8) to 37.2 (+/- 2.3). Group III showed a rise from 27.8 (+/- 1.2) to 34.2 (+/- 2.4). Group IV rose significantly (p=0.005) from 28.3 (+/- 2.7) to 33.1 (+/- 2.6). Group V
rose from 27.7 (+/- 1.9) to 33.5 (+/- 2.6), and in Group VI average PCP rose from 29.9 (+/- 2.2) to 35.0 (+/- 1.6) following treatment.

Figure 14. The Passive Closing Pressures (PCP) of the Intranasal Approach Groups. The PCP of the Eustachian tube refers to the pressure at which the Eustachian tube closes, visualized by cessation of monometer level change following opening. The measurements are organized by experimental subgroup including the two evaluation points for the subgroups. The blue bars refer to the measurement taken on day 29 following sensitization and challenges and the yellow bars refer to the second measurement taken on day 44 following treatment dates and a final TT challenge. Star, statistical significance.

C. Active Clearance of Negative Pressure

The percentage ACNP for the IN group is shown below in Figure 15.

Group I demonstrated a significant drop (p=0.046) following treatment from 42.6 (+/- 6.0) to 31.7 (+/- 3.4). Group II experienced an increase in clearance from
30.3 (+/- 8.5) to 38.0 (+/- 11.1). Of the four treatment groups, both Group III and Group V showed an increase in clearance following treatment. Group III average percentage rose from 50.9 (+/- 7.2) to 62.6 (+/- 9.1) following treatment. Group IV dropped in clearance from 64.0 (+/- 15.1) to 50.0 (+/- 15.5). Group V displayed an increase in clearance following treatment from 38.0 (+/- 9.3) to 59.5 (+/- 11.3). Finally, the high dosage treatment group, group VI, had a drop in ACNP from 31.0 (+/- 12.8) to 28.7 (+/- 6.5).

**Figure 15. The Active Clearance of Negative Pressure (ACNP) of the Intranasal Approach Groups.** The ACNP refers to the percentage of -10cm H₂O released by stimulated swallowing during light anesthesia with 100% being the maximum. The measurements are organized by experimental subgroup including the two evaluation points for the subgroups. The blue bars refer to the measurement taken on day 29 following sensitization and challenges and the yellow bars refer to a final measurement taken on day 44 following treatment dates and a final TT challenge. Star, statistical significance.
D. Mucociliary Transit Time

The IN subgroups showed no significant differences between evaluations in terms of their MCTT, F (5,26)= 0.370, p=0.160. Group I experienced an average MCTT of 206.7 seconds (+/- 34.9). Group II had a quicker average MCTT at 122.5 seconds (+/- 22.4). The lowest treatment group, Group III, had an average MCTT of 216.0 seconds (+/- 101.4). Group IV average was 194.0 (+/- 61.7). Next, Group V MCTT average was 153.8 (+/- 46.2). The largest dosage treatment group, Group VI, had the highest average MCTT at 247.5 (+/- 122.3)

Figure 16. The Mucociliary Transit Time (MCTT) of the Transtympanic Approach Groups. The MCTT refers to the amount of time for blue dye injected transtympanically to travel from the middle ear to the opening of the ET in the nasopharynx via ciliary action. The MCTT measurement was taken on the final, post-treatment evaluation (day 43). The means of the six subgroups are displayed with standard error bars included.
DISCUSSION

Systemically sensitizing the rats to OVA and delivering challenges of the allergen to the Eustachian tube via the middle ear or the nasopharynx was expected to produce an inflammatory allergic response affecting functions of the Eustachian tube. One function of the ET is to carry debris away from the middle ear. Cilia line the Eustachian tube connecting the middle ear to the nasopharynx and have a natural tendency to carry foreign material away from the middle ear toward the nasopharynx (Sade, 1966). The expectations were for the sensitization and challenge with ovalbumin to decrease the effectiveness of this important function and betahistine would improve the ET function in a dose-dependent manner. Improved ET function is characterized by lower POP, PCP, and MCTT and higher ACNP in our study. However, the negative pressure clearance from the middle ear has been shown in previous studies to be the most reliable measure of ET function (Miller, 1965; Takahashi et al., 1989; Hardy et al., 2001; White et al., 2002).

1. Effects of Drug Treatment

   No convincing, significant changes in ET function were found between pre- and post-treatment measures regardless of delivery method or dose level. However, the 50 mg/mL treatment group (Group IV, TT) showed a trend in the predicted direction in regards to POP and ACNP suggesting possible improvement in ET function following treatment at this dose. Because ET
function does not improve with the 10 mg/mL dose (Group III, TT) it is proposed the agent is not pharmacologically effective at this concentration. The 200 mg/mL dose (Group V, TT) did not show any signs of improvement in ET function perhaps due to increased effect on the H₁ receptor. For the most part, the intranasal results were not very telling, though there were a few significant data measures. The PBS control group (Group I, IN) showed ET function improved with regard to POP. There is evidence suggesting saline alone can improve congestion via intranasal application, which supports the POP results (Tomooka et al., 2000). However, the ACNP for Group I, IN suggests the contrary with Group IV, IN showing a significant result as well in regards to PCP. Because the sample sizes were small in this study (10 per group, TT; 6-7 per group, IN), tests for normality were not possible giving less credibility to the p-value.

2. Limitations in Method and Effects on Future Investigations

*Multiple TM puncture.* The possibility of ET function being affected by repeated TM puncture during myringotomy is a concern based on the suggested impairment of function in the PBS control group of with TT delivery. By performing fewer myringotomies in the IN delivery subjects, it was hoped the potential for myringotomy-induced dysfunction of the ET would be limited.

*Masking.* After completion of the TT delivery timeline, it was decided to adjust the IN timetable based on the premise that during the final challenge in the TT group, some or all beneficial drug effects might have been reversed by
repeated OVA challenge. Unfortunately, the IN data did not show much variability among the groups, nor have we yet tested this for the TT delivery route.

Allergen exposure. By changing the challenge schedule between TT and IN delivery regimens, the ET received less exposure to the allergen under the IN conditions. In nature, repeated exposure to allergens is common due to the subject’s environment, therefore it could be that a single spray of allergen via an intranasal path simply is not sufficient to evoke a substantial allergic response, or it could be activating the EPR but not the LPR necessary for longer lasting effects of allergic response. Previous studies showed the direct application of histamine to the nasal mucosa was sufficient to elicit LPR, but it could be the case that an isolated exposure to allergen is not enough to initiate the LPR (Ebert et al., 2002). This factor is especially important when considering the MCTT. Cilia line the entirety of the ET into the middle ear and by only delivering the allergen into the nasopharynx, it is unlikely many cilia will be affected. Therefore the IN MCTT should remain within the subject-to-subject natural variability. For further studies, it would likely be more beneficial to make the delivery of allergic challenge consistent with TT delivery, only altering the delivery of the drug.

Time. Another issue affecting the results could be the time elapsed prior to the post-challenge evaluation. By waiting two days with no further exposure of allergen, the end of the LPR is approached which could be leading to the natural return of normal ET function. In future evaluations, it would be reasonable to evaluate ET function one day following the final challenge.
**Drug Combinations.** Recently, use of antibiotic therapy has been shown to lead to quicker clearance of fluid in the middle ear in combination with systemic steroids than antibiotic therapy alone (Simpson et al., 2011). Similar studies with H₃ receptor blockers, rather than antibiotics, should be conducted to test the viability of further steroid use as synergistic therapy. Alternative delivery methods should also be considered. Current research shows the feasibility of a non-invasive transtympanic delivery of drug using magnetized nanoparticles, allowing a more sustained and targeted delivery to the affected ET (Sarwar, 2013).

### 3. Final Remarks

While the results presented are not entirely persuasive there is evidence that the 50 mg/mL dose may be beneficial when considering Group IV, TT. Given the shortcomings in the methods discussed above and the small size of our samples it seems that continued investigation with betahistine is warranted, especially in light of information from the National Ambulatory Medical Care survey. This survey showed that rates of otitis media visits in the ER significantly dropped from 1995-1996 to 2005-2006, but the proportion of visits that result in antibiotic prescriptions has remained constant around 80% of cases in the same time period suggesting alternative treatment options for otitis media should be further assessed (Grijalva et al., 2009).
REFERENCES


CURRICULUM VITAE

JAMES DAVID WILSON
daavidw1189@gmail.com
Born 1989

Permanent Address:
901 Mill Road
Goldsboro, NC 27534
Cell: 919-920-6594

EDUCATION

Boston University School of Medicine
M.S. Candidate, Medical Sciences
Expected: May 2015

University of North Carolina at Chapel Hill
B.S. Biology, B.A. Chemistry
Cognitive Science Minor
May 2012

CLINICAL AND LAB EXPERIENCE

UNC School of Medicine; Department of Otolaryngology/HNS
Research Assistant
Chapel Hill, NC
July 2014-Present

Central Dermatology Center
Medical Office Assistant
Chapel Hill, NC
October 2012-July 2013

UNC McAllister Heart Institute; Runge Lab
Research Assistant
Chapel Hill, NC
May 2011-May 2012

UNC Hospitals
Emergency Department Aide
Chapel Hill, NC
January 2011-May 2011