Published in final edited form as:

Hear Res. 2017 July ; 350: 22–31. doi:10.1016/j.heares.2017.04.003.

Factors Affecting Sound Energy Absorbance in Acute Otitis Media Model of Chinchilla

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Abstract

Acute otitis media (AOM) is a rapid-onset infection of the middle ear which results in middle ear pressure (MEP), middle ear effusion (MEE), and structural changes in middle ear tissues. Previous studies from our laboratory have identified that MEP, MEE, and middle ear structural changes are three factors affecting tympanic membrane (TM) mobility and hearing levels (Guan et al. 2014; 2013). Sound energy reflectance or absorbance (EA) is a diagnostic tool increasingly used in clinical settings for the identification of middle ear diseases. However, it is unclear whether EA can differentiate these three factors in an AOM ear. Here we report wideband EA measurements in the AOM model of chinchilla at three experimental stages: unopened, pressure released, and effusion removed. These correspond to the combined and individual effects of the three factors on sound energy transmission. AOM was produced by transbullar injection of *Haemophilus influenzae* in two treatment groups: 4 days (4D) and 8 days (8D) post inoculation. These time points represent the relatively early and later phase of AOM. In each group of chinchillas, EA at 250–8000 Hz was measured using a wideband tympanometer at three experimental stages. Results show that the effects of MEP, MEE, and tissue structural changes over the frequency range varied with the disease time course. MEP was the primary contributor to reduction of EA in 4D AOM ears and had a smaller effect in 8D ears. MEE reduced the EA at 6–8 kHz in 4D ears and 2–8 kHz in 8D ears and was responsible for the EA peak in both 4D and 8D ears. The residual EA loss due to structural changes was observed over the frequency range in 8D ears and only at high frequencies in 4D ears. The EA measurements were also compared with the published TM mobility loss in chinchilla AOM ears.

Keywords

wideband tympanometry; acute otitis media; energy absorbance; power reflectance; *Haemophilus influenzae*

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INTRODUCTION

Wideband sound energy absorbance (EA) describes the sound power transmitted into the middle ear over a broad frequency range (Feeney et al. 2003; Keefe et al. 2012). EA is sensitive to middle ear diseases, and the patterns of EA or energy reflectance ($ER=1-EA$) have been characterized for various pathological conditions. These include tympanosclerosis (Rosowski et al. 2012), ossicular discontinuity (Feeney et al. 2003; 2009; Nakajima et al. 2012; Voss et al. 2012), and otosclerosis (Allen et al. 2005; Shahnaz 2009A; 2009B; Sanford et al. 2012; Nakajima et al. 2012; Voss et al. 2012).

Otitis media, defined as inflammation of the middle ear, is the most common disease in young children (Gould et al. 2010; Hoberman et al. 2011). Two major types of otitis media occur: otitis media with effusion (OME), and acute otitis media (AOM). In OME, middle ear effusion (MEE) accumulates in the ear cavity in the absence of signs of infection (Bluestone and Klein 1983; Kurs-Lasky et al. 2011). EA associated with OME are characterized by low absorbance amplitudes at both low and middle frequencies, and by a single peak at high frequencies ($f > 2$ kHz), though sharpness and value of the high-frequency peak varies considerably (Piskorski et al. 1999; Allen et al. 2005; Beers et al. 2010; Feeney et al. 2003; Ellison et al. 2012; Hunter and Margolis 1997). The mechanism of such changes was further illustrated by Voss et al. (2012). In contrast, AOM arises from rapid infection onset within the middle ear. Such infections are accompanied by MEE production, middle ear pressure (MEP) reduction, and structural changes in middle ear tissues (Guan and Gan 2013; Guan et al. 2014). While EA in OME has been well studied, the effects of AOM on EA remain unclear.

The mechanoacoustic properties of the middle ear that accompany AOM differ from those occurring in OME. Bacterial infection can produce distinctive changes in the middle ear including profound structural alterations of the tympanic membrane (TM) (Guan et al. 2015; Grote and van Blitterswijk 1984; Larsson et al. 2003; Magnuson and Hellstrom 1994; Schmidt and Hellstrom 1993), mucosal thickening (Lim and Klainer 1971; Fulghum et al. 1982), and ossicular adhesions (Guan et al. 2014; von Unge et al. 1997; Caye-Thomasen and Tos 2000). In guinea pig and chinchilla models of AOM, we identified that MEP, MEE, and middle ear structural changes are three factors contributing to the loss of umbo mobility and hearing (Guan et al. 2015; Guan et al. 2014; Guan and Gan 2013). However, it remains unclear whether sound ER or EA, an increasingly used clinical tool for diagnosis of middle ear diseases (Allen et al. 2016; Pitaro et al. 2016; Hunter et al. 2010), can differentiate these three factors in infected ears from patients with AOM.

In this paper, we measured wideband EA in the AOM model of chinchilla. Here we report EA instead of ER values since sound absorption is more directly relevant to hearing. EA in the chinchilla ear with AOM was measured sequentially at three stages: the unopened ear, the pressure-released ear, and the ear after effusion removal. This approach allowed us to assess both the combined and individual effects of these three factors on sound energy absorption, and permitted us to investigate whether MEP, MEE, and structural changes induced EA changes in the ear with AOM could be differentiated in the same ear specimen. Two AOM disease time courses, 4 and 8 days post inoculation with *Haemophilus influenzae*

(Guan et al. 2014), were established in chinchillas. EA was measured across these two disease courses at three experimental stages and the measurements were compared with the published TM mobility loss in chinchilla AOM ears.

METHODS

A. Animal preparation

Fifteen adult chinchillas (*Chinchilla lanigera*) weighing between 600–780 g were included in this study. These animals were purchased from Moulton Chinchilla Ranch (Rochester, MN). The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma and met the guideline of the National Institutes of Health. All animals were free from middle ear disease (as evaluated by otoscopic examination) at the beginning of the study.

The animals were divided into uninfected controls (n=6) and two AOM groups, one evaluated at 4 days (4D) post bacterial challenge (n=5) and the other evaluated at 8 days (8D) post challenge (n=4). AOM was produced by transbullar injection of a *H. influenzae* 86–028NP suspension into both ears following the procedure described by Morton et al. (2012). Under general anesthesia [ketamine (10 mg/kg) plus xylazine (2 mg/kg)], 0.3 ml bacterial suspension containing 3000 colony-forming units (CFU) was injected into the superior bulla bilaterally using a 1 cc syringe equipped with a 26 gauge needle. Control animals were untreated.

On the 4th or 8th day post-inoculation, animals were anesthetized with a mixture of ketamine (10 mg/kg) plus xylazine (2 mg/kg). Additional anesthesia was administered as needed to maintain areflexia. To expose the entrance of the ear canal, the pinna and the skin covering the ear canal were removed surgically. Then the TM was examined microscopically to identify signs of AOM such as inflammation or MEE. In each animal, the experiment was conducted bilaterally. Body temperature of both control and AOM animals was maintained throughout the experiment at ~38° C by placing the animal in a prone position on a thermo-regulated surgical heating blanket.

B. EA measurements

EA was measured with a wideband tympanometer (Model AT235h, Interacoustic, MN) with ReFlwin PC software. The measurement probe with commercial tips (outer diameter of 8 mm) was pressed to the bony rim of the entrance of the ear canal and held by hand to achieve a pressure seal. Using click stimuli, the EA was measured at 60 frequencies between 0.25 and 8 kHz while the ear canal air pressure was swept from –300 to +200 daPa in the descending direction. EA values at ambient pressure were extracted from these measurements at 0 daPa. Instead of simply measuring EA at ambient pressure in the ear canal, this type of EA tympanometry measurement was performed to estimate the MEP of both the control and the AOM ears. The surface area of the probe within the ear canal was used for calculation of EA (Keefe et al. 1993). In this study, the diameter at the entrance of the bony part of chinchilla ear canal was determined to be 4–6 mm. The system was calibrated with a set of two rigidly terminated tubes with an inner diameter of 4.5 mm.

EA measurements in ears with AOM were performed in three experimental stages: OM-1, unopened bulla containing the MEP and MEE; OM-2, pressure released from the middle ear; and OM-3, effusion removed from the middle ear. At each experimental stage, the EA measurement was conducted bilaterally in a given animal.

After completion of the EA measurements in stage OM-1, further surgery was performed following the procedure by Guan et al. (2014). Briefly, the skin of the superior temporal bone was removed to expose the middle ear bony wall on top of the temporal bone. Then, a 1 mm diameter hole was drilled into the roof of the middle ear to release the middle ear pressure. After sealing the hole with dental cement (PD-135, Pac-Dent, CA), the OM-2 test was performed.

Upon completing stage OM-2 EA measurements, the hole on top of the temporal bone was re-opened and enlarged with a drill to a diameter of 3–4 mm. Assisted by microscopic visualization, a silicone tube then was inserted to the bottom of the middle ear cavity through this hole. The middle ear effusion was then aspirated manually from the cavity with a 1 ml syringe. The aspiration process was repeated as necessary until no additional fluid could be drained from the tympanic cavity. The total effusion volume obtained from each ear was recorded. Ossicular adhesions were frequently found on the malleus head and between the manubrium and the cochlear promontory when the top cavity was opened for stage OM-3. These ossicular adhesions were not disturbed during the aspiration of the effusion. Thereafter, the opening on top of the temporal bone was covered by a thin glass sheet, and sealed with dental cement. Then, measurement of EA was conducted (stage OM-3). After measurements were completed at the three OM stages, the bulla was harvested. To confirm total MEE removal from the middle ear, another hole, diameter ~4 mm, was opened in the posterior area from the medial side to allow microscopic examination of the middle ear.

Control ears were prepared in the same manner as described above for ears with AOM. To exclude the effect of middle ear pressure in anesthetized animals (Guinan and Peake 1967), a 1 mm diameter hole was drilled through the top of the upper cavity of the bulla to release any preexisting pressure. EA measurements then were performed.

RESULTS

A. Wideband EA tympanogram

Wideband EA tympanograms were plotted with EA as a function of ear-canal pressure and frequency. Because the focus of the present study is to assess the effects of AOM on EA-frequency relationship at ambient pressure, we only show the representative three-dimensional EA tympanograms and two-dimensional EA-pressure curves measured from the control and unopened AOM ears (stage OM-1) in Figs. 1 and 2.

Figure 1A shows a typical three-dimensional EA tympanogram from an uninfected control chinchilla ear. At the lowest evaluated frequency (250 Hz), a single peak was observed at approximately ambient ear canal pressure ($p=0$ daPa). The magnitude and width of the peak gradually increased as the frequency increased to 2 kHz. A “ridge” was observed near ambient pressure at low-to-mid frequencies. At higher frequencies, EA exhibited an M-like

shape with the valley of the M at ~50 daPa. As frequency increased, EA increased in two pressure regions, -300 to -100 daPa and 100 to 200 daPa.

Figure 1B shows a typical EA tympanogram from an unopened (i.e. stage OM-1) 4D AOM ear with 0.4 ml MEE. The EA maximum was near -200 daPa at low-to-mid sound frequencies. The peak was more rounded than that in the control ear. EA in the region of positive pressure began to increase at 1 kHz. At higher frequencies ($f > 2$ kHz), EA peaked at two pressures: -200 and +200 daPa.

A typical EA tympanogram from an unopened 8D ear containing an effusion volume of 0.9 ml is shown in Fig. 1C. A small rounded peak was observed near -200 daPa at low frequencies ($f < 0.5$ kHz). EA at -200 daPa was maximal at 1 kHz, and the local EA peaks were distributed mainly in the negative pressure range across the frequencies tested. Overall EA in ears on day 8 of infection was lower than that observed in ears of 4 days duration.

B. EA-pressure curve

To better describe EA variation with ear-canal pressure at different frequencies, the EA-pressure curves were extracted at 0.5, 1, and 4 kHz, representing a low, middle, and high frequency, respectively, from the non-infected control, 4D, and 8D tympanograms in Fig. 1. Figure 2A shows the EA-pressure curve at three frequencies for the control ear. A single peak was observed near the ambient pressure (0 daPa) at both 0.5 and 1 kHz similar to conventional tympanograms at 226 Hz and 1 kHz. Maximum EA was reached at -25 daPa. The reason why the EA peaked at a pressure slightly lower than the ambient is addressed in the discussion section. At 4 kHz the EA-pressure curve exhibited two peaks, one at 0 and a second at 170 daPa.

Figure 2B displays the EA-pressure curves of the 4D ear at stage OM-1. In both the 0.5 and 1 kHz curves, a rounded peak was observed at -220 daPa, and EA was generally flat at positive pressures. At 4 kHz the EA value reached local peaks in the region > 100 daPa and < -200 daPa.

Figure 2C shows the EA-pressure curves of the unopened 8D ear. The EA curve at 500 Hz was almost flat with a shallow peak near -200 daPa. Absorbance at 1 kHz was greater than that at 500 Hz or 4 kHz. A large peak was observed at -150 daPa. The EA curve at 4 kHz exhibited an “U” shape, and the absorbance between -150 to 50 daPa was close to zero.

C. MEP, MEE, and ossicular adhesions in AOM ears

The MEP in unopened AOM ears (stage OM-1) was read at the pressure where the EA reached its peak in EA-pressure curve at 500 Hz. The MEP of each 4D AOM ear was negative and had a mean value of -184 daPa (± 40 daPa, standard deviation, SD). In the 8D AOM group, three ears exhibited a flat EA-pressure curve, and the MEP could not be identified. The MEP of the remaining ears ($n=5$) in this group were all negative (mean value -145 ± 65 daPa).

The MEE volume in 4D AOM ears ranged from 0.3–0.6 ml and had a mean value of 0.4 ml (± 0.13 ml, SD) when the MEE was aspirated for experimental stage OM-3. The effusion

filled ~half of the tympanic cavity in these ears. MEE volume in 8D ears ranged from 0.7–0.95 ml with an average value of 0.8 ml (± 0.14 ml). MEE filled almost the entire tympanic cavity in these ears. The MEP level and volume of MEE in these two groups of infected animals did not differ significantly from the values we reported in a previous study using the same species. (Guan et al. 2014).

It should be noted that ossicular adhesions are a pathological finding commonly occurring in chinchilla ears with AOM. After the MEE was aspirated (stage OM-3), ossicular adhesions were observed in all of the infected animals, but none of the uninfected controls used in this study.

D. Ambient wideband EA in 4D AOM ears

Figure 3 shows the individual curves (dashed lines) of ambient EA over frequencies of 0.25–8 kHz measured from 10 ears after establishment of AOM for 4 days and compares these outcomes to those of 12 control (uninfected) ears. Shaded areas represent ± 1 SD around the mean values. In each AOM ear, absorbance was measured at three experimental stages: OM-1, OM-2, and OM-3 as shown in Figure 3.

In stage OM-1 (Fig. 3A), the middle ear was unopened, and EA was affected by the combination of the MEP, MEE, and structural changes in the middle ear. EA was generally flat with some fluctuation at low frequencies, peaked between 2 to 4 kHz, and decreased at higher frequencies ($f > 4$ kHz). EA in AOM with 4 days infection resembled the pattern usually observed in human OME ears (Beers et al. 2010; Ellison et al. 2012).

In stage OM-2 (Fig. 3B), EA gradually increased over the 0.25–2 kHz range and then decreased at higher frequencies. Large individual variation was observed at low frequencies due to a local EA peak between 500 to 800 Hz that occurred in four ears.

In stage OM-3 (Fig. 3C), the effusion was removed but the ossicular adhesions remained unaltered. Inter-individual variations of EA curves were reduced compared with those in OM-2. EA increased at low frequencies, maintained its maximal value at 1–3 kHz with a small drop near 2.2 kHz, and decreased at frequencies greater than 3 kHz. As can be seen in Fig. 3C, there are no local peaks at low frequencies after the effusion was removed. Figure 3D displays the ambient EA curves determined from 12 control ears. Absorbance continually increased from 250 Hz to 1 kHz, was flat between 1.5 and 4 kHz (with a notch at 2.5 kHz), and then decreased at higher frequencies.

The mean EA-frequency curves from uninfected control ears and three OM stages in Fig. 3 were extracted and displayed with error bars (± 1 SD) in Figure 4. The statistical results (P -values) for the EA data in Fig. 4 are listed in Table 1. Repeated-Measures ANOVA and Tukey post-hoc tests were used to compare the three OM stages since EA were measured from the same population. An unpaired t-test was used to compare OM-3 values with control ears because the data came from different populations. Statistical analyses were performed with Prism software (Graphpad, La Jolla, CA). The P -values of post-hoc tests were reported as inequalities by the software.

As shown in Figure 4 and Table 1, significant changes in absorbance occurred among the three OM stages (Table 1, column 2). The EA of OM-3 was significantly greater than that of OM-1 over frequencies of 250 Hz to 8 kHz except over the range of 4–6 kHz (column 3 of Table 1). After pressure in the middle ear was released (OM-2), the absorbance increased significantly at low frequencies (0.25–2 kHz, see column 4 of Table 1). As the effusion was removed from the cavity (OM-3), EA increased significantly compared to OM-2 at approximately 1 kHz and at 6–8 kHz (column 5 of Table 1). When the OM-3 values were compared with those of control ears, a significant difference at frequencies > 1 kHz was identified (column 6 of Table 1).

E. Ambient wideband EA in 8D AOM ears

Figures 5A–C show the individual curves (dashed lines) of ambient EA over frequencies from 0.25 to 8 kHz measured in eight AOM ears with 8 days of infection at our three standard experimental stages. Curves for control (non-infected) ears are shown in Fig. 5D and are the same as those in Fig. 3D. In stage OM-1 (Fig. 5A), the absorbance was generally < 0.2 at frequencies below 700 Hz. Absorbance peaked at 1–3 kHz but quickly dropped to its lowest value at 4–5 kHz. Thereafter, absorbance increased again at higher frequencies. Each individual curve exhibited a narrow peak between 1 and 3 kHz. The exact location of the peak varied between ears. As a consequence, the inter-individual variation of EA was large at 1–3 kHz.

In the OM-2 preparations, large EA variations between individuals were observed at low-to-mid frequencies (Fig. 5B). Six of eight ears exhibited a single low-frequency peak at 0.25–1 kHz. The location of the peak varied between individuals. The width of the peak was broader than those observed in stage OM-1. EA declined to a minimum at 3–4 kHz and increased slightly at higher frequencies in most of 8D ears. In stage OM-3 (Fig. 5C), individual variation was observed at low frequencies, and EA curves showed the same pattern over the frequency range. EA increased at low frequencies, became maximal between 1–2 kHz, and decreased at higher frequencies. The individual differences that we noted may reflect different levels, or time courses, of ossicular and tissue structural changes occurring between these ears during 8 days of infection.

Mean values for the EA curves from uninfected controls and the three OM stages of 8D ears are shown in Fig. 6. Statistical analyses of these data are summarized in Table 2. Figure 6 and Table 2 indicate that significant changes in EA occurred in the three stages of AOM (column 2 of Table 2) except at 250 Hz. EA in 8D ears increased within a narrow range of low frequencies after pressure was released (column 4, Table 2). In contrast, the release of MEP significantly increased the EA over 0.25 to 2 kHz in 4D ears (column 4, Table 1). Once the effusion was removed, the EA in 8D ears increased significantly at frequencies greater than 1 kHz (column 5, Table 2). In these infected ears, EA of OM-3 was significantly different from controls over most frequencies except 1 kHz (column 6, Table 2).

F. Comparison of 4D AOM and 8D AOM in each experimental stage

To investigate whether EA can identify AOM progression, a statistical comparison (Unpaired t-test) of ambient EA was made between infected ears at 4 and 8 days after infection (Table 3).

In the unopened ear (OM-1), there was no significant difference between the 4D and 8D ears except near 4 kHz (column 2, Table 3). For OM-2 (after release of MEP), EA in 4D ears differed significantly at 2–4 kHz from that in 8D ears. At those two stages, EA was differentiable between 4D and 8D only at high frequencies. This finding suggests that the effective mass of the middle ear was different between the two post-infection time periods. This should relate to the increase of MEE from the shorter to the longer duration of AOM since the mass of the MEE contributed to the effective mass of the middle ear.

In OM-3 (i.e. upon removal of the MEE), there was a significant difference in response between the 4D and 8D ears over the entire tested frequency range except at 2 and 8 kHz. Such changes in EA suggest that the infection-induced structural changes of the middle ear progressed markedly from 4 to 8 days post challenge with *H. influenzae*.

DISCUSSION

A. Effect of ear-canal pressure sweep on estimation of MEP and ambient EA

The pressure in the middle ear of the control or AOM ear was taken as the pressure value where EA reached a peak in the EA-pressure curve extracted at 500 Hz. This estimation could be different from the true MEP because where the EA peaks in a pressure sweep is affected by the direction of the sweep. Liu et al. (2008) reported that in human adults with normal hearing the EA peak shifted positively with ascending sweeps and negatively with descending sweeps. In this study, a descending sweep was performed in the measurement. This may explain why EA in the control (uninfected) chinchilla ears reached maximum at a pressure slightly lower than 0 daPa (Fig. 2A). In the AOM ears, the estimated MEP may also be lower than their true value.

Another set of data influenced by the ear-canal pressure sweep was the ambient EA extracted at 0 daPa. Because the sweep can shift the overall three-dimensional EA tympanogram towards the negative-pressure side, EA at 0 daPa may differ from the EA measured at ambient pressure. Nonetheless, the comparison in the control and AOM ears is valid because EA data of all the tested ears were obtained using the same measurement conditions.

B. Cause of MEP in AOM ears

It has been reported that negative MEP can be produced in normal ears of anesthetized animals because opening of the Eustachian tube is absent after administration of anesthesia (Guinan and Peake 1967). The mucosal layer in the middle ear absorbs gas, which results in negative MEP in the cavity when there is no ventilation (Bluestone and Doyle, 1988). Gas absorption rate is affected by the pressure gradient between the air in the middle ear and mucosal blood-tissue gases. Once the MEP is almost in equilibrium with the pressure of

mucosal gases, MEP would not change significantly because the pressure gradient is relatively small as is the gas absorption rate (Cantekin et al. 1980). The process to reach the equilibrium is relatively fast: it takes a few hours for MEP to reach a stationary value when ventilation of the middle ear is absent (Cantekin et al. 1980).

Such pressure equilibria were expected to have been established in our experimental AOM ears before anesthesia. Therefore, anesthesia should not contribute to the MEP in AOM ears. In the AOM model, the middle ear fluid was detectable as early as day 2 post-infection (Morton et al. 2004). On day 4 and 8, a substantial amount of fluid had accumulated in the middle ear. This finding suggests that the Eustachian tube had been blocked for a significant period due to infection. From this viewpoint, the pressure equilibrium already should have become established, and MEP in the AOM ears should be constant. Additionally, anesthesia did not change the status of the Eustachian tube in ears with AOM. Therefore, infection was likely to be the only contributor to MEP in these AOM ears.

C. Comparison with published data

Very few studies have reported EA or ER measurements in chinchillas. Margolis et al. (2001) reported the ER of the normal and the obstructed Eustachian tube in the chinchilla ear with varying sound frequency and ear canal pressure. Reflectance tympanograms (reflectance-pressure curve with a fixed frequency) and ambient ER (reflectance-frequency curve at ambient pressure) were described. These investigators also reported the ER in neonatal and adult chinchillas (Hsu et al. 2001). Akinpelu et al. (2015) compared ambient ER of normal chinchillas with those injected with middle ear fluid. In the present study, the pattern of our EA tympanograms in uninfected normal ears is consistent with those reported by Margolis et al. (2001) and Hsu et al. (2001). The EA tympanogram in the normal chinchilla ear exhibited an inverted “V” shape at low frequencies (< 2 kHz) and a “M” shape at higher frequencies (Fig. 1A and 2A). However, there are differences in the baseline measurements of ambient ER or EA between our present study and previous studies. To establish a better comparison, we extracted and converted ER into the ambient EA of normal ears from the data of Margolis et al. (2001) and Akinpelu et al. (2015). Then we plotted these data together with our control EA values. The outcome of this comparison is shown in Fig. 7. The greatest discrepancy occurs at low frequencies. The ambient EA of normal chinchillas reported by Margolis et al. was ~ 0 (i.e. 1 for ER) at frequencies below 1 kHz. In the publication by Akinpelu et al. (2015), the EA of normal chinchillas ranged from 0.2–0.4 (0.6–0.8 for ER) at $f < 1$ kHz. In our present study, the ambient EA of control ears at low frequencies ranged from 0.4–0.8, much larger values than those in the other studies. At higher frequencies, the EAs in the two previous studies agree in general with each other and were lower than our measurements by about ~ 0.2 .

One possible cause of this discrepancy is that the cross-sectional area of the ear canal used to calculate EA or ER in this study differed from that used in the previous studies. Calculation of EA depends on cross-sectional area of the ear canal. The area of the ear-canal at the measurement location should be used in the calculation (Keefe et al. 1993). In our current study, EA was measured at the entrance of the osseous portion of the ear canal. This opening technically had an oval shape but with only small differences between the long and short

axis. Therefore, it was estimated as a circular area. The diameter varied from 4 to 6 mm across the ears. A constant area with a diameter of 4.5 mm was used to calculate EA in wideband tympanometry. The difference between the area used for calculation and the actual area would produce error in the calculation of EA, which can contribute to the inter-ear variation. For example, use of larger values of ear-canal area decreases EA at low frequencies (Voss et al. 2008). The area used to calculate the ER in Akinpelu et al. (2015) was not reported. A constant area with diameter of 8 mm was used by Margolis et al. (2001). An acoustically estimated ear-canal area was used by Hsu et al. (2001). Voss et al. (2008) reported that the acoustically measured ear-canal area was usually greater than the physical measured ear-canal area. The relatively large area used for the calculation in the earlier publications may contribute to their smaller EA at low frequencies, although the area difference alone may not account for that much difference in EA between the present study and their studies.

Another factor more likely to reduce the ambient EA in the normal ear is MEP. Anesthesia can cause negative pressure in the middle ear (Guinan and Peake 1967). In chinchilla ears without MEP, the EA-pressure curve at low frequencies peaks at ambient pressure (0 daPa) and decreases rapidly in the region adjacent to ambient pressure (see Fig. 2A). When MEP is present, the shape of the EA-pressure curve would maintain, but the peak shifts away from zero pressure. The low frequency EA-pressure curve is steep near its peak. A small shift towards left or right due to MEP would result in a substantial decrease of EA measured at ambient pressure. To clarify the effect of MEP on EA, we conducted a pilot study in which we measured EA in anesthetized chinchillas with normal ears without releasing MEP before the measurement. A typical result of such pilot experiments is shown in Fig. 8. The top panel shows the EA tympanogram at 500 Hz. The lower panel shows the EA-frequency curves extracted at ambient and -75 daPa. Relevant findings from other published studies are included in this panel for comparison purposes. In Fig. 8A the EA tympanogram indicates that in this uninfected chinchilla ear there was a negative pressure in the middle ear. The shape of EA-pressure curve shifted towards the negative side, and EA peaked at -75 daPa. As a result, the ambient EA (dotted line in Fig. 8B) started with a low value (~0.2) at low frequencies, which was consistent with that reported by Akinpelu et al. (grey line in Fig. 8B). In contrast, the EA extracted at -75 daPa (black line in Fig. 8B) was much higher than that at ambient in this ear, but similar to those ambient EAs measured upon release of MEP in normal ears (see control curves in Fig. 3).

In the present study, the pre-existing MEP in control ears was eliminated before measurements were made. The admittance tympanograms of normal chinchillas reported by Margolis et al. (Fig. 14 in their paper) and Hsu et al. (Fig. 2 in their paper) suggested that the MEP existed in some animals in their studies. In the study by Akinpelu et al. (2015), a distortion product otoacoustic emission measurement, which was time-consuming, preceded the ER measurement. Negative MEP could be produced during the otoacoustic emission measurement and affect the control ER results. In sum, anesthesia-induced MEP may occur depending on the experimental conditions. If so, it can contribute primarily to lowering ambient EA (producing higher ER) in previously published studies of the normal chinchilla ear.

D. Factors affecting EA at ambient pressure in AOM ears

In our study, EA at ambient pressure in chinchilla AOM ears was investigated at early (4D) and later (8D) periods of AOM. The effects of middle ear pressure, effusion, and structural changes on EA at these two AOM time points following bacterial challenge were evaluated by comparing their EA-frequency curves at an OM stage with respect to the previous stage (Figs. 3 and 5) together with their statistical analysis (Tables 1 and 2). Three factors - MEP, MEE, and middle ear tissue structural changes - affected ambient EA at the early infection in a manner different from that at the longer infection.

D1. Effect of MEP on ambient EA—In unopened AOM ears (OM-1), the EA of 4D and 8D ears was generally flat at low frequencies and reached a maximum value abruptly at mid-high frequencies. This pattern is consistent with the ER results observed in patients with OME by Beers et al. (2010) and Feeney et al. (2003). After MEP was released, EA substantially increased at low frequencies in both 4 day- and 8 day-infected ears. Stiffness of the TM dominated ambient EA at low frequencies as reported by Voss et al. (2012). Thus, release of MEP reduced TM stiffness and increased absorbance at low-frequencies.

The increase of ambient EA upon releasing MEP in 4D ears was greater than that in 8D ears (compare blue line and red line in Figures 4 and 6). This observation is consistent with our previous study of umbo mobility loss in the chinchilla AOM model which indicated that the increase of umbo displacement in 4D ears was more prominent than that in 8D ears when the pressure was released (Guan et al. 2014). The difference in the level of MEE between 4D and 8D ears results in a significant difference in middle ear airspace remaining in those infected ears. The tympanic cavity of 4D ears was nearly half-filled with effusion while that of 8D ears was almost fully filled. Thus, the effect of MEP on EA in the early phase of AOM is greater than that in the later phase, and MEP was the primary contributor to reduction of EA at low frequencies in AOM ears on day 4.

D2. Effect of MEE on ambient EA—Upon removal of the effusion, EA increased at high frequencies (> 2 kHz) in both 4D and 8D ears due to a reduction of TM mass. This finding is similar to the changes of umbo displacement curve from stage OM-2 to OM-3 observed by Guan et al. (2014). However, at low frequencies the effect of MEE on EA was not the same as that on umbo movement. MEE resulted in a single EA peak between 250 and 1000 Hz in some 4D ears and in most 8D ears (Figs. 3B and 5B, respectively). No such well defined peak occurred at low frequencies in the umbo displacement curves (Guan et al. 2014).

Voss et al. (2012) reported that when over half of the cavity in human temporal bone was filled with fluid, ER increased (i.e. EA decreased) substantially at high frequencies. In addition, a low-frequency notch in the ER curve was identified (Fig. 6 in their paper). Akinpelu et al. (2015) reported that when amniotic fluid was injected into the chinchilla ear, ER increased (i.e. EA decreased) mainly at high frequencies and was little-changed at low frequencies. Our findings in the present study are more consistent with the findings reported by Voss et al.

The EA peak located between 250 and 1000 Hz in stage OM-2 (Figs. 3B and 5B) may be caused by the resonant vibration of the whole TM when a large fluid volume is present in

the middle ear. It seems possible that when the mechanical stiffness of the TM was reduced by release of MEP, the resonance frequency of TM vibration decreased, and the narrow EA peak at the mid-high frequencies in stage OM-1 shifted to the low frequencies in OM-2. In each AOM group, the location of the peak varied across ears, causing individual variation in EA curves in OM-2. After the effusion was removed, the low-frequency peak disappeared in both 4D and 8D ears (see Figures 3B, 3C, 5B and 5C). The inter-ear variation observed in OM-2 may relate to the different amounts of middle ear fluid accumulated in individual ears. In summary, MEE contributed to reduction of EA at high frequencies and was responsible for the single EA peak in 4D and 8D ears.

D3. Effect of structural changes on ambient EA—After the pressure and effusion were removed from the middle ear (stage OM-3), residual reduction of ambient EA was observed at high frequencies in 4D ears and over all the frequencies in 8D ears when compared with EA curves of uninfected control ears (green line with squares vs black line in Figures 4 and 6). The residual reduction of EA suggests that the infection-induced structural changes, including ossicular adhesions and mechanical property changes of middle ear soft tissues, also contributed to the changes of EA. The ossicular adhesions on the malleus head, between the manubrium and the cochlear promontory, and around the stapes in the round window niche, were found in both 4D and 8D ears in our present and previous studies (Guan et al., 2014) and are an important factor in the reduction of umbo movement.

The effect of middle ear structural changes on EA has not been reported previously in the literature. Margolis et al. (2001) reported the results of multi-frequency tympanograms obtained from one chinchilla ear with stapes adhesion, but the EA or ER of that ear was not shown in their paper. Shahnaz et al. (2009A) showed that there was an increase of ER (decrease of EA) below 1 kHz in patients with confirmed otosclerosis. However, there was a considerable overlap between the range of ER for the patients and that for normal ears. Voss et al. (2012) reported the ER in five cadaveric ears with stapes fixed by dental cement. Their results showed that in three of five ears the stapes fixation led to small increases of ER at low frequencies, and in the other two ears the ER was not affected much by stapes fixation (Fig. 7 in their paper). Based on those published data, the effect of ossicular fixation on EA or ER seems to be relatively small compared with its effect on the umbo movement as measured in our previous study (Guan et al. 2014).

If we assume that EA was minimally affected by adhesion or fixation of the ossicular chain in the chinchilla AOM ear, the structural changes of middle ear tissues, particularly changes of the TM, are likely to be the main cause for the residual reduction of EA in stage OM-3. Mechanical properties of the TM are believed to be crucial to sound energy absorbance since EA measures the mechanoacoustic response of the entire TM. TM properties are dependent on membrane micro-structure. In the gerbil ear, AOM markedly altered TM structural and mechanical properties (von Unge et al. 1997; Larsson et al. 2003). Our histologic study on the chinchilla model indicated that the TM of 4D AOM was thicker than that of the uninfected control animals, and that the thickening occurred mainly in the outer epithelial layer (Guan et al. 2015). Persistence of the middle ear infection to 8D led to more prominent thickening of the TM due to the edema and infiltration of inflammatory cells into the outer epithelium and inner mucosa (Guan et al. 2015). Those morphological changes suggest that

the mechanical stiffness and mass of TM may differ between the uninfected control, 4D, and 8D ears. The residual loss of EA in 4D ears occurred at frequencies at or above 3 kHz, while the residual reduction of EA in 8D ears was observed over all the frequencies. This difference may relate to different degrees of TM structural changes between early and later phases of AOM. Such TM microstructure variation might be the primary contributor to the residual loss of EA in AOM ears.

E. Future studies

The present study focused on the EA changes over the frequency range induced by MEP, MEE, and structural changes in AOM ears. However, the EA-pressure curves were not fully investigated. The EA tympanograms extracted at single frequencies displayed in Fig. 2 are similar to the traditional middle ear recordings from animals and clinical patients. The EA-pressure curves at low frequencies in both 4D and 8D reflect the type B or C tympanograms consistent with results from chinchilla AOM models measured by traditional low-frequency tympanometry (Doyle et al. 1982; Hoa et al. 2009). But the EA-pressure curves at high frequencies in 8D ears were substantially different from those in 4D ears due to longer infection period. Thus, wideband EA tympanograms may offer more insight into the physiology of AOM than conventional tympanometry. The EA-pressure relationship requires further examination to assess its practical applicability and clinic relevance. In addition, to fully understand the reflectance or absorbance measurements associated with AOM, the phase data require analysis in future studies.

CONCLUSION

The effects of MEP, MEE, and middle ear structural changes on ambient EA in the chinchilla AOM ear were examined over the course of the disease (4 days and 8 days post challenge with the bacterium *H. influenzae*). Results show that the MEP was the primary contributor to EA reduction in 4D AOM ears and had smaller effects on EA in 8D ears. MEE contributed to reduction of EA at high frequencies and was responsible for the narrow EA peak in both 4D and 8D ears. After release of MEP and removal of MEE, there was residual EA loss over all frequencies in 8D ears and only at high frequencies in 4D ears, which was probably associated with TM structural changes. This study characterizes the roles of MEP, MEE, middle ear structural changes in the reduction of EA in chinchilla AOM ears.

Acknowledgments

This work was supported by NIH grant R01DC011585.

List of Abbreviation

AOM	acute otitis media
CHL	conductive hearing loss
EA	energy absorbance
ER	energy reflectance

LDV	laser Doppler vibrometry
MEE	middle ear effusion
MEP	middle ear pressure
OME	otitis media with effusion
SPL	sound pressure level
TM	tympanic membrane

References

- Allen JB, Jeng PS, Levitt H. Evaluation of human middle ear function via an acoustic power assessment. *J Rehabil Res Dev*. 2005; 42(4 Suppl 2):63–78. [PubMed: 16470465]
- Allen, JB., Robinson, SR., Miller, JAL., Jeng, PS., Levitt, H. Middle-ear reflectance: concepts and clinical applications. In: Cacace, AT.de Kleine, E.Holt, AG., van Dijk, P., editors. *Scientific foundations of audiology: perspectives from physics, biology, modeling, and medicine*. Plural Publishing, Inc.; San Diego, CA: 2016. p. 1-41.
- Akinpelu OV, Funnell WR, Daniel SJ. Detection of otoacoustic emissions in chinchilla when the middle ear contains amniotic fluid. *Laryngoscope*. 2015; 125(4):138–42.
- Beers AN, Shahnaz N, Westerberg BD, Kozak FK. Wideband reflectance in normal Caucasian and Chinese school-aged children and in children with otitis media with effusion. *Ear Hear*. 2010; 31(2): 221–33. [PubMed: 19858721]
- Bluestone, CD., Klein, JO. Otitis media with effusion, atelectasis, and Eustachian tube dysfunction. In: Bluestone, CD., Stool, SE., editors. *Pediatric Otolaryngology*. W.B. Saunders Company; Philadelphia, PA: 1983. p. 419-432.
- Bluestone CD, Doyle WJ. Anatomy and physiology of eustachian tube and middle ear related to otitis media. *J Allergy Clin Immunol*. 1988; 81(5 Pt 2):997–1003. [PubMed: 3286738]
- Cantekin EI, Doyle WJ, Phillips DC, Bluestone CD. Gas absorption in the middle ear. *Ann Otol Rhinol Laryngol Suppl*. 1980; 89(3 Pt 2):71–75. [PubMed: 6778356]
- Caye-Thomasen P, Tos M. Polyp and fibrous adhesion formation in acute otitis media caused by non-typeable or type b *Haemophilus influenzae* or *Moraxella catarrhalis*. *Acta Otolaryngol*. 2000; 120(7):810–814. [PubMed: 11132712]
- Doyle WJ, Supance JS, Marshak G, Cantekin EI, Bluestone CD, Rohn DD. An animal model of acute otitis media consequent to beta-lactamase-producing nontypable *Haemophilus influenzae*. *Otolaryngol Head Neck Surg*. 1982; 90(6):831–6. [PubMed: 10994438]
- Ellison JC, Gorga M, Cohn E, Fitzpatrick D, Sanford CA, et al. Wideband acoustic transfer functions predict middle-ear effusion. *Laryngoscope*. 2012; 122(4):887–94. [PubMed: 22374909]
- Feeney MP, Grant IL, Marryott LP. Wideband energy reflectance measurements in adults with middle-ear disorders. *J Speech Lang Hear Res*. 2003; 46(4):901–11. [PubMed: 12959468]
- Feeney MP, Grant IL, Mills DM. Wideband energy reflectance measurements of ossicular chain discontinuity and repair in human temporal bone. *Ear Hear*. 2009; 30(4):391–400. [PubMed: 19424071]
- Fulghum RS, Brinn JE, Smith AM, Daniel HJ, Loesche PJ. Experimental otitis media in gerbils and chinchillas with *Streptococcus pneumoniae*, *Haemophilus influenzae*, and other aerobic and anaerobic bacteria. *Infect Immun*. 1982; 36(2):802–10. [PubMed: 6979517]
- Grote JJ, van Blitterswijk CA. Acute otitis media: An animal experimental study. *Acta Otolaryngol*. 1984; 98:239–49. [PubMed: 6496060]
- Guan X, Jiang S, Seale TW, Hitt BM, Gan RZ. Morphological changes in the tympanic membrane associated with *Haemophilus influenzae*-induced acute otitis media in the chinchilla. *Int J Pediatr Otorhinolaryngol*. 2015; 79(9):1462–71. [PubMed: 26183006]

- Guan X, Chen Y, Gan RZ. Factors affecting loss of tympanic membrane mobility in acute otitis media model of chinchilla. *Hear Res.* 2014; 309:136–46. [PubMed: 24406734]
- Guan X, Gan RZ. Mechanisms of tympanic membrane and incus mobility loss in acute otitis media model of guinea pig. *J Assoc Res Otolaryngol.* 2013; 14:295–307. [PubMed: 23483330]
- Guinan JJ Jr, Peake WT. Middle-ear characteristics of anesthetized cats. *J Acoust Soc Am.* 1967; 41(5):1237–61. [PubMed: 6074788]
- Gould JM, Matz PS. Otitis media. *Pediatr Rev.* 2010; 31(3):102–116. [PubMed: 20194902]
- Hoa M, Syamal M, Sachdeva L, Berk R, Coticchia J. Demonstration of nasopharyngeal and middle ear mucosal biofilms in an animal model of acute otitis media. *Ann Otol Rhinol Laryngol.* 2009; 118(4):292–298. [PubMed: 19462851]
- Hoberman A, Paradise JL, Rockette HE, Shaikh N, Wald ER, Kearney DH, Colborn DK, Kurs-Lasky M, Bhatnagar S, Haralam MA, Zoffel LM, Jenkins C, Pope MA, Balentine TL, Barbadora KA. Treatment of acute otitis media in children under 2 years of age. *N Engl J Med.* 2011; 364(2):105–115. [PubMed: 21226576]
- Hsu RW, Margolis RH, Schachern PA, Javel E. The development of the middle ear in neonatal chinchillas II. Two weeks to adulthood. *Acta Otolaryngol.* 2001; 121(6):679–88. [PubMed: 11678166]
- Hunter LL, Margolis RH. Effects of tympanic membrane abnormalities on auditory function. *J Am Acad Audiol.* 1997; 8(6):431–46. [PubMed: 9433689]
- Hunter LL, Feeney MP, Miller JA, Jeng PS, Bohning S. Wideband reflectance in newborns: Normative regions and relationship to hearing screening results. *Ear Hear.* 2010; 31(5):599–610. [PubMed: 20520553]
- Keefe DH, Bulen JC, Arehart KH, Burns EM. Ear-canal impedance and reflection coefficient in human infants and adults. *J Acoust Soc Am.* 1993; 94(5):2617–38. [PubMed: 8270739]
- Keefe DH, Sanford CA, Ellison JC, Fitzpatrick DF, Gorga MP. Wideband aural acoustic absorbance predicts conductive hearing loss in children. *Int J Audiol.* 2012; 51(12):880–91. [PubMed: 23072655]
- Larsson C, Dirckx JJ, Decraemer WF, Bagger-Sjoberg D, von Unge M. Pars flaccida displacement pattern in purulent otitis media in the gerbil. *Otol Neurotol.* 2003; 24:358–64. [PubMed: 12806284]
- Lim DJ, Klainer A. Cellular reactions in acute otitis media—scanning and transmission electron microscopy. *Laryngoscope.* 1971; 81(11):1772–86. [PubMed: 5117718]
- Liu Y, Sanford CA, Ellison JC, Fitzpatrick DF, Gorga MP, Keefe DH. Wideband absorbance tympanometry using pressure sweeps: System development and results on adults with normal hearing. *J Acoust Soc Am.* 2008; 124(6):3708–3719. [PubMed: 19206798]
- Magnuson K, Hellstrom S. Early structural changes in the rat tympanic membrane during pneumococcal otitis media. *Eur Arch Otorhinolaryngol.* 1994; 251:393–8. [PubMed: 7857626]
- Margolis RH, Paul S, Saly GL, Schachern PA, Keefe DH. Wideband reflectance tympanometry in chinchillas and human. *J Acoust Soc Am.* 2001; 110(3 Pt 1):1453–64. [PubMed: 11572356]
- Morton DJ, Bakaletz LO, Jurcisek JA, VanWagoner TM, Seale TW, Whitby PW, Stull TL. Reduced severity of middle ear infection caused by nontypeable *Haemophilus influenzae* lacking the hemoglobin/hemoglobin-haptoglobin binding proteins (Hgp) in a chinchilla model of otitis media. *Microb Pathog.* 2004; 36:25–33. [PubMed: 14643637]
- Morton DJ, Hempel RJ, Seale TW, Whitby PW, Stull TL. A functional tonB gene is required for both virulence and competitive fitness in a chinchilla model of *Haemophilus influenzae* otitis media. *BMC research notes.* 2012; 5:327. [PubMed: 22731867]
- Nakajima HH, Pisano DV, Roosli C, Hamade MA, Merchant GR, et al. Comparison of ear-canal reflectance and umbo velocity in patients with conductive hearing loss: a preliminary study. *Ear Hear.* 2012; 33(1):35–43. [PubMed: 21857516]
- Piskorski P, Keefe DH, Simmons JL, Gorga JL. Prediction of conductive hearing loss based on acoustic ear-canal response using a multivariate clinical decision theory. *J Acoust Soc Am.* 1999; 105(3):1749–64. [PubMed: 10089599]

- Pitaro J, Al Masaoudi L, Motallebzadeh H, Funnell WRJ, Daniel SJ. Wideband reflectance measurements in newborns: Relationship to otoscopic findings. *Int J Pediatr Otorhinolaryngol*. 2016; 86:156–160. [PubMed: 27260599]
- Rosowski JJ, Nakajima HH, Hamade MA, Mahfoud L, Merchant GR, et al. Ear-canal reflectance, umbo velocity, and tympanometry in normal-hearing adults. *Ear Hear*. 2012; 33(1):19–34. [PubMed: 21857517]
- Sanford CA, Schooling T, Frymark T. Determining the presence or absence of middle ear disorders: an evidence-based systematic review on the diagnostic accuracy of selected assessment instruments. *Am J Audiol*. 2012; 21(2):251–68. [PubMed: 22585938]
- Schmidt SH, Hellstrom S. Phenol anesthesia of the tympanic membrane in purulent otitis media: a structural analysis in the rat. *Eur Arch Otorhinolaryngol*. 1993; 249:470–2. [PubMed: 8442942]
- Shahnaz N, Bork K, Polka L, Longridge N, Bell D, et al. Energy reflectance and tympanometry in normal and otosclerotic ears. *Ear Hear*. 2009; 30(2):219–33. [PubMed: 19194289]
- Shahnaz N, Longridge N, Bell D. Wideband energy reflectance patterns in preoperative and post-operative otosclerotic ears. *Int J Audiol*. 2009; 48(5):240–7. [PubMed: 19842799]
- von Unge M, Decraemer WF, Bagger-Sjoberg D, Van den Berghe D. Tympanic membrane changes in experimental purulent otitis media. *Hear Res*. 1997; 106:123–36. [PubMed: 9112112]
- Voss SE, Horton NJ, Woodbury RR, Sheffield KN. Sources of variability in reflectance measurements on normal cadaver ears. *Ear Hear*. 2008; 29(4):651–65. [PubMed: 18600136]
- Voss SE, Merchant GR, Horton NJ. Effects of middle-ear disorders on power reflectance measured in cadaveric ear canals. *Ear Hear*. 2012; 33(2):195–208. [PubMed: 22037477]

Research Highlights

- Three middle ear factors contributing to changes of EA in AOM were identified
- Middle ear pressure reduced EA mainly at $f < 2$ kHz in early and late AOM phases
- Middle ear effusion was responsible for large EA peaks in both AOM phases
- Effusion also caused reduction of EA at high frequencies in both phases
- Middle ear structural changes resulted in residual reduction of EA in both phases

Figure 1.

Energy absorbance tympanogram (as a function of ear-canal pressure and frequencies) in (A) an uninfected control ear, (B) an unopened 4 day AOM ear, and (C) an unopened AOM ear 8 days post challenge.

Figure 2.
EA-pressure curve at 0.5 (solid line), 1 (thin dashed line), and 4 kHz (thick dashed line) in
(A) control, (B) unopened 4 day, and (C) unopened 8 day AOM ear.

Figure 3. Ambient EA-frequency curve at 4 days (A) OM-1, (B) OM-2, (C) OM-3, and (D) control ears. The dotted lines represent the individual curves. The shaded area represents ± 1 SD around the mean.

Figure 4. Mean $\pm$ SD ambient EA-frequency curve at 4 days OM-1 (red line with circles), OM-2 (blue line with triangles), OM-3 (green line with squares), and uninfected control ears (black line).

Figure 5. Ambient EA-frequency curve at 8 days (A) OM-1, (B) OM-2, (C) OM-3, and (D) control ears. The dotted lines represent the individual curves. The shaded area represents ± 1 SD around the mean.

Figure 6.

Mean ambient EA-frequency curve with SD at 8 days: OM-1 (red line with circles), OM-2 (blue line with triangles), OM-3 (green line with squares), and control ears (black line).

Figure 7. Ambient EA-frequency curve of normal chinchilla ears in Margolis et al. 2001 (dashed black line), Akinpelu et al. 2015 (grey line), and our present study (solid black line).

Figure 8.

An example of EA measured in a normal ear of an anesthetized chinchilla without releasing the middle ear pressure. (A) EA-pressure curve at 500 Hz. (B) EA-frequency curve at ambient pressure (dotted line) and -75 daPa (solid black line) in comparison with ambient EA-frequency curve of normal chinchilla ears in Margolis et al. 2001 (dashed black line) and Akinpelu et al. 2015 (grey line).

Table 1

List of P values derived from: (1) One way Repeated-Measures ANOVA test and Tukey post-hoc test on the ambient EA data between three 4D OM stages; (2) Unpaired Student t-test on the ambient EA data between OM-3 and control.

Frequency (kHz)	ANOVA P value	Tukey P value OM-1 vs OM-3	Tukey P value OM-1 vs OM-2	Tukey P value OM-2 vs OM-3	Unpaired t-test P value OM-3 vs Control
0.25	0.0014	< 0.01	< 0.01	> 0.05	0.2812
0.5	0.0001	< 0.001	< 0.001	> 0.05	0.4658
0.75	0.0001	< 0.001	< 0.001	< 0.05	0.3548
1	0.0001	< 0.001	< 0.001	< 0.01	0.0005
2	0.0026	< 0.01	< 0.01	> 0.05	0.0000
4	0.1084	> 0.05	> 0.05	> 0.05	0.0000
6	0.0115	> 0.05	> 0.05	< 0.01	0.0000
8	0.0027	< 0.01	> 0.05	< 0.01	0.0000

Note: The P values with grey background represent significant difference between groups ($P < 0.05$). The P values of Tukey post-hoc test are shown as inequalities since no exact value is reported by the software.

Table 2

List of P values derived from: (1) One way Repeated-Measures ANOVA test and Tukey post-hoc test on the ambient EA data between three 8D OM stages; (2) Unpaired Student t-test on the ambient EA data between OM-3 and control.

Frequency (kHz)	ANOVA P value	Tukey P value OM-1 vs OM-3	Tukey P value OM-1 vs OM-2	Tukey P value OM-2 vs OM-3	Unpaired t-test P value OM-3 vs Control
0.25	0.0906	> 0.05	> 0.05	> 0.05	0.0000
0.5	0.0013	< 0.05	< 0.01	> 0.05	0.0022
0.75	0.0008	< 0.01	< 0.01	> 0.05	0.0150
1	0.0137	< 0.05	> 0.05	> 0.05	0.0604
2	0.0223	> 0.05	> 0.05	< 0.05	0.0044
4	0.0002	< 0.001	> 0.05	< 0.01	0.0000
6	0.0313	> 0.05	> 0.05	< 0.05	0.0000
8	0.0318	> 0.05	> 0.05	< 0.05	0.0006

Note: The P values with grey background represent significant difference between groups ($P < 0.05$). The P values of Tukey post-hoc test are shown as inequalities since no exact value is reported by the software.

Table 3

List of P values derived from unpaired Student t-test on the ambient EA data at each experimental stage between 4D and 8D.

Frequency(kHz)	OM-1 4D vs 8D	OM-2 4D vs 8D	OM-3 4D vs 8D
0.25	0.6932	0.0740	0.0001
0.5	0.4863	0.0899	0.0011
0.75	0.9203	0.2397	0.0090
1	0.2543	0.1942	0.0156
2	0.3788	0.0019	0.7611
4	0.0000	0.0004	0.0070
6	0.1003	0.3513	0.0045
8	0.9562	0.9244	0.3664

Note: The P values with grey background represent significant difference between groups ($P < 0.05$).