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Research

Ex-Vivo Cholesteatoma Staining: A Feasibility Study for Intraoperative Staining and Identification of Occult Cholesteatoma

Stuart Curtis[1] and David Foyt[2*]

[1]*Department of Otolaryngology, Albany Medical Center, Albany, New York, USA*

[2]*Department of Otolaryngology, Neurotology/Lateral Skull Base Surgery, Albany Medical Center, Albany, New York, USA*

Summary

Complete cholesteatoma removal from the middle ear is a difficult task especially in the face of widespread disease. The challenge often lies in intraoperative identification of the cholesteatoma within the confined and difficult to visualize spaces of the middle ear amidst surrounding inflammatory mucosa. To this effect there can be a high rate of disease recidivism after attempted resection. Visualization of the middle ear has improved with use of endoscopes; however, residual and recurrent disease remains a concern. Cholesteatoma is an unbalanced proliferation of keratinizing squamous epithelium which leads to surrounding tissue inflammation with cytokine and enzyme activation causing local tissue destruction. This abnormal composition of epithelial differentiation and keratin debris serves as a potential target of preferential tissue staining which could lead to improved intraoperative removal of cholesteatoma. Vital dyes such as Indocyanine green and Lissamine green have been employed in other medical specialties but its application in otologic surgery has not been investigated.

This study is an *ex-vivo* study testing the feasibility of an intraoperative dye in cholesteatoma removal using Indocyanine green and Lissamine green with a secondary goal of assessing dye effects on surrounding mucosa. Results of the study showed that cholesteatoma stains with both Indocyanine green and Lissamine green. Completeness of stain was directly correlated with concentration of stain and duration of stain applied. We also observed a difference in staining between cholesteatomaand mucosal control specimens. Lower concentrations provided better differentiation between these two groups with Indocyanine green being more favorable.

Abstract

Introduction

Intraoperative removal of cholesteatoma can be challenging with a high rate of recidivism. A large component of cholesteatoma is abnormal keratin tissue and can be a potential source of staining to facilitate visualization and removal. We propose an *ex-vivo* study looking at the ability of Lissamine Green and Indocyanine Green to stain cholesteatoma and allow improved visualization and removal of diseased tissue.

Methods

Ex-vivo staining of cholesteatoma was performed using specimens from 10 patients. Specimens were stained with Lissamine Green and Indocyanine Green after intraoperative removal. Normal mucosa was used as a control. Specimens were stained using various concentrations of ophthalmic dyes with different application times. Dye concentrations of Lissamine Green were: 1%, 0.5%, 0.25%, 0.1% and 0.01%. Indocyanine Green concentrations were: 5 mg/mL and 0.5 mg/mL.Stains were applied for either 15 or 30 seconds. Pictures of pre- and post-stain specimens were obtained and analyzed for effectiveness of stain.

***Corresponding Author:** David Foyt, Albany Medical Center, 35 Hackett Blvd. 2nd Floor, Albany, NY 12208, Fax: 518-262-8091, Tel: 518-262-5575; E-mail: albanyeardoc@yahoo.com

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Results

A total of 32 stains were performed on specimens obtained from 10 patients including 23 stains performed on cholesteatoma and 9 stains performed on normal tissue. In general, we observed a higher degree of staining in the cholesteatoma specimens versus the normal tissue; however, both tissues stained with some degree by the dyes used in this study. Cholesteatoma stained with both the 0.5 mg/mL and 5 mg/mL concentrations of Indocyanine Green and adequate staining with Lissamine Green required concentrations above 0.01%. Indocyanine green had less affinity to the normal mucosa compared to Lissamine Green.

Conclusions

Indocyanine and Lissamine Green are vital dyes which show affinity for staining Keratin and could potentially be used for intraoperative identification and removal of occult cholesteatoma during otologic surgery. Indocyanine Green 5mg/ml concentration showed the most robust staining of cholesteatoma over mucosal control. Preparations are being made for *in-vivo* trials.

Key Words: Cholesteatoma; Stain; IndocyanineGreen; Lissamine-Green; Otologic Surgery

Introduction

Cholesteatoma surgery can be challenging for several different factors and is notorious for having a relatively high rate of recidivism ranging up to 50%[1-6].Common sites for recurrent or residual disease include the sinus tympani and anterior epitympanum[5,7].Various strategies have been employed to reduce the rate of residual or recurrent disease in patients undergoing cholesteatoma surgery. Techniques such as endoscopic assisted surgery have been employed to improve visualization and removal of cholesteatoma[3].While techniques have been successful in reducing the rate of residual disease there is still significant recidivism[2,8].The challenge is not only the visualization of cholesteatoma, but distinguishing it from the surrounding inflammatory tissue and normal middle ear mucosa. Optical imaging using immunofluorescence, high resolution microscopy and other methods have been explored as possible techniques for the intraoperative identification of cholesteatoma allowing more complete removal[9-12].Some notable disadvantages of these techniques are the cost and need for specialized equipment which is not widely available or affordable to most practitioners.

Dyes and stains are utilized by medical specialties such as pathology to distinguish between different tissue types allowing identification of abnormalities and aiding in diagnosis. Based on a tissue's composition it will exhibit varying amount of stain uptake. Cholesteatoma is described as an expanding lesion of stratified squamous epithelium with a large component of abnormal desquamated keratin[13-16]. Since a large component of cholesteatoma is keratin, this is a potential source for staining to facilitate visualization and removal of the diseased tissue intraoperatively.

Vital stains such as fluorescein, rose bengal, and Indocyanine green, and Lissamine green have been employed within the specialty of ophthalmology to reveal ocular abnormalities including corneal epithelial defects and keratitis[17-19].Indocyanine green is a stain with a wide range of applications including intravascular use and angiography[20,21]. The use of these agents has been shown to be safe and effective[22,23].The purpose of this study is to identify potential stains which could be used intraoperatively to facilitate identification and removal of cholesteatoma. We present an *ex-vivo* study looking at the effectiveness of cholesteatoma staining with two vital stains: Indocyanine green and Lissamine green. Concentrations and time of exposure were varied in order to obtain maximal keratin staining over background mucosal control.

Methods

Tissue specimens were removed from patients undergoing cholesteatoma surgery by a single neurotologist in an academic medical institution. Approval for this study was obtained from the Institutional Review Board at Albany Medical Center (IRB# 5025). The study was designed to include acquisition of at least 10 cholesteatoma specimens for *ex-vivo* staining using Indocyanine Green or Lissamine Green. Consent was obtained from the patient at the time of surgery. Cholesteatoma was removed from patients and set aside for routine histopathology review and an additional portion of the specimen was set aside for inclusion in the study. Presence of cholesteatoma was confirmed on pathologic examination. Normal mucosa, if removed as a necessary part of the procedure, was also obtained and used as a control specimen.

Acquired specimens were labeled as cholesteatoma or mucosa and stained in the frozen section pathology room using either Lissamine green or Indocyanine green. Staining of the specimens either occurred immediately after removal or stored and processed later. Indocyanine green was prepared using a 25 mg/vial powder mixed with 5 mL of sterile water. Specimens were tested in concentrations of either 5 mg/mL or 0.5 mg/mL.Lissamine green solution was prepared using Lissamine green ophthalmic strips and a previously documented method of acquiring 1% concentration solution[24].The 1% solution was then diluted to concentrations of 0.5%, 0.25%, 0.1% and 0.01%.

Specimens were stained with the prepared concentrations of dye solution for either 15 or 30 seconds and then rinsed of excess dye in

order to mirror actual surgical technique. Pre- and post-stain pictures were taken for gross analysis of the effectiveness of the dyes. Microscopic evaluation of the specimen was also performed. Confirmation of the diagnosis of cholesteatoma specimen was achieved through the final description provided by the pathology department on the routinely sent specimen.

Results

A total of 32 stains were performed on specimens obtained from 10 patients undergoing either a tympanomastoidectomy or tympanoplasty procedure. A majority of the specimens available for staining were cholesteatoma. Out of 32 specimens, 23 stains were performed on cholesteatoma and 9 stains performed on middle ear mucosa. All specimens marked as cholesteatomaintraoperatively were confirmed based on the final pathology report.

Both stains produced significant staining of the cholesteatoma specimens and could easily be visualized both microscopically and macroscopically. Intensity and completeness of the post-stained specimens both increased with the higher concentration of dye used and with the longer application time. Lissamine Green produced adequate staining of specimens using concentrations greater than 0.01%, and 15s was sufficient to stain the specimen (Table 1). Indocyanine green required at least 30s for substantially visible staining using the 0.5 mg/mL solution. At the standard concentration of 5 mg/mL, both 15s and 30s durations resulted in moderate staining (Table 2).

Table 1: Concentration and time variations of stains with Lissamine Green

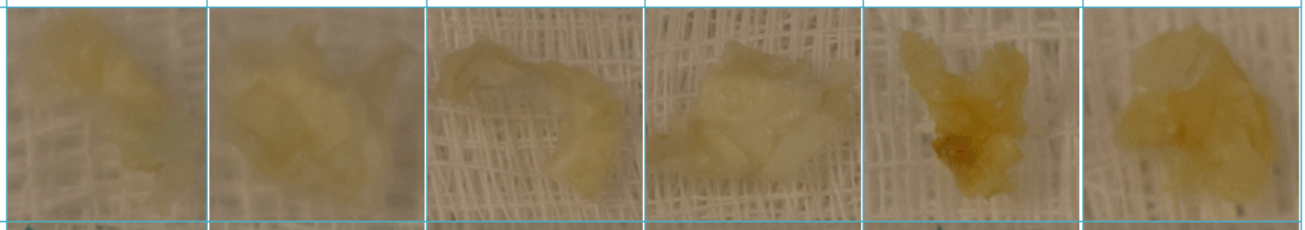
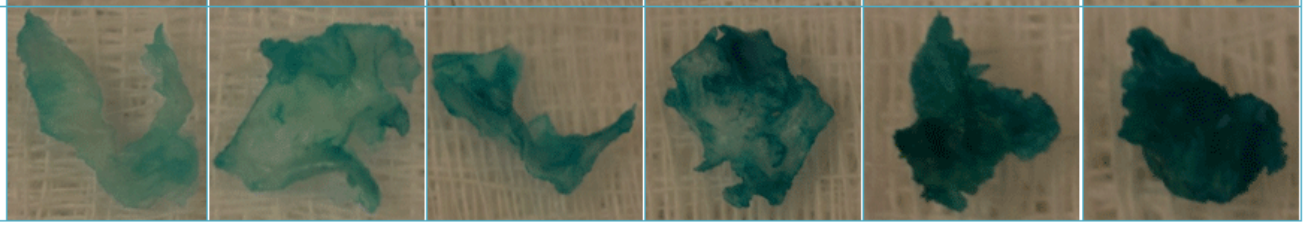
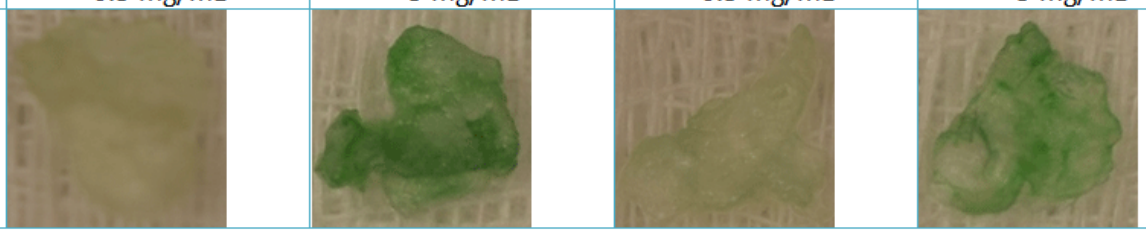
Time	15seconds				30 seconds	
Solution	0.1%	0.25%	0.5%	1%	0.5%	1%
Pre-Stain						
Post-Stain						

Table 2: Concentration and time variations of stains with Indocyanine Green

Time	15seconds		30 seconds	
Solution	0.5 mg/mL	5 mg/mL	0.5 mg/mL	5 mg/mL
Post-Stain				

While the quantity and quality of non-cholesteatoma specimens were limited, we did observe a trend toward higher degree of staining in the cholesteatoma specimens versus normal tissue; however, both tissues stained with some degree by the dyes used in this study (Tables 3 & 4). Cholesteatoma stained with both the 0.5 mg/mL and 5 mg/mL concentrations of Indocyanine Green and adequate staining was seen using Lissamine Green concentrations greater than 0.01%. Indocyanine green had less affinity to the normal mucosa compared to Lissamine Green and there was noticeable difference between cholesteatoma and mucosa. For the Lissamine Green at 0.01% concentration we did not appreciate a difference in staining.

Table 3: Indocyanine Green stains of cholesteatoma versus control

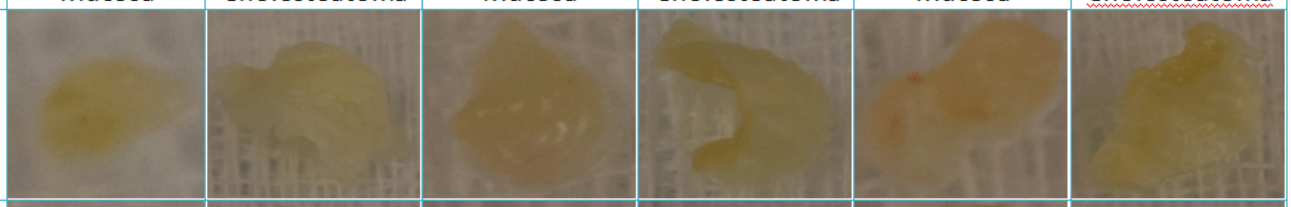
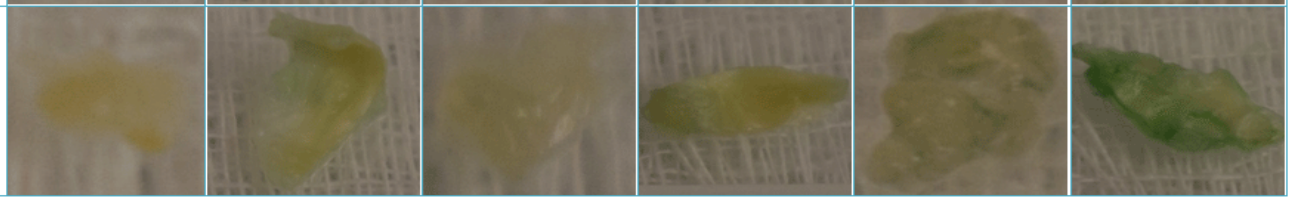
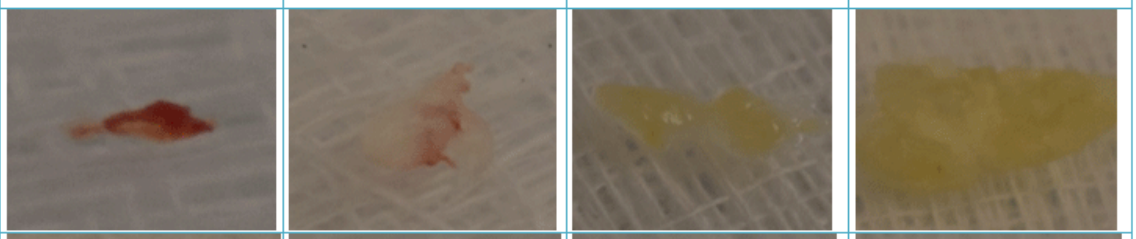
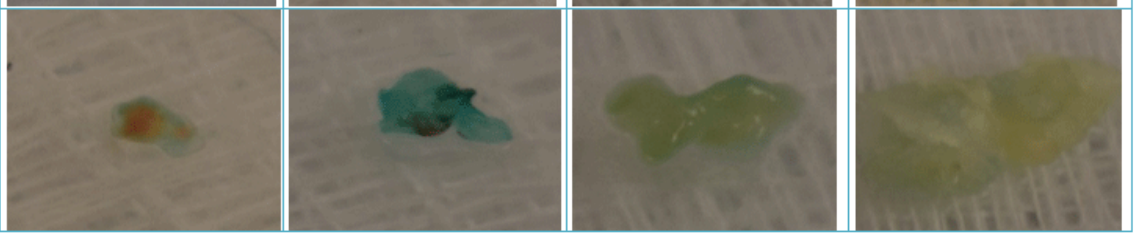
Time	15 seconds		30 seconds			
Solution	0.5 mg/mL		0.5 mg/mL		5 mg/mL	
Type	Mucosa	Cholesteatoma	Mucosa	Cholesteatoma	Mucosa	Cholesteatoma
Pre-Stain						
Post-Stain						

Table 4: Lissamine green staining of cholesteatoma and control

Solution	1%		0.01%	
Type	Mucosa	Cholesteatoma	Mucosa	Cholesteatoma
Pre-Stain				
Post-Stain				

Discussion

With the high rate of cholesteatoma recidivism despite current techniques of eradication, additional measures are needed to improve pathology identification and allow more complete removal. Methods for intraoperative identification of cholesteatoma have been investigated to help reduce the rate of residual disease and our current study illustrates the feasibility of applying a stain intraoperativelyto improve surgical resection. Other techniques appear promising but are not yet able to be used routinely in the operating room. Zou et al.[9] presented a study on coherent anti-Stokes Raman spectroscopy (CARS) microscopy as a potential method of identifying cholesteatomaintraoperatively. This relies on the resonant signal from the vibration of molecular bonds. They looked at CH2 bonds present in the accumulated lipids found in cholesteatoma and CH3 bonds found in peptides from proteins found in inflammatory tissue. They found that they were able to detect a strong distinguishable resonant signal in cholesteatoma separate from that generated in inflammatory

tissue. Based on this they suggest a CARS microendoscope could be developed to distinguish cholesteatoma from inflammatory tissue intraoperatively. While an interesting concept, CARS is not routinely accessible to the standard practitioner.

Other studies have looked at high-resolution microendoscopy (HRME) as a possible intraoperative tool. Bradley et al.[11] presented a study on the use of HRME and proflavine in an ex-vivo study evaluating the ability for otolaryngologists to classify specimens as cholesteatoma versus normal middle ear tissue. They used proflavine as a stain following cancer studies showing a high affinity to keratinized tissue which is also found in cholesteatoma. A previous study by the same group validated the use of this system[25].Presenting cholesteatoma and normal middle ear mucosa visualized with HRME, participants were able to correctly identify specimens 95% of the time with a sensitivity and specificity of 98% and 92% respectively. This method is highly sensitive and specific but also requires specialized equipment. Our study showed a potential way of recognizing cholesteatoma using easily obtained and relatively inexpensive intraoperative stains visualized with a standard operating microscope.

Immunofluorescence has also been tested as an avenue for cholesteatoma detection. A study by Takagi[12] et al. looked at galectin-7, a protein found in stratified epithelia, as possible target. They obtained cholesteatoma specimens and targeted galectin-7 with the appropriate antibody over time periods of 10, 30 and 60 minutes and then observed the specimens under a confocal laser microscope.Visualization of specimens showed fluorescence in the cholesteatoma specimen with little response in the middle ear mucosa and granulation tissue. The cholesteatoma stained at all time periods of staining with more complete staining with longer duration of stain application.Likewise, we saw a similar increase in stain completeness in our study; however, one potential benefit that we observed during our study is the ability to stain cholesteatoma in a matter of seconds as opposed to minutes to an hour thus potentially reducing patient anesthesia and operative time. Fluorescence does have promise and while Indocyanine Green does not have antibody mediated binding to cholesteatoma, it does fluoresce at near infrared wavelengths which could be a potential way to augment visualization of tissue absorbance. The disadvantage of this would be the need for an imaging source with near infrared capabilities.

There are several limitations to this study including the small sample size and limited number of control specimens. While this was a feasibility study to assess the ability to stain cholesteatoma, more robust control samples of normal middle ear mucosa are needed to identify degree of staining and titration of the stains to an optimal level. Interesting our results showed that staining with Lissamine Green at higher concentrations showed a higher uptake in the cholesteatoma but at lower concentration we saw increased uptake in the mucosal specimen. It is unclear if this phenomenon will be seen with further stains or if this was the result of the tissues themselves. It is possible that inflammatory tissue within the middle ear may react differently to staining than normal middle ear tissue. Timing of stain may have also affected stain uptake. When possible, specimens were stained on the day of extraction; however, some specimens were stored and stained at later dates. An additional limitation is that specimens may stain differently when performed *in vivo*. During the study the specimen was saturated in the stain for duration of time and exposed circumferentially to the dye. *In vivo* staining will likely only contact the superficial surface and may take up the stain differently.

Conclusions

Indocyanine Green at a concentration of 5mg/ml and thirty second tissue exposure time was ideal in identifying keratin debris in a background of uninvolved mucosa. Indocyanine Green is a potential intraoperative stain to aid the surgeon more identifying occult cholesteatoma. Indocyanine and Lissamine Green are dyes currently employed clinically with a proven safety record in neurophthalmologic surgery. While animal safety studies need to be performed, the safety profile of these dyes should be similar as has been proven for ophthalmologic applications. Use of Indocyanine Green during cholesteatoma surgery, especially in situations of severe mucosal disease may significantly reduce the incidence of recurrence.

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