RESEARCH PROTOCOL

STUDY TITLE

Sore throat secondary to endotracheal intubation: the role of neutrophil activation

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1. SYNOPSIS

Study Title	Sore throat secondary to endotracheal intubation: the role of neutrophil activation
Objective	The study is aimed at identifying neutrophil surface changes that occur during tracheal injury secondary to endotracheal intubation and to correlate them with the development of sore throat.
Study Period	Planned enrollment duration: 1 year Planned study duration: Subjects will participate from time of consent prior to surgery through approximately 120 minutes following extubation.
Number of Patients	60 evaluable patients and 25 evaluable healthy controls
Study Treatment	Patients will undergo placement of an endotracheal tube (ETT) as standard of care. Tracheal lavage will be conducted using 5 mL of sterile saline solution by a push/suction technique. Specimen samples obtained by wall suction will be collected at 2 time points following intubation. Blood samples will be obtained at 2 time points, simultaneous with the collection of the tracheal specimens. Healthy volunteers will have a blood draw.
Study Design	This study is prospective.
Inclusion and Exclusion Criteria	Inclusion: 1. Ages 18 to 65 2. American Society of Anesthesiologists Physical Status Classification (ASA) I and II as determined by the study physician as this is a subjective measure. 3. Required to have endotracheal intubation 4. Scheduled for short admission (<23 hrs), or ambulatory orthopedic surgery (ankle, shoulder, or extremity surgery); general surgery (exploratory laparotomy, cholecystectomy, ventral or inguinal hernia repair); obstetric/gynecological surgery(total abdominal hysterectomy, bilateral salpingo-oophorectomy); urologic surgery (nephrectomy, prostatectomy), lumbar spine surgery(discectomy, one or two level lumbar fusion) procedures

	5. Provide informed consent. Exclusion: 1. Active pulmonary disease within 5 days prior to enrollment 2. On immunosuppressive medications 3. Previous tracheal surgery 4. On azithromycin 5. Diabetes 6. Pregnancy 7. Smoking history of less than 6 weeks prior to surgery 8. Surgery or intubation within 5 days of surgery 9. Planned surgical procedure involving neck or thoracic regions 10. Active pulmonary disease 11. Autoimmune disease (HIV, AIDS, Rheumatoid Arthritis 12. Hepatitis B or C, or active Hepatitis A 13. Cancer Healthy Controls: Inclusion: 1. Ages 18 to 65 2. Provide informed consent.
Measurements	**Lavage Processing** Tracheal lavage fluid specimens will be placed on ice and transported immediately to our laboratory. A sample of 0.25cc or less will be removed for cell count and differential count. We will do total cell counts using tryptan blue to check viability of cells. Remaining whole fluid will be centrifuged at 1500 rpm for 15 minutes at 4° C and supernatants frozen in aliquots. **Neutrophil assessment and purification** Neutrophils will be counted in the tracheal lavage and peripheral blood with a HEMAVET analyzer (Drew Scientific). Neutrophils will be purified by immunomagnetic bead-mediated negative selection with biotin-labeled antibodies (e-biosciences) specific for CD11b, CD11c, CD14, CD16 CD62L, CD54, CD32, CD88, and CD66b. Flow cytometry will be used for assessment of neutrophil surface receptor presence. **Reactive Oxygen Species (R0S) determination** Neutrophil production of ROS will be assessed with the fluoro-probe dihydrorhodamine. **Interleukin Assays** Accumulation of interleukin 1 (IL-1), IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23p19, (transformin growth factor 1 (TGF β-1) and tumor necrosis factor alpha (TNF-α) in culture will follow stimulation with formylated peptides by immunosorbent assay (ELISA) for human

	cytokines (R&D Systems, Minneapolis, MN). **Toll-like Receptors (TLR)** Determination of TLR-2, TLR-4 and TLR-9 and Mitochondrial DNA will be done by PCR. **Neutrophil extracellular traps (NETs) formation** Neutrophil cells will be stained and photographic documentation of the NET presence will be documented.
Statistical Methodology	The primary aim of this study is to describe the inflammatory changes that result in a neutrophil surface receptor expression unique to the interaction between the trachea and ETT resulting in sore throat. Blood samples will be obtained and neutrophil cells (PMN) will be isolated for analysis at the same time intervals as the tracheal lavage samples. Individual indicators of inflammation (PMN, cytokines, clusters of differentiation –CD, TLR's as described above) will be compared to baseline values of these variables measured at time zero. We will compare neutrophil (PMN) changes between patients who developed sore throat and those who do not by using the Wilcoxon rank sum test, since we expect the data to be non-normally distributed. With a sample size of 60 patients, we will have at least 80% power to detect large differences (Cohen's D≥O.8) between the sore throat and non-sore throat groups with an alpha level of 0.05 and assuming that half of the participants report a sore throat following intubation. As our pilot study is exploratory, we did not inflate our sample size to account for multiple testing. We will confirm any identified associations found in our pilot study with a future larger study. Subjects who undergo intubation attempts x 2 or greater will be considered a subset population and not part of the 60 evaluable patients. Assuming that lack of sore throat relates to anti-inflammation, measuring a biomarker such as IL-10, may provide information on the regulatory function of this cytokine. We have observed that IL-10 at a concentration of 200 pg/ml generated by PMNs inhibited reactive oxygen species burst from alveolar macrophages challenged by LPS. Power analysis was conducted for two tailed T-test using the following assumptions: a standard deviation of 100 pg/ml. A difference of 75 pg/ml between the two groups will be considered a significant difference. We will need approximately 30 patients per group for an alpha 0.05 and Beta of 0.2.

2. STUDY PROTOCOL

2.1 Background and Significance
Background and preliminary Studies:

Endotracheal intubation results in a high incidence of sore throat. Placement of an endotracheal tube (ETT) is essential for the administration of general anesthesia. Approximately 60 thousand surgical cases are performed every day in the United States that require general anesthesia. The ETT has been implicated in local tracheal injury, development of postoperative sore throat (POST)[1,2,3,4,5,6], tracheitis and pneumonia[7]. POST is ranked high by patients along with pain and nausea as an undesirable outcome following general surgery[1,2,3,4]. Although sore throat tends to be more evident in the first 24 hours, its presence has a negative influence on patient satisfaction post-surgery[2,4]. The incidence of POST has been reported around 50% in several studies following general endotracheal anesthesia and appears to affect a broad range of patients[1,2,3,4]. Some studies have shown a greater incidence of sore throat in young female patients who received succinylcholine, and underwent gynecological procedures; other studies have not shown a significant gender difference[4]. Although the exact pathophysiology of POST has not been elucidated, we recently showed that various arms of the innate immune system are activated following ETT placement[5,6]. Notably, we observed the expression of inflammatory mediators, complement C5a activation and neutrophil infiltration in the mucosal tissues and the trachea in the absence of bacterial infection. However, we did observe mucosal surface injury. As it has been clearly shown that neutrophil infiltration and activation can be driven in response to sterile cell death[8], our preliminary data suggest that the link between ETT placement and neutrophil accumulation is through the injury to mucosal cells.

PMN infiltration found significant in our previous studies of tracheal inflammation. In a pilot swine study[5] we documented a reproducible pattern of inflammation in the peritracheal area in which all markers measured displayed a positive increase over a period of time (6 hours). A similar pattern was documented in our human study[6] in which we found that despite intubating under controled conditions by an experienced practitioner, tracheal injury was still demonstrated (Figure 1). In both studies (swine and human), we measured cellular concentration (polymorphonuclear cells) and a group of proteins (cytokines) that indicate cellular responses to injury. In both groups, a significant neutrophilic reaction was documented, hence the reason for the study.

Significance of PMN phenotype in local tracheitis induced by endotracheal intubation. If our hypothesis is correct we predict that patients with sore throat will have a different PMN pro-inflammatory activation phenotype compared to patients with no sore throat, and we anticipate that the neutrophils will have a regulatory phenotype (IL-10 expression). Determination of PMN phenotypes will be essential in future research planning as sore throat may represent a group of patients more prone to developing active airway inflammation and bacterial seeding in different segments of the lower airways. PMN activation is a desirable effect to clean up acute inflammatory products but unopposed inflammatory activity may be more detrimental to local tissue homeostasis preventing the resolution of inflammation.

Endotracheal intubation relates to Ventilator-associated pneumonia. The exact etiology between tracheal injury and lower airway inflammation is quite unclear. The prevailing view is that intubation-induced inflammation is caused by bacterial pathogens introduced by the endotracheal tube. However, our previous studies have suggested little evidence of bacterial pathogens in the upper airway, with ample evidence of upper tracheal epithelial injury, neutrophil recruitment and inflammatory cytokine release during short term intubation. Thus, we hypothesize that intubation-induced inflammation is predominantly a sterile tracheal injury response that may compromise homeostatic barriers important in preventing subsequent infection. Interestingly, recent studies have suggested the existence of different neutrophil subtypes with pro- and anti-inflammatory (immunosuppressive) functions[12]. Some patients may avoid the consequences of upper airway injury by recruiting neutrophil populations with immunosuppressive factors that promote tissue regeneration and the resolution of inflammation.

Importantly, several reports have suggested there is a relationship between endotracheal intubation and subsequent development of ventilator-associated pneumonia (VAP) with clear implications on overall lung function and patient morbidity. In particular, studies of ventilator-associated tracheobronchitis (VAT), indicate that 10- 32% of intubated patients diagnosed with VAT developed VAP[33]. The use of non-invasive positive pressure ventilation in COPD, respiratory insufficiency, immuno-compromised and congestive heart failure patients have shown to decrease the incidence of VAP dramatically, supporting the notion that the endotracheal tube is a major culprit in respiratory inflammation and infection[34].

The exact pathophysiology of endotracheal tube-associated pneumonia has not been elucidated although the endotracheal tube has been viewed as a major factor in development of VAP. A variety of endotracheal tube modifications (different cuff design, antibiotic coating, and addition of suction channels) have been designed to limit bacterial colonization and VAP have shown only small improvement in outcome for our patients [35] suggesting the need to better understand how endotracheal tubes promote tracheal inflammation to better prevent distal airway diseases such as VAT and VAP.

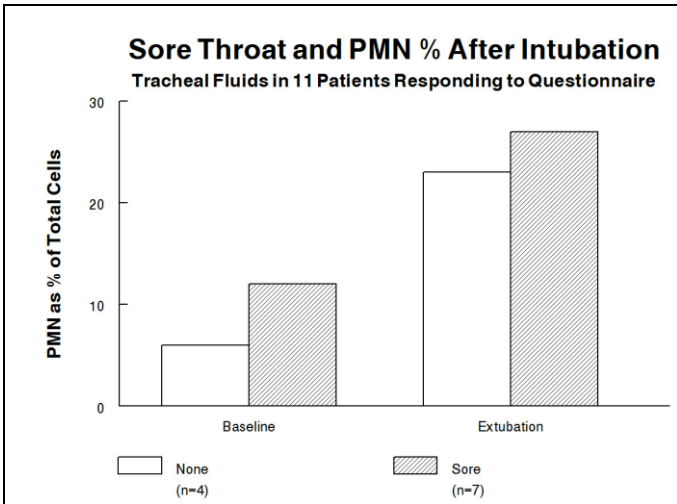

Figure 1. Polymorphonuclear cells (PMNs) as a percentage of total cells in human trachea lavages obtained from 11subjects who responded to post-operative sore throat questionnaire. Median time to end of surgery was 3 hours.

Sore throat and PMN elevation correlated in intubated patients. We conducted a human study of tracheal intubation and asked patients to described the presence or absence of sore throat after surgery, and found that the PMN elevation (figure 1) was slightly different between symptomatic and non-symptomatic subjects, albeit the population number was

small. The initial markers of inflammation detected, such as PMN and protein, increased steadily ($p<0.01$) over time (average 3.2 hours) in human subjects. Neutrophils and cytokines are important components of an organized inflammatory reaction and since several of the cytokines documented may have their origin in the PMN cells, we decided to investigate PMN activation. There are studies evaluating the airway response to injury or infection, but to our knowledge, none have been related to upper tracheal inflammation and in particular the activation of PMN cells in the development of sore throat.

Cytokines produced by PMNs participate in modulation of inflammation and anti-inflammation. Cytokines are proteins produced by a variety of cells including neutrophils[9]. We measured several cytokines (TNF-α, IL-1β, IL-6, and IL-8) in the human tracheal specimens obtained. Our findings included a progressive elevation of all cytokines measured. TNF-α was not found initially but after the first hour it became significant ($p<0.01$). Importantly, some cytokines can delay neutrophil apoptosis, which can lead to substantial increases in mucosal inflammation[10]. One particular cytokine TNF-α has pro or anti-apoptotic actions in neutrophils depending on whether Nfk-β is activated, a key transcriptional regulator that inhibits cell death[11]. Besides controlling survival, cytokines released by neutrophils can promote chemotaxis (IL-8), stimulate revascularization (VEGF), promote activation of other myeloid cells (GM-CSF, IL-6, IL-1b) and resolve inflammation (IL-10)[12]. Our observation regarding PMN derived cytokines and their participation in an inflammatory response is consistent with findings described in several studies[9,10,11,12] .

PMN activation occurs in response to bacterial and non-bacterial stimulants. Neutrophils are mobilized from the bone marrow and travel to a site of injury or infection following specific chemoattractant gradients released by injured tissue. Several chemoattractants such as interleukin 8 (IL-8), leukotrienes, prostaglandins, complement 5a (C5a), platelet activating factor (PAF) and formylated peptides (fMLP) are known to control local neutrophil accumulation[13]. Lipopolysaccharide (LPS) is present in gram-negative bacteria and may promote the release of inflammatory cytokines such as tumor necrosis factor (TNF-α) and IL-1β. Neutrophils can be activated not only due to bacterial infiltration but also may be activated under sterile conditions[14]. Danger associated molecular patterns (DAMPS) released by injured cells (e.g. mitochondrial DNA, ATP, Heat shock proteins) following ischemia-reperfusion injury, trauma or chemical injury are recognized by pattern recognition receptors (PRR) which in turn generate innate immune responses[15]. One well characterized class of PRRs, are Toll-like receptors (TLRs). TLR2 and TLR4, for example recognize the DAMP heat shock protein 60 while TLR9 recognizes mitochondrial DNA. Interestingly, DAMPS mimic the effects of microbial pathogen-associated molecular patterns (PAMPS)[15,16] such as LPS and can drive neutrophilic infiltration without the presence of microbial antigens[15]. Sterile inflammation results when tissue injury occurs in the absence of infectious organisms and presents a clinical picture characterized by local redness, swelling, heat and pain.

Surface expression of encoded proteins is present in PMN cell during injury. Continuous exposure to an antigen will result in neutrophil surface expression –*Clusters of Differentiation* (CD)-, mediated by β2-integrins which are expressed only in neutrophil cells[17]. Cellular adhesion to plasma proteins such as intracellular adhesion molecule-1 (ICAM-1)[18] (CD54) is mediated by integrins in

response to inflammatory cytokines. Clusters of differentiation are used for identification of neutrophils, to determine migration, phagocytosis and activation. Some of the CDs expressed in the neutrophil surface include those related to neutrophil adhesion[19] (CD11b, CD11c, CD16 CD62L, and CD54); Immunoglobulins (CD32) and anaphylatoxins (CD88, CD66b)[20]. The effects of granulocyte-macrophage colony stimulating factor (GM-CSF), are well described regarding encoding of CDs in response to bacteria and DAMPs[21]; with significant enhancement of the pro-inflammatory activity of PMNs, and is consistent with the concept of a significant biosynthetic capacity for PMN cells.

Neutrophils produce large quantities of reactive oxygen species. Although reactive oxygen species (ROS) are generated by neutrophils to control infection, this activity may also alter local healthy tissue. Hydrogen peroxide and superoxide are generated during phagocytosis of particles or elevated concentration of chemoattractants[22]. A sign of neutrophil activation is an acute increase in oxygen consumption (respiratory burst) that is mediated by a membrane-bound complex, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase[23]. NADPH mediates formation of superoxide anion after dismutation to hydrogen peroxide, which in turn is involved in promoting formation of secondary reactive products, such as singlet oxygen, hypochlorous acid and hydroxyl radicals. The end point of this process is neutrophil degranulation with subsequent enzyme degradation of host and microbial tissues.

Regulatory PMN cells are important for host protection against injury. PMN cells have demonstrated dual properties in regulating a specific response to sterile and non-sterile injury by using different mechanisms to induce inflammation or anti-inflammation (immunosuppression)[24]. During infectious injury mediated by gram positive, gram negative bacteria, fungus, PMN may influence activation of T cells which produce interferon γ (IFN- γ), and in turn IFN- γ acts as a potent activator of PMN locally[25]; or can involve Toll –like Receptors (TLRs) such as TLR-2, TLR-4 and TLR-9[26]. We are interested in studying the response of PMNs to injury secondary to the ETT, by analyzing several indicators of PMN activation regarding inflammation and anti-inflammation.

Several systemic diseases and certain antibiotics may alter PMN responsiveness to injury. A variety of systemic diseases such as diabetes, rheumatoid arthritis, acute lung injury and several others may affect local PMN responses to injury by altering inflammation, apoptosis and proper anti-inflammation. In the case of diabetes with microangiopathy surface receptor CD11b was elevated and CD62L was decreased[27]. The impact of macrolide antibiotics and in particular azithromycin in the improved outcome of respiratory diseases such as acute lung injury (ALI), community-acquired pneumonia (CAP) and ventilator associated pneumonia (VAP) appear to be mediated by its anti-inflammatory properties. In particular azithromycin may affect PMN activation, PMN survival, oxidative burst, and attenuation of nuclear factor-κβ[28], therefore modulating the inflammatory response. It is not known the exact effect of these systemic factors in the local expression of neutrophils at the tracheal level, therefore our interest to compare local and systemic PMN activation in the healthy subject.

A variety of biomaterials activate PMN in blood. The endotracheal tubes contain a biomaterial known as di-(2-ethyl-hexyl)-phthalate or DEHP as it is commonly known. Studies in human and rat blood have shown its ability to increased CD-11b expression in PMN cells[29]. The effects on PMN of neonates also include inhibition of chemotaxis, stimulation of oxidative metabolism, and upregulation of NADPH-oxidase-1[30]. DEHP is the only plasticizer approved for human devices by the U.S. Food and Drug Administration. Studies of materials involved either human blood samples, or *in vitro,* or rat models, none have *in vivo* local activation of PMN cells.

Significance of intubation related sore-throat in airway inflammation VAT/VAP:
Placement of an endotracheal tube results in tracheal injury. We hypothesize that intubation-related sore throat is associated with neutrophil activation in the airway. To the best of our knowledge the association of neutrophil activation in the subglottic area and intubation-related sore throat has not been studied. We contend this is a relevant clinical phenomenon, which has implications beyond the larynx and pharynx because studies have shown that there is a link between upper (endotracheal intubation) and lower respiratory inflammation or infection (VAP)[36]. Although not all patients develop sore throat following intubation they do have elevated neutrophils in the subglottic area, suggesting the possibility of the existence of different neutrophils phenotypes regulating inflammation in the trachea. Therefore, to provide better insight into the pathophysiology of upper airway inflammation and progression to VAT and VAP we propose to analyze PMN-mediated inflammation in the trachea through characterizing neutrophil phenotype and function in our patient population.
Neutrophils have a fundamental role in inflammation as effectors cells, capable of responding to sterile tissue injury. Expression of surface receptors on the neutrophils under certain conditions may result in an activated phenotype that will enhance their ability to migrate, produce more reactive oxygen species and delay apoptosis. Unique PMN receptors –cluster of differentiation CD- had been found in blood of patients with respiratory diseases (asthma, COPD) and had been correlated with severity of clinical manifestations. Neutrophil activity has been extensively investigated during systemic inflammation; however, the characterization of neutrophil phenotype and function in local sites of inflammation is less well defined. Placement of an endotracheal tube (ETT) is performed for a variety of medical conditions including short term general anesthesia. We are interested in analyzing the neutrophil phenotype *in vivo* while the ETT is in direct contact with the tracheal tissue in an attempt to correlate them with the occurrence of sore throat. A better understanding of neutrophil trafficking and CD expression in the upper airway may offer new and more precise therapeutic targets to prevent local and distant disease.
The glutathione-S-transferase mu1 (GMSTM1) null genotype is fairly common in the general population (40-43%) and in all ethnicities (Montero R. et al. 2007. Hum Biol). The presence of GMSTM1 is associated with increased risk for cancer (Zhong S, et al. 1993. Carcinogenesis) and inflammatory diseases of the airway and lungs (Alexis NE, et al. 2009. J Allergy Clin Immunol; Wu W, et al. 2012. Free Radic Biol Med). Studies have shown that reactive oxygen species (ROS) are abnormally generated in GMSTM1-null phenotype (Yang Y, et al. 2009. Hypertension). Since neutrophil activity is closely link to ROS activity and we have demonstrated in our studies of human tracheal lavages that ROS is significantly elevated in those with tracheal injury, we would want to

analyze the presence of GMSTM1 in all the specimens collected and in the prospective human donors.

Effect of ATG16L1 allelic variation on neutrophil longevity. Autophagy 16-like 1 (ATG16L1) is an essential part of a protein complex responsible for initiating and carrying out autophagy. Autophagy is an essential intracellular process necessary for intracellular degradation and involves autophagosome and lysosome formation. Most people carry non-mutant, or "hypermorphic," alleles for ATG16L1 allowing for the proper degradation of unneeded intracellular components. However, some carry mutant, or "hypomorphic," alleles that disrupt normal autophagic processes. This disruption in proper autophagy leads to improper and excessive inflammatory responses by the innate immune system. Patients both heterozygous and homozygous for these hypomorphic alleles have a markedly increased risk of developing inflammatory bowel diseases, particularly Crohn's disease.
It has been shown that the presence of the hypomorphic ATG16L1 alleles also contribute to the development of ventilator associated pneumonia and other postoperative inflammatory complications32. However, the effect these alleles have on postoperative responses by the most prominent inflammatory immune cell the neutrophil, have yet to be elucidated. By isolation and subsequent sequencing of these alleles in neutrophils from patients with and without postoperative inflammatory complications, we can further understand how this gene plays a role in thoracic immune response.
Mitochondrial DNA released during cellular injury or trauma has been implicated in sterile inflammation. Injured cells release a variety of molecules capable of eliciting an innate immune response to non-bacterial products. The presence of mitochondrial DNA (mtDNA) released during cell injury has been implicated in development of non-bacterial or sterile inflammation by activation of human neutrophils leading to increased migration, activation and degranulation mediated by Ca^{2+} and mitogen-activated protein kinases (MAPK). Studies of trauma patients with evidence of systemic inflammation indicate that DAMPs are present in the serum of affected patients and in particular mtDNA has been detected at levels several times higher than patients without trauma. Less is known of local cellular injury and in particular in the tracheal area during active injury such as endotracheal tube presence. The mechanisms involved in PMN activation resulting in tracheal and airway injury have not been elucidated, hence our interest in studying mtDNA locally with the goal to determine its impact on PMN activation and thus design therapies directed to specific anti-inflammatory targets using anti-mitocondrial DNA[37].
Neutrophil are essential cells of the innate immune system and have multiple defensive functions intended to protect tissue homeostasis. As part of the extracellular neutrophil activity a release of a substance call neutrophil extracellular traps (NETs)[38] may occur and appears to have a phagocytic function. NETs are also regarded as a form of cell death different than apoptosis or necrosis. We are asking for approval to stain the neutrophil human cell to determine the level of NET formation under the conditions previously requested in the initial protocol. No extra specimens are requested.

Innovation:

The use of endotracheal intubation as a procedure to facilitate general anesthesia and provide airway protection in the intensive care unit and may result in a variety of airway injuries including sore throat. There is no current therapy universally accepted for prevention of sore throat despite a multitude of pharmacological and non-pharmacological interventions. Tracheal research is in itself innovative and analysis of local PMN activation in the context of acute tracheal injury represents a new approach in the quest to understand the pathophysiology of sore throat. Patient satisfaction after surgery is negatively influenced in part by sore throat. Identification of specific PMN surface receptors will provide therapeutic targets that can be used to modulate the inflammatory reaction and facilitate healing. The primary aim of this research is to improve understanding of the cellular mechanisms involved in sore throat following endotracheal intubation. If neutrophil activation patterns are correlated with occurrence of sore throat, more precise treatment(s) and preventive measurements can be taken to improve quality of patient care, satisfaction, safety and outcomes. This study may provide sufficient evidence to support new treatments such as anti-IL-6, anti-TNF-alpha, Apyrase and other anti-inflammatory medications to coat the endotracheal tube and thus prevent sore throat and inflammation.

Healthy Volunteers

We found inflammation in the tracheal lavages of healthy humans indicating that neutrophil activation is occurring while the endotracheal tube is in place. We hypothesize that the inflammation is mainly mediated by sterile products in particular necrotic tissue or cellular mitochondrial DNA resulting from local cell injury. Now we would like to recreate similar neutrophil response by looking in the blood of healthy volunteers and challenge the neutrophils with necrotic tissue or labeled mitochondrial DNA obtained from laboratory cultured cells and look for markers of neutrophil activation that also will include CD35/CD49b, connexin43 and the previously approved CD markers. By exploring the role of CD35/CD49b39 and possibly connexin4340 we could attempt to understand how the neutrophil cell respond to exposure to necrotic tissue and what possible mechanisms are involved in the responses. Determination of NET's (neutrophil extracellular traps) will be also done as proposed previously for airway neutrophils. If recreation of the cellular injury in the blood neutrophil results in similar cellular changes as the ones seen in the trachea, then we will have a good human model to study airway inflammation.

Approach

2.1.1 Preliminary Data

2.2 Objective

The study is aimed at identifying neutrophil surface changes that occur during tracheal injury secondary to endotracheal intubation and to correlate them with the development of sore throat.

2.3 Patient Selection

2.3.1 Inclusion Criteria

1. Ages 18 to 65
2. American Society of Anesthesiologists Physical Status Classification (ASA) I and II as determined by the study physician as this is a subjective measure.
3. Required to have endotracheal intubation
4. Scheduled for short admission (<23 hrs), or ambulatory orthopedic surgery (ankle, shoulder, or extremity surgery); general surgery (exploratory laparotomy, cholecystectomy, hernia repairs); obstetric/gynecological surgery(total abdominal hysterectomy, bilateral salpingo-Oophorectomy); urologic surgery (nephrectomy, prostatectomy), lumbar spine surgery (discectomy, one or two level lumbar fusion) procedures.
5.
6. Provide informed consent.

2.3.2 Exclusion Criteria

1. Active pulmonary disease within 5 days prior to enrollment
2. On immunosuppressive medications
3. Previous tracheal surgery
4. On azithromycin
5. Diabetes
6. Pregnancy
7. Smoking history of less than 6 weeks prior to surgery
8. Surgery or intubation within 5 days of surgery
9. Planned surgical procedure involving neck or thoracic regions
10. Active pulmonary disease
11. Autoimmune disease (HIV, AIDS, Rheumatoid Arthritis
12. Hepatitis B or C, or active Hepatitis A
13. Cancer

Healthy Controls:

Inclusion:

1. Ages 18 to 65
2. Provide informed consent

2.4 Design and Procedures

2.4.1 Study Design

This study is observational and prospective.

2.4.2 Minimization of Bias

There will be no specific gender or ethnic background for enrollment and the decision to place the endotracheal tube will be at the discretion of the attending anesthesiologist. Addition of CD35/CD49b and connexin43 will be done to determine neutrophil activity and subpopulation of neutrophils active during different conditions and may result in local inflammation or immune-suppression.

2.4.3 Pre-Study Period

Subjects who are potential candidates for the study will be educated as to the study procedures, benefits, and potential risks. Each subject who qualifies for entry into the study on the basis of inclusion/exclusion criteria, and completion of an informed consent will be assigned the next available patient number. This indicates enrollment in the study. Subjects who drop out of the study prior to completing the study will be replaced by using the next available patient number.

2.4.4 Study Period

Subjects will participate from intubation time to the immediate post operative period.
After the placement of the endotracheal tube (Hi-Lo™ Evac Mallinckrodt), tracheal lavages will be performed using 5 milliliters of sterile saline solution by a push/suction technique. Wall suction will be set to a minimum in order to minimize tracheal injury and to comply with standards of medical care. The same procedure for collection of tracheal sample will be repeated at the end of the surgical procedure,immediately prior to removal of the ETT. The specimen will be collected in a sterile container and placed in a laden ice container for transportation and immediate processing.

A simultaneous blood sample of approx 5cc, will be collected by venipuncture or by sampling from intravenous or arterial line if available at the end of the surgical procedure,immediately prior to removal of the ETT. A total of approx 5cc blood will be collected for research purposes.

Review of the medical record will be conducted for data acquisition of medical history, and preoperative blood test results to determine enrollment in the study according to the specified inclusion or exclusion criteria protocol. Physiologic and demographic measurements such as temperature, oxygenation, and blood pressure, gender, body mass index and coexisting diseases will be recorded as per anesthesia records. All data will be de- identified. All patients will be asked to respond to presence or absence of sore throat and its degree of intensity using a Visual Analogue Scale (VAS) sliding scale at 120 minutes post extubation and following a specific cognitive assessment performed by the recovery room nurses as part of their discharge protocol. Cognitive evaluation will include patient interview to determine the following: Orientation to time, place, person and situational (Why are you here?).

Healthy volunteers
A blood sample of approx 20cc, will be collected by venipuncture for research purposes to challenge the neutrophils with necrotic tissue obtained from laboratory cultured cells and look for markers of neutrophil activation that also will include CD35/CD49b and the previously approved CD markers

2.5 Observations and Measurements

2.5.1 Lavage Processing
Tracheal lavage fluid will be placed on ice and transported immediately to our laboratory. A sample of 0.25cc or less will be removed for cell count and differential count. We will do total cell counts using tryptan blue to check viability of cells. Remaining whole fluid will be centrifuged at 1500 rpm for 15 minutes at 4° C and supernatants frozen in aliquots.

2.5.2 Neutrophil assessment and purification
Neutrophils will be counted in the tracheal lavage and peripheral blood with a HEMAVET analyzer (Drew Scientific). Neutrophils will be purified by immunomagnetic bead-mediated negative selection with biotin-labeled antibodies (e-biosciences) specific for CD11b, CD62L, CD54, CD32, CD88, and CD66b. Flow cytometry will be use for assessment of neutrophil surface receptor presence.

2.5.3 Reactive Oxygen Species (R0S) determination
Neutrophil production of ROS will be assessed with the fluoro probe dihydrorhodamine.

2.5.4 Interleukin Assays
Accumulation of interleukin 1 (IL-1), IL-4, IL-6, IL-8, IL-10, IL-17, IL-23p19, transformin growth factor 1 (TGF β-1) and tumor necrosis factor alpha (TNF-α) in culture will follow stimulation with formylated peptides by immunosorbent assay (ELISA) for human cytokines (R&D Systems, Minneapolis, MN).

2.5.5 Toll-like Receptors (TLR)
Determination of TLR-2, TLR-4, TLR-9 and mitochondrial DNA will be done by PCR.

2.5.6 Neutrophil extracellular traps (NETs) formation
Neutrophil cells will be stained and photographic documentation of the NET presence will be documented.

2.5.7 Primary Outcome Measures
The study is aimed at identifying neutrophil surface changes that occur during tracheal injury secondary to endotracheal intubation and to correlate them with the development of sore throat.

2.5.8 Exploratory ~~Secondary~~ Outcome Measures
To analyze the determine the presence and role of GMSTM1 in all the specimens collected and in the prospective human donors.

Evaluate the role of autophagy 1 [ATG16L1] genetic polymorphisms of neutrophils in patients with and without postoperative airway inflammation or ventilator-associated pneumonia. ¶

2.5.9 Statistical Methods

The primary aim of this study is to describe the inflammatory changes that result in a neutrophil surface receptor expression unique to the interaction between the trachea and the ETT and result in sore throat. Blood samples will be obtained and neutrophil cells (PMN) will be isolated for analysis at the same time intervals as the tracheal lavage samples. Individual indicators of inflammation (PMN, cytokines, clusters of differentiation –CD, TLR's as described above) will be collected at specific pre-determined times and will be compared to baseline values of these variables measured at time zero. We will compare neutrophil (PMN) changes between patients who developed sore throat and those who do not by using the Wilcoxon rank sum test, since we expect the data to be non-normally distributed.

2.5.10 Sample Size

With a sample size of 60 evaluable patients, we will have at least 80% power to detect large differences (Cohen's D≥O.8) between the sore throat and non-sore throat groups with an alpha level of 0.05 and assuming that half of the participants report a sore throat following intubation. As our pilot study is exploratory, we did not inflate our sample size to account for multiple testing. We will confirm any identified associations found in our pilot study with a future larger study. All subjects that undergo intubation attempts times 2 or more, will not be considered evaluable for the primary aim of this research. However, these patients will be considered as a subset and of possible interest for analysis and outcome measures.

Data analysis of the blood neutrophil changes occurring under different laboratory conditions will be analyzed using a Student-t test to compare them with changes observed in the injured airway neutrophils. A total of 25 evaluable patients will provide sufficient blood neutrophils to determine differences.

3.0 Management of Intercurrent Events

3.1 Adverse Experiences

The investigator will closely monitor subjects for evidence of adverse events. All adverse events will be reported and followed until satisfactory resolution. The description of the adverse experience will include the time of onset, duration, intensity, etiology, relationship to the study procedures (none, unlikely, possible, probable, highly probable), and any follow up treatment if required.

3.2 Premature Discontinuation

If a subject withdraws from the study, the subject will be replaced in order to provide the required number of evaluable subjects. Subjects will be withdrawn if the investigator decides that discontinuation is in the best interest of the subject, or the subject requests withdrawal from the study.

3.3 Potential Risks

3.3.1 Potential risks from ETT saline lavage

By using sterile normal saline solution we minimized the impact of the irrigation on the trachea since the osmolality of the saline is similar to the osmolality of the serum. The collection of tracheal

secretions will be performed by trained personnel. Wall suction will be set to a minimum in order to minimize tracheal injury and to comply with standards of medical care. Although the endotracheal tube does not contain any particular medication, its presence may induce sore throat (reason for the study). The ET tube is being placed as standard of care for patients undergoing general anesthesia. The intrinsic risks related to the placement of the endotracheal tube are beyond the scope of our research.

3.3.2 Potential risks from blood draw

Discomfort, bruising, and/or bleeding at the site of the needle injection. Every attempt will be made to collect the blood sample from an indwelling venous or arterial catheter. All samples will be collected using standard medical techniques by trained personnel.

3.3.3 Potential risk from loss of confidentiality

One risk of participating in this study is that confidential information may be accidentally disclosed. We will use our best efforts to keep the information secure, and the risk of accidental disclosure is very small. All samples and health information will be de-identified and assigned a study ID number. Statisticians involved in the project will have access only to de-identified data for the purposes of analysis. The original informed consents will be kept by the PI and a copy will be given to all subjects. Neutrophil surface activation data samples will be kept confidentially. Any information obtained from medical records will be coded, with a key to the code linking code numbers to names kept at a separate location, under lock and key. 1) The link to identifiers will be destroyed at the end of the study. We have no evidence to suggest that testing will provide evidence of previously undiagnosed or unrecognized illness, or susceptibility to illness. 2) We will not use samples for any purpose other than to perform tracheal and blood neutrophil analysis. 3) Data will be stored under lock and key (office, file cabinet) and only the investigators will have access. If data are published, there will be no link to identifiers. Study data will not be revealed to any organization or individuals.

3.4 Procedures to Minimize Potential Risks

Study related procedures will be conducted in the operating room under the general supervision of the PI, a board-certified anesthesiologist, thoroughly trained and experienced in intubation, tracheal lavage, and blood collection. Subjects will be monitored through-out the study period by trained personnel. Subjects will be informed that participation is voluntary and they may refuse to participate and may withdraw from the study at any time without penalty. Subjects will be told that in the event of a physical injury as the direct result of study procedures, they will be cared for by a member of the investigating team at no cost, within the limits of the Washington University compensation plan.

3.5 Data and Safety Monitoring Plan

The principal investigator will monitor the study for any adverse events and serious adverse events per the institutional guidelines. All unanticipated problems will be reported to the HRPO. Should there be a serious adverse event that occurs that increases the risks to the participants, the study will be stopped, an investigation will be conducted, and a findings report will be generated before the study is resumed. The specific monitoring plan for this investigation is commensurate with the risks

and the size and complexity of the investigations planned. These risks, as described above, are small. Based on these considerations, we will utilize a monitoring plan that involves:

(1) Engaging a colleague not involved in the study to serve in a monitoring capacity. Based on the small size and low-risk, we envision only a third person (the colleague), not a full DSMB.
(2) A specific plan for submitting Adverse Event Reports to the IRB. That plan is detailed in the department's SOP for Adverse Event Reporting (attached).

4. HUMAN SUBJECTS RESEARCH

4.1 Protection of Human Subjects

The study will be conducted under appropriate Washington University Institutional Review Board protocols and consent forms approvals. The study will be conducted under the supervision of the PI, a Board-Certified and GCP-certified anesthesiologist, and a mentor with several years experience in the conduct of human volunteer studies.

4.2 Sources of Materials

Data will be collected from the subject's electronic medical record. Blood and tracheal specimens will be obtained for research purposes only. Patients will be asked to report presence or absence of sore throat and to score the severity of pain using a sliding VAS ruler.

4.3 Recruitment and Informed Consent

Subjects will be recruited from the PI's patient population when they are admitted to the surgical holding area prior to ambulatory surgery. Healthy volunteers will be recruited using flyers posted around the medical school campus. Subjects will be provided verbal and written descriptions of the study aims, procedures, risks, and benefits, and will be required to give written informed consent. A member of the investigative team provides all study descriptions, informed consent, and answers all questions. No deception is required for the purposes of this study. Subjects are informed verbally and in writing that participation is voluntary and they may refuse to participate and may withdraw from the study at any time without penalty.

4.4 Potential Benefits of the Proposed Research to the Subjects and Others

There is no immediate benefit to individual subjects in this study. This study seeks to identify neutrophil surface changes that may occur related to sore throat and may serve as reliable biomarker(s) for designing possible therapies that would benefit future patients.

4.4.1 Importance of the knowledge to be gained

The incidence of sore throat is significant and the pathophysiology is still unknown. There is no reliable way to diagnose or treat sore throat following placement of ETT. Many patients rate sore throat as a significant issue following general surgery and is viewed negatively by patients. If a patient requires endotracheal intubation for several days the inflammation documented in our previous studies may represent only the beginning of a more distal and severe form of respiratory

disease such as bronchitis, possible pneumonia. Finding and treating early sore throat may improve and prevent other respiratory disease. If our research demonstrates the surface changes in the neutrophils, we may be able to design an endotracheal tube coated with a specific anti-inflammatory receptor blocker and thus prevent occurrence of upper respiratory diseases.

4.5 Inclusion of Women

Studies and their advertisements actively encourage the participation of women in the research. As a matter of operational policy, our studies of healthy volunteers routinely and deliberately include equivalent numbers of women and men. To ensure sufficient enrollment of women, we typically close enrollment to men once their quota has been filled. This approach has been highly successful.

4.6 Inclusion of Minorities

All of our studies and their advertisements actively encourage the participation of minorities in research. Our minority recruiting typically matches the demographic composition of the Washington University community from which subjects will be recruited (78% white, 21% Black, <1 % Hispanic). However, based on the higher incidence of *CYP2B6*6* allele in African-Americans, we anticipate a higher enrollment of minorities related to increased incidence of genotype.

4.7 Inclusion of Children

Children <18 yr will not be studied in this investigation.

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Appendix A

Date

Address

To Whom It May Concern:

On behalf of the research team at Washington University in Saint Louis School of Medicine and the Department of Anesthesiology and Critical Care, we would like to thank you for your participation in research study # ___ - **Sore throat secondary to endotracheal intubation: the role of neutrophil activation**

If you have any questions about your participation in this research study, please feel free to contact Dr. Carlos Puyo at 314-747-0259 or e-mail: puyoc@wustl.edu.

Thank you,

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