Optical Clearing Agents for Optical Imaging Through Cartilage Tympanoplasties: A Preclinical Feasibility Study

*Junzhe Wang, †Gaurav Chawdhary, *‡Xiaojie Yang, §Francis Morin, ||Mamoona Khalid-Raja, *Joshua Farrell, *Dan MacDougall, ‡Fangyi Chen, ¶David P. Morris, and *¶#Robert B. A. Adamson

*School of Biomedical Engineering, Dalhousie University; †Department of Otolaryngology, Royal Hallamshire Hospital, Sheffield, U.K.; ‡Department of Biomedical Engineering, Southern University of Science and Technology, Shenzhen, Guangdong, China; §Family Medicine, McGill University, Montreal, Quebec; ||Stepping Hill Hospital, Stockport NHS Foundation Trust, Great Manchester, England, U.K.; ¶Division of Otolaryngology Head & Neck Surgery, Department of Surgery; and #Electrical and Computer Engineering Department, Dalhousie University, Halifax, Nova Scotia, Canada

Hypothesis: Optical clearing agents (OCAs) can render cartilage tympanoplasty grafts sufficiently transparent to permit visualization of middle ear structures in an operated ear using optical coherence tomography (OCT) imaging.
Methods: Pieces of human tragal cartilage were treated with glycerol, a commonly used OCA. A reference reflector was imaged with OCT through the tympanoplasty as it cleared and the optical attenuation of the graft was measured. The reversibility of clearing and the dimensional changes associated with glycerol absorption were also measured. In a separate experiment, a human cadaveric temporal bone was prepared to simulate an ossiculoplasty surgery with cartilage replacement of the tympanic membrane. A partial ossicular replacement prosthesis (PORP) inserted in the ear was imaged with OCT through a 0.4mm cartilage graft optically cleared with glycerol.
Main Outcome Measure: The optical attenuation of 0.4mm cartilage grafts decreased at 2.3+/−1.1 dB/min following treatment with glycerol, reaching a total decrease in attenuation of 13.6+/−5.9 dB after 7 minutes. The optical and dimensional effects of glycerol absorption were reversable following saline washout. In the temporal bone preparation, treatment of a cartilage graft with glycerol resulted in a 13 dB increase in signal-to-noise ratio and a 13 dB increase in contrast for visualizing the PORP through the graft with OCT.
Conclusions: Optical clearing agents offer a potential pathway towards optical coherence tomography imaging of the middle ear in post-surgical ears with cartilage grafts.
Key Words: Cartilage tympanoplasty—Middle ear—Noninvasive visualization—Optical clearing agents—Optical coherence tomography—Prosthesis migration.

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OPTICAL COHERENCE TOMOGRAPHY OF THE MIDDLE EAR

Optical coherence tomography (OCT) is a promising new modality for middle ear imaging that can visualize anatomy at higher resolution and with better soft tissue contrast than computed tomography (CT) and can measure the vibration of middle ear structures in response to sound (1). Middle ear OCT is real-time, portable, can be deployed in the clinic at point of care and does not expose patients to ionizing radiation. While still an emerging clinical technology, middle ear OCT has been successfully used to detect cholesteatomas and dimeric segments of the tympanic membrane (2), to guide middle ear surgery (3), to measure tympanic membrane thickness (4), to grade otitis media (4–7), to assess the success of tympanoplasty surgery (8), and to detect stapes fixation (9). One of the most promising applications of middle ear OCT is in postsurgical assessments of middle ear function following tympanoplasty, with or without middle ear reconstruction. In many procedures that involve tympanoplasty there is a risk of postsurgical complication or of poor outcomes. For example, in ossiculoplasty, which usually involves tympanoplasty as a final step, only 72% of patients obtain a postsurgical air-bone gap less than 20 dB (10). When hearing is assessed postoperatively, clinicians are keen to know

Address correspondence and reprint requests to Robert B. A. Adamson, Ph.D., School of Biomedical Engineering, Dalhousie University, 5981 University Avenue, PO BOX 15000, Halifax, NS B3H 4R2, Canada; E-mail: rob.adamson@dal.ca

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whether any persisting hearing loss is due to implant displacement, poor contact, or the effects of fluid or scarring. This information is useful as it impacts the likelihood of success with revision surgery (11). Computed tomography (CT) is of limited use in evaluating such cases because of its poor contrast for soft tissue and fluid and because of artefacts caused by the presence of implants in reconstructed ears.

Tympanoplasty often includes surgery to repair or replace the tympanic membrane with a graft. Tympanoplasty is a common treatment for tympanic membrane perforation and is often the final step after middle ear reconstructions requiring the replacement or reinforcement of the tympanic membrane to prevent prosthesis extrusion. Autologous cartilage is a robust and widely used material for tympanoplasty. Cartilage offers lower rates of retraction and resorption without compromising hearing outcomes as compared with more traditional graft materials like fascia (12,13). Cartilage is usually harvested from the patient's tragus or cymba concha and is cut and carved to the desired dimensions and thickness in the operating room.

In cases where tympanoplasty results are sub-optimal, optical coherence tomography's high soft tissue contrast, high resolution, and ability to detect sound-induced motion could be very helpful in investigating the issue. Unfortunately, the optical opacity of autologous graft materials, particularly cartilage, prevents us from obtaining OCT images of structures medial to the graft.

Figure 1 illustrates this problem. Images (A) and (B) show, respectively a microscope image of a normal tympanic membrane and an optical coherence tomography B-mode image of the middle ear seen through it. The malleus, incus, and cochlear promontory are all clearly visible and accessible to OCT-based vibrometric measurements to assess mobility (14). Images (C) and (D) show, respectively a microscope image of an ear with a cartilage tympanoplasty and the OCT image of the ear. In the OCT image the cartilage graft and its medial mucosalized surface can be visualized, but the opacity of the graft precludes visualization of any middle ear structures medial to the tympanic membrane due to the cartilage graft's thickness and high optical scattering cross-section.

In this study we explore a potential solution to this problem using optical clearing agents (OCAs). OCAs are a class of biocompatible chemicals which are applied to turbid tissue to reduce its optical scattering coefficient and increase its transparency (15). Most OCAs improve tissue transparency through three physical mechanisms (16). First, they cause osmotic dehydration of tissue resulting in more densely packed tissue. Second, OCAs infiltrate the interstitial space and dehydrate both the interstitial space and intracellular fluids, elevating the index of refraction of tissue and improving index matching to fibrous and lipid microstructures. Third, in collagen-containing tissues like cartilage, OCAs cause partial dissociation of collagen fibrils into microfibrils, thereby achieving reduced scattering through a smaller mean scatterer size (16). For in-vivo applications, in addition to achieving good optical clearing, OCAs must achieve high levels of biocompatibility, must be reversible and must be capable of being resorbed and excreted from the body. The most commonly-used in-vivo optical clearing agents are aqueous solutions of glycerol, polyethylene glycol (PEG), glucose, fructose and other sugars, polypropylene glycol (PPG), and acetic acid or various mixtures of these agents (17). Most in-vivo use of optical clearing agents has focused on clearing of the skin (16), although a recent study of possible relevance to the present work examined optical clearing of articular cartilage using the low-osmolality CT contrast agent iohexol (18).

For the present study we selected anhydrous glycerol for optical clearing as glycerol is a widely used component of commercially marketed eardrops for softening and dispersing cerumen (19), is used at 1,000 mg/mL concentration to treat acute otitis media (20), and is a component in nasal sprays (21). The long and widespread use of glycerol in these applications gives some confidence in glycerol's general safety and capacity to be tolerated when used in the external ear canal. To our knowledge, while optical clearing agents have previous been used for increasing the transparency of the tympanic membrane to improve infrared otoscopy (22), OCAs have not previously been used to aid middle ear OCT imaging. We envision a scenario where an optical clearing agent would be administered topically to the post-tympanoplasty tympanic membrane in clinic as an eardrop. Patients would wait for the clearing effect to occur, and OCT imaging would be conducted. Following

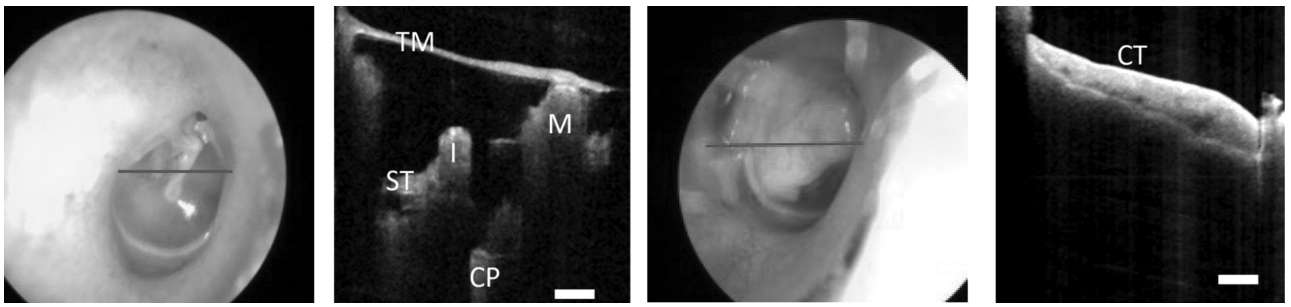

FIG. 1. From left to right, (*A*) microscopic image of a normal tympanic membrane. The blue line indicates the plane of optical coherence tomography (OCT) image (*B*) OCT image of a normal middle ear showing the tympanic membrane (TM), malleus (M), incus (I), stapedius tendon (ST), and cochlear promontory (CP) (*C*) microscopic image of a cartilage tympanoplasty, (*D*) optical coherence tomography image of a cartilage tympanoplasty (CT). Scale bars are 1 mm.

imaging, the optical clearing agent would be rinsed off or resorbed to return the tympanic membrane and graft to their original state.

In the present preclinical feasibility study, we focus on assessing the rate of clearing and the degree of clearing that can be achieved in tragal cartilage (which is used for autologous grafts in our institution) in vitro. We also focus on demonstrating the potential clinical utility of OCAs in combination with OCT imaging in a cadaveric model as a first step toward clinical studies. The experimental outcomes relevant to this assessment are the amount and rate of clearing achieved, the time needed to achieve full clearing and the improvement in OCT image quality that results from the clearing. As an initial assessment of safety of the approach, we also investigate the amount of tissue shrinkage caused by glycerol absorption (1) and the reversibility of the clearing process (23).

METHODS AND RESULTS

Tragal cartilage samples were obtained from excess material harvested from consented patients during cartilage tympanoplasty surgery (N = 5). All human tissues were collected in accordance with a protocol approved by the Nova Scotia Health Authority Research Ethics Board (REB FILE# 1022981) and with the Helsinki Declaration (JAMA 2000; 284:3043–3049). Samples were cut into 5 mm × 5 mm sections, refrigerated, and kept in a 0.9% saline until used. Subcutaneous fat and pericondrium were removed from the samples to expose only the cartilage. To measure the reversibility of glycerol absorption, N = 7 pieces of excess cartilage obtained from trimming the samples were treated with undiluted glycerol for 90 minutes and washed in saline for 10 minutes. Samples were imaged under white-light microscopy before and after treatment. ImageJ (24) was used for estimating the surface area of the samples. Following preparation, the samples were cut to 400 μm thickness using a Kurz precise cartilage knife (25).

Measurements of cartilage opacity were made using a custom swept-source OCT system designed for in vivo imaging of the human middle ear operating at a center wavelength of 1550 nm with a sweep range of 40 nm, a sweep rate of 100 kHz and a maximum output power of 9.3 mW. The system achieved a lateral resolution of 50 μm and an axial resolution in air of 40 μm and a sensitivity of 105 dB and could image over a depth range of 15 mm, allowing it to image the full depth of the middle ear in 2D (512 × 330 pixels) at 20 frames per second or 3D (512 × 512 × 330 voxels) at one volume per 5 seconds. The system also has vibrometric measurements capabilities (14), but these were not used in this study. The system is a more advanced prototype of the system described in detail in a previous publication from our group (14) with the same system architecture and similar performance.

A reference reflector consisting of a glass petri dish whose bottom was painted white was used as reference target. The reflector was imaged through the cartilage samples as they cleared. At each time point, the cartilage samples were treated topically with anhydrous glycerol and the glycerol was allowed to diffuse into the cartilage for 60 seconds. The glycerol was then wiped off the sample with a swab and an OCT B-mode scan of the sample and target was collected. This produced a set of OCT images taken at the same location at intervals of 1 minute. The process was repeated until no further significant increase in transparency was observed (Fig. 2).

Figure 3A shows a typical cartilage graft lying on a printed paper sheet before and after an immersion in glycerol for 6 minutes. The increased transparency of the cartilage and the increased contrast of the text behind it is readily apparent in the photograph. Figure 3B shows a sequence of OCT B-mode images of the reference target taken at 1-minute intervals in the clearing process for one of the samples. In each OCT B-mode image, the white arrow indicates the location of the graft where both the proximal and distal sides of the cartilage graft can be easily identified. The orange arrow points out the top glass surface of the petri dish and the red arrow shows the painted bottom surface of petri dish. The brightness of the target (indicated by the red arrow) progressively increases as the cartilage clears over time. Figure 3C shows the mean image reflectance in the region of interest (ROI) indicated by the red box in the B-mode images at 1-minute intervals. The red dashed line indicates the baseline ROI mean intensity when the target is imaged directly with no cartilage between it and the OCT optics. The purple dashed line indicates the ROI mean intensity observed before glycerol is applied to the cartilage sample.

Following treatment with glycerol, the intensity of the ROI increased roughly linearly at 2.3 (±1.1) dB/min and reached saturation in less than 7 minutes, with a mean reflectance increase of 13.6 (±5.9 dB). Following clearing, the samples were bathed in saline for 10 minutes and the reflectance remeasured and found to be 0.52 (±0.50 dB) consistent with a return to baseline transparency with no significant difference in mean intensity before treatment and after washout ($p > 0.5$). This demonstrates that the optical clearing caused by glycerol can be reversed.

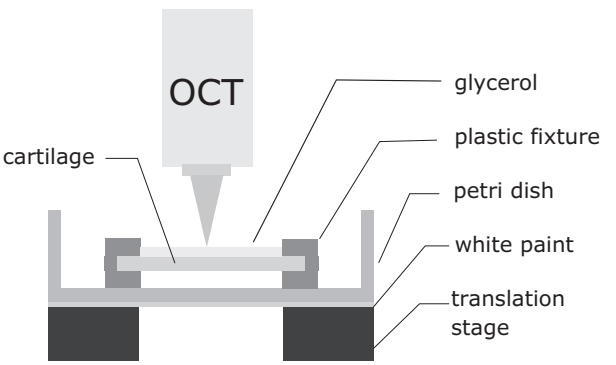

FIG. 2. Experimental setup for measuring the reflectance of a reference reflector through cartilage samples.

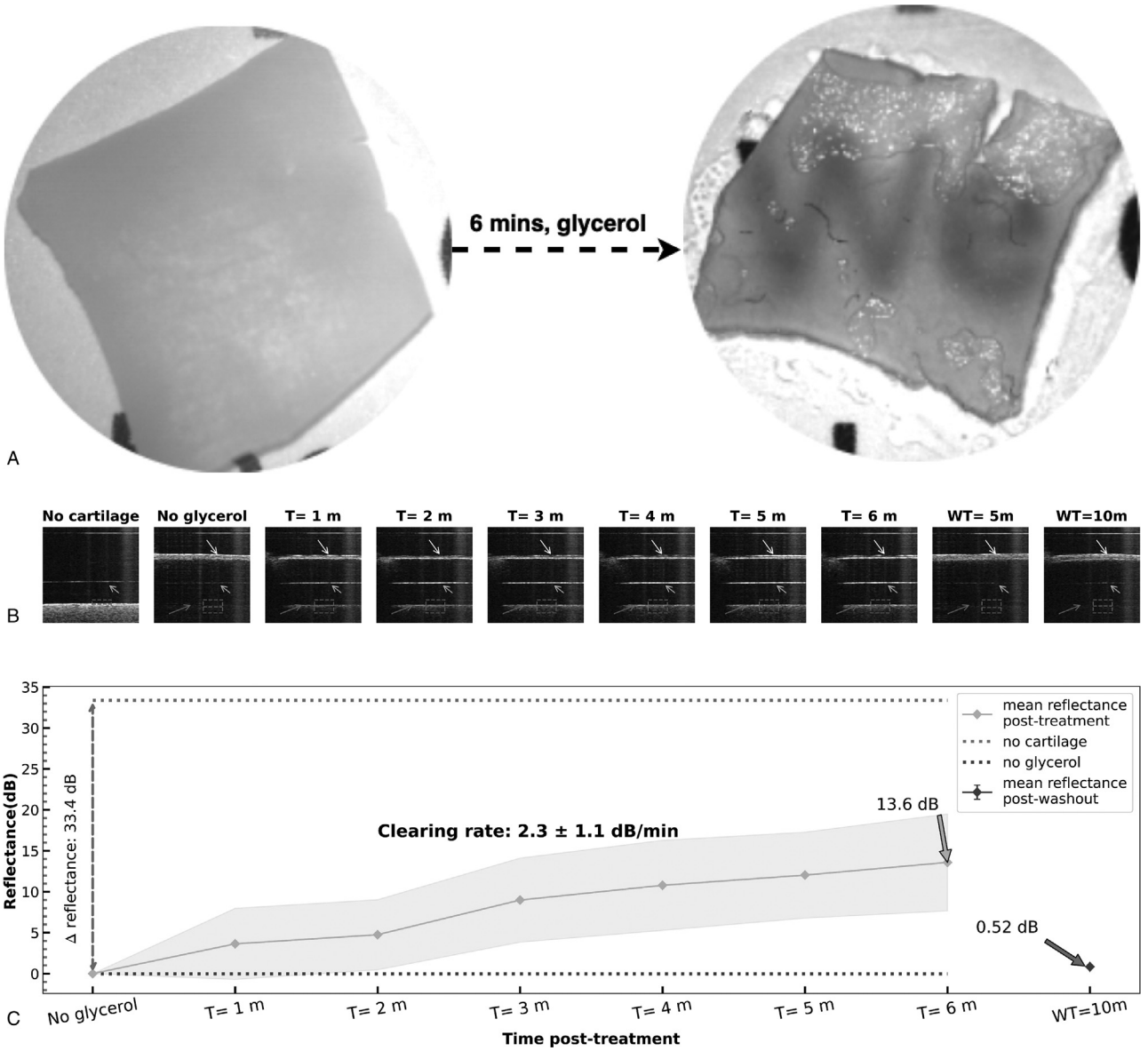

FIG. 3. (*A*) Photograph of cartilage graft pre- and post-optical clearing with glycerol. (*B*) A series of OCT B-mode images of cartilage with a white paint reference reflector distal to it taken at 1 minute intervals during optical clearing and at 5 minute intervals following washout in saline. The white, orange, and red arrows indicate the proximal side of the graft, the distal side of the graft, and the reference reflector. The red box indicates an ROI in the reference reflector and a dotted red line traces out the top surface of the petri dish (*C*) plot of reference reflector reflectance during clearing. The red dotted line indicates the reflectance observed in the absence of the graft, and the purple dotted line indicates the baseline reflectance before glycerol treatment. The solid purple line indicates the reflectance after washout.

Figure 4 shows the results of investigation into glycerol-induced shrinkage of the cartilage samples. N = 7 small cartilage samples trimmed from the cartilage harvested from patients were immersed in undiluted glycerol for 90 minutes to achieve full glycerol absorption. A mean reduction in sample area of 31 (±6)% was observed under optical microscopy, presumably due to hyperosmotic dehydration. Following immersion in a saline bath for 10 minutes, the samples returned to 102 (±2)% of the original area indicating that the dimensional changes associated with optical clearing are also reversible.

Figure 5 shows the result of a preclinical validation of the use of optical clearing for postoperative visualization of the middle ear in a cadaveric temporal bone model. A cadaveric temporal bone was prepared by removing the pinna and cartilaginous ear canal to improve access. The incudostapedial joint was disarticulated and the tympanic membrane and incus were removed through the canal. A partial ossicular replacement prosthesis (PORP) was placed on the stapes superstructure and covered with a tragal cartilage graft harvested from the same cadaveric temporal bone, prepared in the same way as in the in vitro

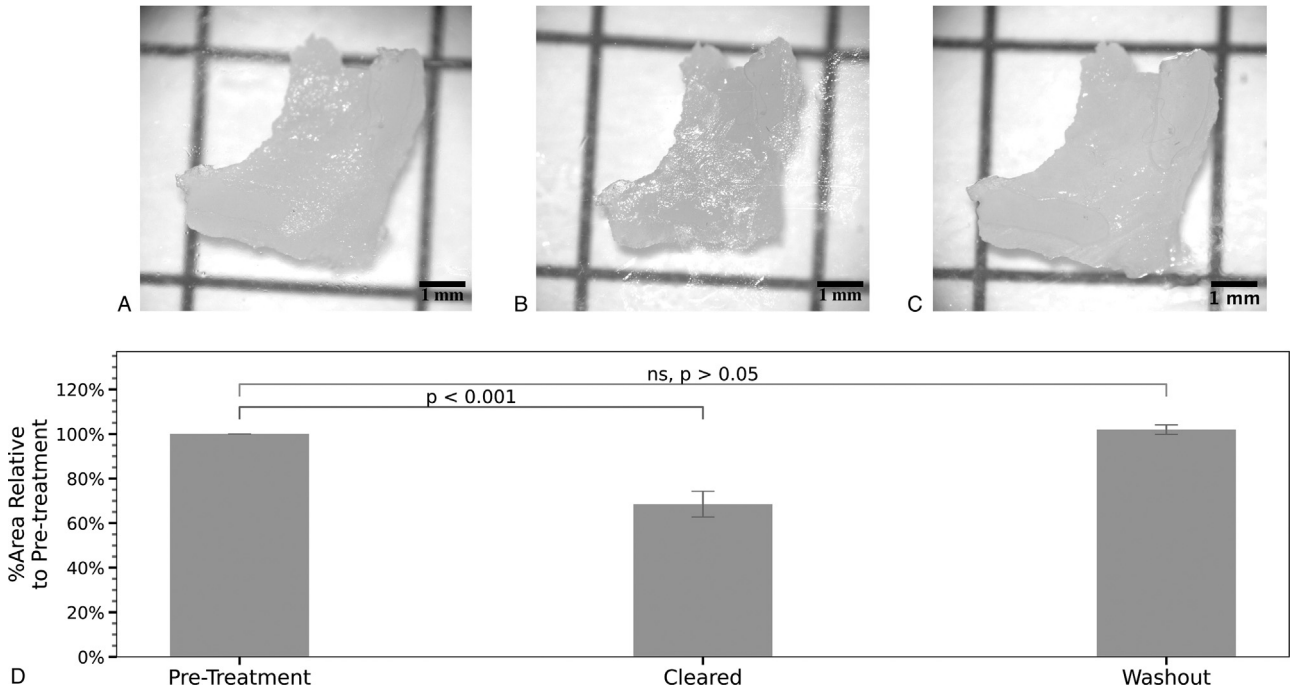

FIG. 4. Photograph of cartilage graft overlaying a 5 × 5 mm grid reference sheet. (*A*) Cartilage graft before treatment with glycerol (*B*) after treatment with glycerol with reduced area (*C*) after saline washout for 10 minutes (*D*) surface area changes for (*A*), (*B*), and (*C*) for n = 7 samples. NS denotes nonsignificant for a two-tailed *t* test.

experiment and cut to fit the dimensions of the tympanic annulus. 3D OCT images, 2D OCT images, and microscopic photos of the middle ear were obtained through the untreated cartilage. In the 2D OCT image, two ROIs were selected in the PORP (solid red boxes), and in the empty background region (solid green box).

Signal to noise ratio (SNR) and contrast between the PORP and background were used to assess the improvement of visibility in the simulated ossiculoplasty model. SNR is defined as

$$\mathrm{SNR} = 10\log_{10}\frac{\mu_s}{\sigma_b}$$

where μ_s is the mean intensity in the PORP (signal) ROI and σ_b is the standard deviation of the intensity in the background ROI. Contrast is defined as

$$C = 10\,\log_{10}\frac{\mu_s}{\mu_b}$$

where μ_b is the mean intensity in the background region. Both SNR and contrast are expressed in decibels.

After obtaining baseline images with uncleared cartilage (Fig. 5A, D, G), the cartilage was removed and placed in a glycerol bath for 10 minutes to achieve full optical clearing, wiped clean of glycerol with a swab and placed onto the annulus. Figure 5H shows the PORP and handle of the malleus with the cartilage removed. In the OCT images of Figure 5A and D, before glycerol treatment, the PORP (indicated with a yellow arrow) is barely visible in the 2D and 3D OCT images. Following treatment, the PORP with its distinctive circular plate with holes is readily visible. The improvement in SNR and contrast between images (D) pretreatment and (E) posttreatment were 13 and 13 dB, respectively. To demonstrate that clinically significant changes could be observed with this technique, the shaft of PORP was detached from the stapes footplate to simulate the postoperative dislocation of the PORP as seen in Figure 5I. The middle ear was again reimaged through the cleared cartilage graft using OCT from the ear canal. In both 2D and 3D OCT images, the PORP is no longer visible at its original location (indicated by the dashed red ellipse for 2D and box in 3D images).

DISCUSSION

While optical clearing of skin has been extensively studied, to our knowledge there has only been one previous study examining optical clearing of cartilage. In this study, a 0.90 mm thick sample of bovine articular cartilage was cleared with iohexol and the OCT loss slope was found to decrease by 30 dB/mm within 50 minutes after treatment (18), this degree of clearing is consistent with the clearing observed in this study of 34 dB/mm following 7 minutes of clearing in a 0.40 mm sample, given the difference in clearing agent and tissue type. Other studies also confirmed the similar improvement in contrast with various combination of tissues and OCAs (1,26).

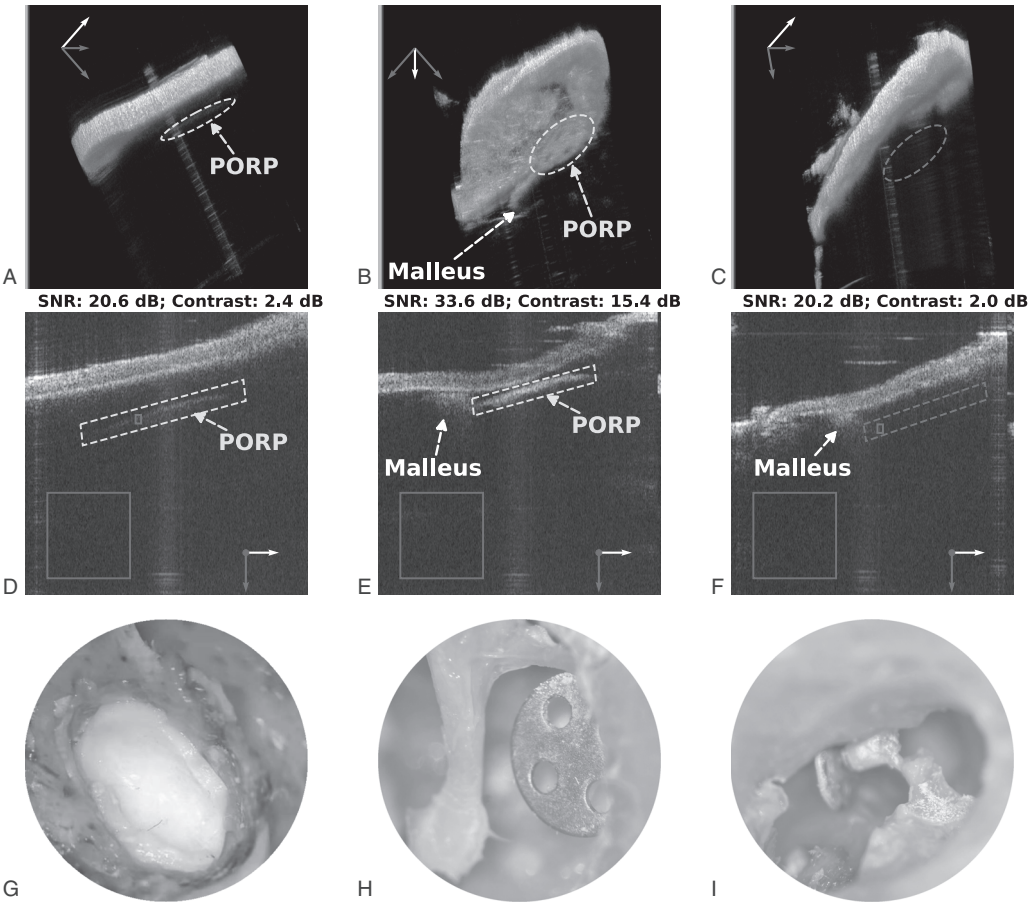

FIG. 5. Imaging of a simulated ossiculoplasty model. (*A*), (*D*), and (*G*) 3D OCT image, B-mode image and microscopic image of the untreated cartilage graft overlaying the PORP; (*B*), (*E*), and (*H*) 3D OCT image and B-mode image of middle ear through cleared cartilage and transcanal microscopic photo with cartilage graft removed to show orientation of PORP; (*C*), (*F*), and (*I*) 3D OCT image and B-mode image through cleared cartilage graft of the middle ear after PORP was deliberately dislodged and otoscopic photo of the middle ear taken through a posterior tympanotomy showing the dislodged PORP. The PORP was not visible in transcanal microscopy after it was dislodged. The 3D images have been oriented so as to highlight the location of the PORP with a different orientation used in each image. A set of cartesian axes are provided to show the orientation. The red arrow points laterally in the direction of the ear canal, the white arrow points superiorly and the green arrow points anteriorly. The yellow ellipse/box in (*A*), (*B*)/(*D*), (*E*) highlight the location of the PORP whereas the red ellipse/box in (*C*)/(*F*) indicates the original position of the PORP before its simulated dislodging in 2D and 3D images. PORP indicates partial ossicular replacement prosthesis.

In a laser Doppler vibrometry study examining the effect of varying cartilage tympanoplasty thickness on acoustic vibration, it was found that thinner tympanoplasties exhibit improved low-frequency sound transmission performance down to 400 μm thickness (27). In practice, the improved acoustic performance from thinner grafts must be weighed against the increased difficulty in handling them during surgery due to the tendency of thin grafts to curl, but 400 μm grafts are generally manageable. The present study offers a second reason to use thinner grafts, namely, to enable improved postsurgical follow-up through OCT-based visualization of the middle ear.

The degree to which cartilage is thinned and whether perichondrium is preserved on one, both or neither surface of the graft is a matter of personal choice and varies between surgeons. Some surgeons augment the lateral surface of their cartilage reconstruction with fascia to facilitate the re-epithelialization of the lateral surface of the graft from the tympanic membrane remnant or deep canal skin. The medial aspect of the cartilage graft usually becomes mucosalized with time.

The present study does not account for the interposition of these additional layers in the reconstruction. As the epithelial covering lateral to an established cartilage repair and the medial mucosal layer are much thinner than the graft itself, it seems likely that they would have little direct optical effect. However, the lateral epithelium may prevent the diffusion of the OCA into the cartilage from the ear canal thereby slowing the optical clearing effects. In a clinical scenario, we imagine an optical clearing agent being applied to the tympanic membrane of a patient followed by a 20 to 30 minutes wait for the clearing to take effect. Whether this is feasible can only be established in an in vivo model in which the graft is epithelialized.

Three important issues relevant to the safety of using OCAs are graft shrinkage, ototoxicity, and reversibility. In our experiments we observed 31 (±6)% areal tissue shrinkage corresponding to a 14% change in linear dimensions. While it is reassuring to see that the cartilage shrinkage seen after treatment with the OCA is reversible, shrinkage can be expected to create stress on the surrounding tympanic membrane tissue that may have safety implications. Given the natural compliance of cartilage and the elasticity of any residual rim of native tympanic membrane we do not anticipate that the observed degree of shrinkage would cause any permanent damage to the tympanic membrane repair. However, careful in vivo studies on well-established, robust, in situ, cartilage tympanoplasty grafts are required in animals or humans to verify this point.

Should shrinkage prove to present risk to patients, OCAs with lower osmolality than glycerol are available that may exhibit lower shrinkage, although the ototoxicity of any candidate OCAs would have be closely evaluated (28). Glycerol can also be diluted with water to decreases its ability to dehydrate and shrink tissue, but dilution also decreases its effectiveness as an optical clearing agent (28).

While we are not aware of any formal studies of the ototoxicity of glycerol, its widespread use in eardrops (19) and nasal sprays argue that it is safe to use in the outer and middle ear. Glycerol is produced endogenously by sebaceous glands and is a naturally occurring component in blood plasma where it plays a role in maintaining epithelial hydration levels (29). As a result, the body is capable of transporting and metabolizing glycerol, although the local uptake rate and mechanisms for glycerol in the tympanic membrane specifically has not previously been investigated. On this basis it appears likely that glycerol used in optical clearing of cartilage grafts would be resorbed naturally by the body, although it is not clear how long this process would take. In our study, the effects of glycerol clearing on the optical and mechanical properties of cartilage grafts appear to be fully reversible following rinsing with saline which provides reason to expect that glycerol absorption would be reversible in vivo.

While this study has focused on the visualization of the middle ear through autologous cartilage tympanoplasties, many other graft materials are available and have been used in this application. Autologous graft materials used for tympanoplasty grafts include fascia, perichondrium, periosteum, vein, fat, and skin (30) with cartilage and fascia being the most common in modern practice. Additionally, new graft biomaterials for tympanic membrane grafts remains an active area of research, with grafts having been demonstrated made from polylactic acid (31), polydimethylsiloxane (31), polycaprolactone (31), and decellularized porcine tissue (32) among other materials (33). The optical clearing techniques we demonstrated in this study for cartilage may be applied to these other materials as well, and some of these materials may have sufficient innate transparency as to permit acceptable middle ear imaging without the need for optical clearing. As middle ear OCT gains adoption, optical transparency (whether innate or induced with OCAs) may become an increasingly important consideration in the selection of graft materials alongside traditional considerations of acoustic performance, biocompatibility, and mechanical strength.

Finally, while the present study focused on the ability of optical clearing agents to improve visualization with optical coherence tomography, our results apply equally well to other optical imaging modalities including traditional optical modalities like optical microscopy/otoscopy and more advanced methods like Raman spectroscopy. And while this study focused on the clearing of tympanoplasties, the same techniques could potentially be used to clear thickened, scarred, calcified or fibrotic tympanic membranes which can also make visualization of the middle ear space difficult.

CONCLUSION

We have measured the optical attenuation of a cartilage tympanoplasty model using optical coherence tomography before and after application of glycerol as an optical clearing agent. We observed a mean reduction in attenuation in cartilage grafts of 13.6 (±5.9) dB at an estimated linear rate of clearing of 2.3 (±1.1) dB/min following topical application of glycerol. We also showed that this degree of clearing was clinically significant for imaging of post-ossiculoplasty ears and that it generated SNR and contrast improvements of 13 and 13 dB in imaging a prosthesis. The process of glycerol absorption appears reversible, at least in terms of the optical clearing and the dimensional changes that it generates. The amount of clearing achieved, and the rate of clearing appear consistent with the clinical application of this technique to facilitate visualization in postoperative ears and provide strong motivation for the extension of this work to in vivo studies in patients.

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