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Dynamic Property Changes in Stapedial Annular Ligament Associated with Acute Otitis Media in the Chinchilla

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Abstract

Located at the end of the ossicular chain, the stapedial annular ligament (SAL) serves as a closed yet mobile boundary between the cochlear fluid and stapes footplate. It is unclear how SAL properties change with acute otitis media (AOM). This paper reports the measurements of SAL dynamic properties in chinchilla AOM model using dynamic mechanical analyzer (DMA) and frequency-temperature superposition (FTS) principle. AOM was analyzed in two infection groups: 4 days (4D) and 8 days (8D) post induction. SAL specimens were measured using DMA at three temperatures: 5, 25, and 37 °C. To extend the testing frequencies to higher levels, FTS principle was employed. Then generalized Maxwell model was utilized to define the constitutive equations of the SAL. The complex shear moduli were obtained from seven samples of control, 4D, and 8D groups. Results show that the storage and loss shear moduli of SALs decreased due to AOM. The storage moduli for 4D and 8D ears were similar below 100 Hz, and the loss modulus for 4D was significantly larger than 8D across the entire frequency range. This study reports data that contributes to ear biomechanics and improves understanding on the effects of AOM in middle ear tissues.

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Conflicts of Interest

None Declared

Ethical Approval

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Conflict of interest

All authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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Keywords

stapedial annular ligament; acute otitis media; chinchilla; complex modulus; frequency-temperature superposition; dynamic mechanical analyzer

1 INTRODUCTION

Connecting the base of the stapes to the oval window of the ear is the stapedial annular ligament (SAL), a ring of fibrous tissue which provides a mobile boundary between the cochlear fluid and stapes footplate. SAL enables the stapes to transfer vibration into the cochlear fluid, stimulating the sensory hair cells in the cochlea which communicate with the brain to process hearing [1]. SAL is mainly composed of mature elastic fibers as shown in histological and transmission electron microscopy (TEM) images [2, 3, 4].

Due to its anatomical location, SAL is a particularly important component in modulating acoustic-mechanical transmission to the cochlea from the middle ear. Recently, there have been several studies on the mechanical properties of SAL reported using human temporal bones [5, 6, 7]. Gan et al. [5] used a quasi-static uniaxial tension test to measure the shear modulus of human SAL and demonstrated that it is a typical viscoelastic material with hysteresis, a non-linear stress-strain relationship, and stress relaxation function. The shear modulus changed from 3.6 to 220 kPa as the shear stress increased from 2 to 140 kPa. Kwacz et al. [8] measured the static stiffness of human SAL via atomic force microscopy and reported that SAL is a linear elastic material for deflections up to approximately 100 nm with a stiffness of approximately 120 N/m and an elastic modulus of approximately 1.1 MPa. Lauxmann et al. [9] investigated the nonlinear stiffness properties of the human SAL and measured the stiffness value as 1050 N/m. These studies provide important data for understanding SAL mechanical behavior in response to force or deformation. However, the SAL works across the auditory frequency range up to 20 kHz and the dynamic properties of the ligament largely determine sound transmission from the middle ear to the cochlea. There is only one recent publication about human SAL dynamic properties by Zhang and Gan [7]. They reported a storage shear modulus of 31.7 kPa at 1 Hz and 61.9 kPa at 3.76 kHz and a mean loss shear modulus of 1.1 kPa at 1 Hz and 6.5 kPa at 3.76 kHz.

Middle ear diseases cause alterations in the morphological and mechanical properties of middle ear tissues, including the tympanic membrane (TM), round window membrane (RWM), and the SAL, as proven in previous investigations with acute otitis media (AOM), an infective disease of the middle ear [10, 11, 12]. To measure variation of any mechanical properties in AOM ears, an animal model is necessary for experimental approach because AOM cannot be induced properly in cadaver temporal bones. Recently, an AOM model was created in the chinchilla and the changes in mechanical properties of TM and RWM were reported by Yokell et al. [13] and Wang et al. [14]. For the SAL, however, biomechanical changes in pathological ears have never been reported. It is also unknown how the mechanical properties of the SAL vary as the AOM infection progresses. The degree of inflammatory response changes during AOM development, but how this affects the mechanical properties of SAL across the infection period remains unclear [13, 14].

Therefore, further investigation of SAL in AOM ears is needed to understand the mechanism of AOM-induced hearing loss.

The aim of this study is to determine the mechanical properties of the SAL and variation of these properties in different phases of AOM in chinchilla ears. Following the method developed by Zhang and Gan [7, 15], a control and two treatment groups that characterize relatively early and later distinct stages of infection, 4 days (4D) and 8 days (8D) post AOM induction, were measured using dynamic mechanical analyzer (DMA) at frequencies from 2 to 40 Hz at 5, 25, and 37 °C. To spread the testing frequencies to higher levels which will better represent the auditory frequency range of both the chinchilla and human, frequency-temperature superposition (FTS) principle was employed. [7]. Next, generalized Maxwell model was later utilized to define the constitutive equations regarding the SAL. The complex modulus, comprised of storage shear modulus G' and loss shear modulus G'' , was attained from both normal and AOM ears. All data reported here contributes to scientific knowledge of biomechanics of the middle ear.

2 METHODS

2.1 SAL Specimen Preparation and Experimental Setup

2.1.1 Chinchilla AOM Model—This study included twelve adult chinchillas (*Chinchilla lanigera*) measuring between 600 and 780 g. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma and meets the guidelines of the National Institutes of Health. All animals had healthy, disease-free ears before AOM inoculation according to otoscopic examination.

Twelve chinchillas were separated into a control group of four animals and an AOM group of eight animals which was further divided into two groups: the 4D group of four animals and 8D group of four animals. AOM infection was induced via transbullar injection of *Haemophilus influenzae* 86-028NP suspension bilaterally following the method described by Morton et al. [16]. Under general anesthesia [ketamine (10 mg/kg) and xylazine (2 mg/kg)], 0.3 ml of a bacterial suspension (3000 CFU) was injected into the superior bulla of each ear using a 26 gauge needle on 1 cc syringe. The control group remained untreated, and daily otoscopic examination was performed on all chinchillas.

At the 4th or 8th day post-inoculation, each animal was anesthetized and the TM was microscopically or otoscopically examined to confirm the presence of AOM before being euthanized in order to harvest the bullae. Similarly, Control ears were examined to confirm the absence of infection before sacrifice.

2.1.2 SAL Specimen Preparation—After euthanasia, both bullae from each animal were excised from the skull. After opening the superior bulla and removing both the TM and malleus-incus complex, each temporal bone was cut into an approximate 2 cm x 2 cm x 1.5 cm cube to reveal the SAL, stapes, and adjacent bony wall following the procedure described by Gan et al. [5]. Near the oval window, the scala vestibule wall was opened to release cochlear pressure and saline solution was applied to the underside of the footplate to help preserve physiological conditions.

2.1.3 Experimental Setup—Figure 1 is the experimental setup schematic for dynamic testing of SAL specimens in DMA and is similar to that reported by Zhang and Gan [7] using DMA measurements in human SALs. Briefly, the bony cochlea portion of the sample was secured onto the X-Y translational platform and positioned inside a temperature chamber 2 cm away from a thermocouple. A negative feedback circuit was installed to maintain a specified temperature with precision of $\pm 1^\circ\text{C}$. The SAL specimen was aligned in the analyzer (ElectroForce 3200, Bose, Eden Prairie, MN) by adjusting the Z-translational column and X-Y translational stage under a surgical microscope (Zeiss, OPMI 1-FC). As shown in Fig. 1, a titanium partial ossicular-replacement prosthesis (PORP, Gyrus, ENT, LLC, Memphis, TN) served as a mounting fixture for the peak of the stapes, which was carefully attached to the PORP with cyanoacrylate gel glue, none of which reached the SAL.

2.2 Measurement of Specimen Dimensions

Since the SAL was hidden between the bony cochlear wall and the stapes footplate, dimensions were taken after testing when the footplate was removed from the oval window, as described by Gan et al. [5]. A digital CCD camera captured still images of the oval window and stapes. Figure 2A depicts the image and dimensions of the stapes footplate length L1 and width L2, while Fig. 2C depicts the oval window length L3 and width L4. Previous histology on the human middle ear suggests that the SAL is thicker in the anterior than in the posterior [17, 18]. However, due to technique limitation, accurate *in vivo* measurement along the entire perimeter of the footplate to measure this is not currently possible. For simplification, SAL thickness was assumed to be uniform in this study. Therefore, the width or length difference between the oval window and stapes footplate, L4-L2 or L3-L1, was calculated and the average value was taken for SAL thickness, d . Potential errors due to this assumption were estimated in a previous study on static properties of human SAL [5].

SAL height h was assumed equal to the footplate edge height and was the average of measurements taken at three different locations (posterior, anterior, and middle areas) from an image, as shown in Fig. 2B. Similarly, the inner rim perimeter of the footplate was calculated from a transferred binary image. SAL dimensions of all specimens are listed in Table 1 with average and standard deviation (SD) for the (A) control, (B) 4D, and (C) 8D groups.

2.3 Viscoelastic Model of SAL Specimen

When a load was applied on the stapes head along the z-direction as shown in Fig. 1, Gan et al. demonstrated that the SAL specimen experienced shear strain [5]. Shear stress τ and shear strain γ are defined below following the descriptions from Zhang and Gan [7]:

$$\tau(t) = \frac{F}{Ch} = \frac{F_0 e^{i(2\pi ft + \delta)}}{Ch} \quad (1)$$

$$\gamma(t) = \frac{u}{d} = \frac{u_0 e^{i(2\pi ft)}}{d} \quad (2)$$

where t is time, f is frequency, C is ligament circumference, h is ligament height, δ is the phase difference between the displacement and force, u and F respectively, while u_0 and F_0 are the corresponding amplitudes. Next, the complex modulus G^* (comprised of storage shear modulus G' and loss shear modulus G'') were calculated for each SAL as follows:

$$|G^*| = \frac{F_0/C h}{u_0/d} \quad (3)$$

$$G' = |G^*| \cos \delta \quad (4)$$

$$G'' = |G^*| \sin \delta \quad (5)$$

$$G^*(f) = G'(f) + iG''(f) \quad (6)$$

In this study, the SAL was considered a homogenous and isotropic material. Maxwell model was utilized to describe the linear viscoelastic behavior of the SAL, and model parameters were fitted from the measured data. For this step, the complex shear modulus can be further articulated by

$$G'(\omega) = G_0 + \sum_{i=1}^n G_i \tau_i^2 \omega^2 / (1 + \tau_i^2 \omega^2) \quad (7)$$

$$G''(\omega) = \sum_{i=1}^n G_i \tau_i \omega / (1 + \tau_i^2 \omega^2) \quad (8)$$

$$\eta(f) = \tan \delta = \frac{G''(f)}{G'(f)} \quad (9)$$

where ω is the circular frequency ($2\pi f$). G_0 , G_i and τ_i are the model parameters. A previous study of TM dynamic properties measured with DMA and FTS principle demonstrated that a two-term linear model is appropriate to characterize the viscoelastic behavior of middle ear tissues [15]. Two spring-dashpot elements ($N=2$) give a total of five parameters: G_0 , G_1 , G_2 , τ_1 , and τ_2 , all of which were determined through theoretical fitting with experimental data from each SAL specimen. Note that the SAL samples exhibited the same nonlinear stress-strain relationship as a typical soft tissue, as previously reported on the quasi-static properties of human SAL by Gan et al. [5]. In this first attempt to quantify the dynamic

properties of AOM chinchilla SAL, a linear viscoelastic model is utilized to simplify the experimental approach by not including the nonlinear components of the shear stress and shear strain.

2.4 Dynamic Test on SAL Specimen

2.4.1 Preconditioning Test—Preconditioning tests were first conducted to stabilize the mechanical properties of the ligament. For each specimen, five cycles of uniaxial vibration (frequency 0.1 Hz and displacement amplitude 0.05 mm) were programmed in DMA. During the dynamic test at each selected frequency, ten or more cycles of sinusoidal vibration were conducted on the sample, meaning the first five cycles doubled as preconditioning for that particular frequency.

2.4.2 Dynamic Test—The dynamic testing was performed sequentially at three temperatures of 5, 25, and 37 °C. At each temperature, the control was tested at 2, 5, 10, 20, and 40 Hz while 4D and 8D groups were tested at 3, 6, 10, 20, 30, and 40 Hz (displacement amplitude 0.05 mm). Samples were kept at physiological moisture levels by administering saline via syringe through the hole opened on the cochlear wall.

2.5 FTS Principle

FTS theories are based on a simple relationship between effects of frequency (time) and temperature on the dynamic properties and molecular behaviors of polymers [24]. Since the 1950s, the application of FTS has been extended from polymers and plastics to composites. Recently, FTS principle has been applied to biological tissues to investigate the frequency-dependent mechanical properties of soft tissues, particularly those in the middle ear [7, 15, 19, 25]. This principle can be expressed as

$$G^*(T_0, f) = G^*\left(T, \frac{f}{\alpha_T}\right) \quad (10)$$

where f is the frequency, T_0 is the reference temperature (37° C in this study), T is the temperature (5 and 25° C in this study), and α_T is the shift factor representing the effect of temperature on the material's complex modulus. Factor α_T must comply with WLF equation as shown:

$$\log \alpha_T = \frac{c_1(T - T_0)}{c_2 + T - T_0} \quad (11)$$

where T and T_0 are absolute temperature, and their unit is Kelvin. Empirical constants c_1 (dimensionless unit) and c_2 (Kelvin) define an equation so that shift factor can be determined at additional temperatures.

For a more detailed procedure on how to use FTS principle to extend complex moduli of SAL samples to higher frequencies, refer to a previous study [7]. Briefly, the complex shear moduli of SAL samples measured at three temperatures (5, 25, and 37 °C) were all

logarithmically plotted as functions of frequency on the same graph. Next, the lower temperature complex modulus curves were shifted horizontally to higher frequencies to align with the higher temperature curves, finally resulting in a master curve at the reference temperature (37 °C). Then shift factor a_T was found using Eq. (10). Using Eq. (11) to calculate constants c_1 and c_2 , consistency between the WLF equation and experimental data was confirmed for each SAL sample.

3 RESULTS

Figure 3 shows the storage shear modulus G' and loss shear modulus G'' acquired from two typical SAL samples from control (Figs. 3A and 3B), 4D AOM (Figs. 3C and 3D), and 8D AOM (Figs. 3E and 3F) over the tested frequencies at three temperatures (5, 25, and 37 °C). The closed symbols mark storage modulus values while the open symbols mark loss modulus values. Statistical analysis was performed via two-variable ANOVA (without repetition for mean values and with repetition for individual samples), and all comparisons (storage vs loss, COL vs 4D, 4D vs 8D, and COL vs 8D) yielded significance of $p < 0.05$. These results demonstrate that the shear modulus increased with a decrease in temperature or an increase in frequency for both storage and loss moduli. In the control samples, however, the value of the loss shear modulus plateaued between approximately 10 and 40 Hz.

Table 2 lists the shift factors and maximum frequencies of the master curves obtained from SAL samples in control, 4D, and 8D groups (7 samples per group). Across all groups, a_5 had significantly higher mean value than a_{25} , as expected based on FTS principle [18, 19]. The shift factor a_T was tested for temperature dependence by fitting experimental data into the WLF equation [Eq. (11)] before determining the two constants c_1 and c_2 .

Figure 5 depicts the master curves of the mean complex shear modulus for samples in each of the (A) control, (B) 4D, and (C) 8D groups. As demonstrated and verified to be statistically significant via ANOVA analysis, the complex shear modulus generally increases as the frequency increases. For control samples, mean G' is 1.21 kPa at 2 Hz and rises to 15.62 kPa at 8 kHz, while mean G'' is 0.15 kPa at 2 Hz and rises to 5.64 kPa at 8 kHz. For 4D SAL samples, mean G' is 0.18 kPa at 3 Hz and rises to 1.88 kPa at 6.4 kHz, mean G'' is 0.12 kPa at 3 Hz and rises to 1.23 kPa at 6.4 kHz. For 8D SAL samples, mean G' is 0.16 kPa at 3 Hz and rises to 0.76 kPa at 4.8 kHz, while mean G'' is 0.05 kPa at 3 Hz and rises to 0.34 kPa at 4.8 kHz. The dimensions of SAL did not have much variation between specimens, which could account for small standard error values.

4 DISCUSSION

4.1 Comparison with Published Mechanical Properties of SAL

As emphasized in the Introduction, there are no published mechanical properties of SAL in healthy or diseased chinchillas in current literature. Zhang and Gan [7] reported the mean storage shear modulus of human SAL ranged from 31.7 to 61.9 kPa as frequency increases from 1 Hz to 3.76 kHz, while the mean loss shear modulus varied from 1.1 to 6.5 kPa. The complex modulus of the SAL in chinchillas was calculated in this study over frequencies of

2 Hz to 8 kHz using the same method as that used by Zhang and Gan [7]. The mean storage shear modulus of SAL in control group was 1.21 kPa at 2 Hz, 11.86 kPa at 4 kHz, and 15.62 kPa at 8 kHz, while loss modulus was 0.15, 4.41, and 5.64 kPa at the corresponding frequencies, respectively. Overall both the storage and loss shear moduli of chinchilla SAL are much lower than those of the human SAL.

On the other hand, interspecies variation of the SAL exists in the ligament's dimensions. The mean thickness of human SAL of 0.25 mm was used to determine the moduli [7]. The mean thickness of chinchilla SAL was 0.12 mm in this study. The average perimeter of the chinchilla SAL was 6.0 mm, corresponding to about 76.6% of the human SAL perimeter used by Zhang and Gan [7]. Because chinchilla SAL showed lower storage modulus with smaller size compared to human SAL, the stiffness of chinchilla SAL was much lower than that of human SAL. This could be reasonably attributed to the morphological differences between these two species in the stapes and the annular ligament.

Wada et al. [26] measured the stapes mobility of guinea pigs and rabbits. They reported that the stiffness of SAL in the guinea pig and rabbit were 16 ± 7 N/m and 115 ± 25 N/m, respectively. Stiffness of the rabbit SAL was over seven times greater than that of the guinea pig, further indicating that there may exist a significant difference in SAL stiffness across different species.

4.2 Effect of AOM on Mechanical Properties of SAL

In the chinchilla SAL samples, dynamic modulus was determined after 4 and 8 days transbullar inoculation with *H. influenzae* bacteria. The storage modulus at 100 Hz was on average 2.02 kPa in normal ears, 0.68 kPa in 4D AOM ears, and 0.43 kPa in 8D AOM ears. Correspondingly, the loss modulus was 0.53 in normal ears, 0.37 kPa in 4D ears, and 0.12 kPa in 8D ears. Overall, both the storage and loss moduli of SAL decreased in AOM ears compared to those of the control. The storage modulus of SAL samples from 4D AOM ears was on an average 66% lower than that of control ears while the loss modulus was 30% lower. In addition, there was an average 79% decrease in storage modulus and a 77% decrease in loss modulus of the SAL from 8D AOM ears.

Comparing the storage shear modulus curves in the 4D ears with those of 8D ears (Fig. 6A), the storage modulus at 4D did not differ much from that of 8D ears at frequencies below 100 Hz. However, the storage modulus of 8D AOM ears decreased about 30–60% more than the 4D AOM ears at frequencies above 100 Hz. In contrast to the storage modulus, the loss modulus of 8D AOM ears decreased about 50–75% more than the 4D ears. This suggests that the main change in storage modulus occurred within the first 4 days of infection, while the large alteration in loss modulus occurred after these first 4 days. All changes in mechanical properties of SAL due to AOM would be correspondingly indicative of changes in the cochlear input.

A quantitative comparison of the measured modulus curves of the infected chinchilla SALs with literature data is limited because there are no published comparable measurements. Recently, dynamic properties of the infected RWM in AOM ears reported by Wang et al. in the chinchilla [14] and Gan et al. in the guinea pig [27]. The results from both chinchilla and

guinea pig RWMs from AOM ears showed that both the storage and loss moduli were lower than those of normal ears. This trend of storage modulus change in the infected SAL is similar to that of infected RWMs in guinea pigs and chinchillas. Moreover, change of dynamic modulus of the chinchilla TM in AOM was reported by Yokell et al. [13]. AOM increased the dynamic modulus of the chinchilla TM, which is opposite of findings in RWM of chinchilla AOM ears [14] and those in this study. The reason for these contrasting changes of the dynamic modulus in AOM ears remains unclear. Even so, it is likely relevant that boundary conditions for the experimental specimens varied between the TM, RWM, and SAL. The RWM and SAL were left intact and fully attached to the bony walls at their peripheries, while the TM strip was sliced from the anterior or posterior portion of the membrane, releasing any residual deformation due to disease of the undamaged structure.

4.3 Calculation Limitations

Because an intact temporal bone was used, this study has obvious advantages over those that involve excised tissues. However, dimensions (especially those of the intact diseased SAL) were more difficult to obtain while the TB was fully intact. It is known that the thickness and width of the SAL are not uniform and the tissue may have direction-dependent properties due to fiber arrangement. The shape of the oval window is also irregular. In the present study, the SAL was assumed to be either homogeneous or isotropic in structure, and was treated as an elliptical ring. Although the same assumption was made by researchers to estimate the SAL elastic modulus [5, 3] and numerous authors have treated the SAL as an elliptical ring having homogeneous or isotropic mechanical properties in finite element modeling of the middle ear, such approaches certainly affect the calculation of elastic modulus of the SAL [28]. Additionally, in this study an axial force was applied at the stapes head along the z-direction, meaning the inhomogeneous stiffness distribution or rotational stiffness of SAL could not be considered, and only the averages of shear moduli were derived. Therefore, the storage and loss moduli reported in this study are applicable only if this same simplified model is applied.

Previous studies demonstrated that the SAL has a nonlinear stress-strain relationship and the shear modulus varies as stress level varies [5, 29, 30]. Nonlinear behavior would produce distortion in the measured stress-frequency curves from loading, and the stress may be no longer perfectly sinusoidal. Under nonlinear conditions, the storage modulus and the loss modulus may lose their precise physical meaning. Thus, the output moduli are the approximate complex moduli at input frequency, ignoring the high order components of the shear stress and shear strain. Otherwise, other methods are necessary for quantifying the nonlinear viscoelastic characteristics.

5 CONCLUSION

This study is the first to discuss the dynamic properties of the SAL across the auditory frequency range in chinchilla AOM ears infected by *H. influenzae*. Dynamic tests were performed at three different temperatures (5, 25, and 37 °C) and at frequencies varying from 2 to 40 Hz in the DMA system. To extend the testing frequencies to higher levels (up to 8 kHz), FTS principle was employed in normal (control), 4D, and 8D AOM ears. Next,

generalized Maxwell model was used to define the viscoelastic behavior of the SAL. The complex shear moduli (storage modulus G' and loss modulus G'') were obtained from seven SAL specimens each from control, 4D (4 days post induction), and 8D (8 days post induction). For control samples, mean G' is 1.21 kPa at 2 Hz and rises to 15.62 kPa at 8 kHz, while mean G'' is 0.15 kPa at 2 Hz and rises to 5.64 kPa at 8 kHz. For 4D SAL samples, mean G' is 0.18 kPa at 3 Hz and rises to 1.88 kPa at 6.4 kHz, mean G'' is 0.12 kPa at 3 Hz and rises to 1.23 kPa at 6.4 kHz. For 8D SAL samples, mean G' is 0.16 kPa at 3 Hz and rises to 0.76 kPa at 4.8 kHz, while mean G'' is 0.05 kPa at 3 Hz and rises to 0.34 kPa at 4.8 kHz. This data contributes to biomechanical understanding of the middle ear tissues and helps to improve the correctness of the finite element model for chinchilla and human ears, both affected and unaffected by AOM.

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Highlights

- Dynamic tests of stapedial annular ligament at otitis media ears were performed
- Complex modulus of the ligament decreased at 4 and 8 days post bacterial challenge
- Moduli were shifted to high frequencies using frequency-temperature superposition
- Storage modulus decreased significantly at 4 days post challenge over frequencies
- Loss modulus of 8 days post challenge was significantly lower than that of 4 days

Figure 1.

The schematic of the experimental setup for dynamic test of the SAL specimen in DMA. The stapes head was affixed to the mounting fixture, and the SAL was located where the stapes met the temporal bone (TB).

Figure 2.
Photographs demonstrating how dimensions were measured for use in calculations on SAL:
(A) stapes footplate, (B) stapes, and (C) oval window.

Figure 3.

Typical complex shear modulus-frequency curves obtained at 5, 25, and 37 °C from two SAL specimens in each group: (A) and (B) control, (C) and (D) 4D, and (E) and (F) 8D. Following the FTS principle described in Section 2.5, the shift factors α_5 and α_{25} were calculated and c_1 and c_2 values were derived for each specimen (see Table 2). The complex shear modulus curves at lower temperatures (5 and 25 °C) were shifted to higher frequencies, adjacent to the curves obtained at the higher temperature (37 °C). In this way, final master curves were constructed for control, 4D, and 8D at reference temperature $T_0 = 37$ °C. Figure 4 shows master curves of the complex shear modulus at 37 °C for two samples from (Figs. 4A and 4B) control, (Figs. 4C and 4D) 4D, and (Figs. 4E and 4F) 8D groups. As shown in Fig. 4, for control specimen (COL-1), the storage shear modulus is 1.21 kPa at 2 Hz and rises to 16.30 kPa at 8 kHz, while loss shear modulus is 0.14 kPa at 2 Hz and rises to 4.27

kPa at 8 kHz. For 4D specimen 4D-1, the storage shear modulus is 0.18 kPa at 3 Hz and rises to 1.93 kPa at 6.4 kHz, while the loss shear modulus is 0.12 kPa at 3 Hz and rises to 1.26 kPa at 6.4 kHz. For 8D specimen 8D-1, the storage shear modulus is 0.16 kPa at 3 Hz and rises to 0.76 kPa at 4.8 kHz, while the loss shear modulus is 0.05 kPa at 3 Hz and rises to 0.33 kPa at 4.8 kHz. The results from 2 samples in each group also show that in 4D ears the value distinction between G' and G'' was reduced compared with those observed in control or 8D ears over the frequency range 3 Hz to 6.4 kHz.

Figure 4.

The FTS resulted complex shear modulus-frequency master curves obtained at 37 °C from six SAL specimens: (A) and (B) two control samples, (C) and (D) two 4D samples, and (E) and (F) two 8D samples.

Figure 5.

The FTS resulted complex shear modulus-frequency curves (mean $\pm$ SE) from 7 SAL samples of each experimental group: (A) control, (B) 4D, and (C) 8D chinchilla ears. The mean values of storage and loss moduli obtained from control and AOM groups are displayed in Figure 6. Storage modulus of 4D samples decreased significantly according to ANOVA from the control samples across frequencies and the loss shear modulus of 4D samples also decreased significantly, but to a lesser degree. On the other hand, the loss shear modulus decreased significantly from the 4D to 8D samples, while the reduction of storage shear modulus from the 4D to 8D samples were only shown at frequencies over 1 kHz. The storage moduli for 4D and 8D ears were similar below 100 Hz, and then 4D became significantly higher in value than 8D, reaching a difference of approximately 300 Pa at 6 kHz. For loss modulus, 4D was significantly larger than 8D across the entire frequency range, reaching a final difference of 600 Pa at 6 kHz.

Figure 6.

Comparison of the mean storage modulus (A) and loss modulus (B) from control, 4D, and 8D chinchilla SAL groups. Note: Complex shear modulus is the combination of both storage and loss moduli, so these images cannot be described as the complex modulus. Generalized Maxwell model was employed to characterize the viscoelastic behavior of the SAL samples; the mean values of complex modulus in control, 4D, and 8D are shown in Fig. 6. Figures 7A, 7B, and 7C display the Maxwell model fitting with the experimental data from control, 4D, and 8D specimens, respectively. Good agreement between the measurement data and Maxwell model was observed with a correlation coefficient of $r > 0.83$ in all three groups of SAL specimens.

Figure 7.
Theoretical fitting of generalized Maxwell model to the experimental complex modulus (mean data) for (A) control, (B) 4D, and (C) 8D chinchilla SALs.

Dimensions of chinchilla SAL samples measured from Control group. Note: FP is an abbreviation of footplate and OW is an abbreviation of oval window.

Table 1A

Specimen	COL-1	COL-2	COL-3	COL-4	COL-5	COL-6	COL-7	Mean ± SD
FP length, <i>L1</i> (mm)	2.50	2.45	2.48	2.55	2.52	2.50	2.51	2.5 ± 0.03
FP width, <i>L2</i> (mm)	0.90	0.86	0.89	0.91	0.95	0.89	0.90	0.90 ± 0.03
OW length, <i>L3</i> (mm)	2.62	2.64	2.59	2.61	2.63	2.66	2.60	2.62 ± 0.02
OW width, <i>L4</i> (mm)	1.05	0.98	1.02	1.03	0.97	1.01	0.99	1.01 ± 0.03
SAL thickness, <i>d</i> (mm)	0.13	0.07	0.07	0.08	0.09	0.10	0.08	0.09 ± 0.02
SAL (FP) height, <i>h</i> (mm)	0.12	0.11	0.12	0.13	0.12	0.12	0.11	0.12 ± 0.01
SAL (FP) perimeter, <i>C</i> (mm)	5.88	6.02	6.01	6.00	5.99	6.06	5.98	5.99 ± 0.06
Stapes height, <i>L5</i> (mm)	1.50	1.40	1.51	1.50	1.40	1.51	1.49	1.47 ± 0.05

Table 1B

Dimensions of chinchilla SAL samples measured from 4D group.

Specimen	4D-1	4D-2	4D-3	4D-4	4D-5	4D-6	4D-7	Mean $\pm$ SD
FP length, <i>L1</i> (mm)	2.51	2.49	2.47	2.50	2.42	2.53	2.50	2.49 $\pm$ 0.04
FP width, <i>L2</i> (mm)	0.86	0.93	0.90	0.91	0.89	0.88	0.91	0.90 $\pm$ 0.02
OW length, <i>L3</i> (mm)	2.61	2.58	2.62	2.64	2.67	2.69	2.59	2.63 $\pm$ 0.04
OW width, <i>L4</i> (mm)	0.93	0.99	1.00	0.98	1.01	0.99	1.02	0.99 $\pm$ 0.03
SAL thickness, <i>d</i> (mm)	0.09	0.12	0.09	0.08	0.13	0.09	0.11	0.10 $\pm$ 0.02
SAL (FP) height, <i>h</i> (mm)	0.12	0.13	0.12	0.12	0.10	0.13	0.15	0.12 $\pm$ 0.02
SAL (FP) perimeter, <i>C</i> (mm)	6.01	5.89	6.02	5.99	6.00	5.99	5.92	5.97 $\pm$ 0.05
Stapes height, <i>L5</i> (mm)	1.48	1.41	1.52	1.50	1.49	1.50	1.48	1.48 $\pm$ 0.03

Table 1C

Dimensions of chinchilla SAL samples measured from 8D group.

Specimen	8D-1	8D-2	8D-3	8D-4	8D-5	8D-6	8D-7	Mean ± SD
FP length, <i>L1</i> (mm)	2.40	2.45	2.48	2.44	2.52	2.50	2.51	2.47 ± 0.04
FP width, <i>L2</i> (mm)	0.90	0.86	0.89	0.91	0.97	0.89	0.87	0.90 ± 0.04
OW length, <i>L3</i> (mm)	2.53	2.54	2.60	2.57	2.61	2.66	2.63	2.59 ± 0.05
OW width, <i>L4</i> (mm)	1.00	0.98	0.96	1.03	0.95	1.01	0.99	0.99 ± 0.03
SAL thickness, <i>d</i> (mm)	0.09	0.11	0.09	0.08	0.15	0.11	0.11	0.11 ± 0.02
SAL (FP) height, <i>h</i> (mm)	0.14	0.11	0.15	0.13	0.12	0.15	0.14	0.13 ± 0.02
SAL (FP) perimeter, <i>C</i> (mm)	5.96	6.02	6.01	6.00	5.94	6.00	5.98	5.99 ± 0.03
Stapes height, <i>L5</i> (mm)	1.50	1.48	1.51	1.50	1.42	1.51	1.49	1.49 ± 0.03

Table 2A
The shift factors, c1 and c2 of WLF equation, and maximum frequency of chinchilla SAL Control samples.

Specimen	COL-1	COL-2	COL-3	COL-4	COL-5	COL-6	COL-7	Mean ± SD
α_{25}	36.0	32.0	28.0	29.0	33.0	26.0	31.0	30.7 ± 3.4
α_5	408	376	389	415	418	394	402	400 ± 15
$\ln(\alpha_{25})$	3.6	3.5	3.3	3.4	3.5	3.3	3.4	3.4 ± 0.1
$\ln(\alpha_5)$	6.0	5.9	6.0	6.0	6.0	6.0	6.0	6.0 ± 0.0
Maximum Frequency (kHz)	8.16	7.52	7.78	8.30	8.36	7.88	8.04	8.01 ± 0.30
c1	5.1	5.1	5.1	5.0	5.0	5.0	5.0	5.00± 0.01
c2 (K)	-5.0	-5.0	-5.0	-5.0	-5.0	-5.0	-5.0	-5.00 ± 0.00

Table 2B
The shift factors, c1 and c2 of WLF equation, and maximum frequency of chinchilla SAL 4D samples.

Specimen	4D-1	4D-2	4D-3	4D-4	4D-5	4D-6	4D-7	Mean ± SD
α_{25}	11.5	10.9	10.5	11.4	10.8	11.3	11.1	11.1 ± 0.4
α_5	153	148	170	169	158	160	162	160 ± 8
$\ln(\alpha_{25})$	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4 ± 0.0
$\ln(\alpha_5)$	5.0	5.0	5.1	5.1	5	5.1	5.1	5.1 ± 0.1
Maximum Frequency (kHz)	6.12	5.92	6.80	6.76	6.32	6.40	6.48	6.40 ± 0.32
c1	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.90 ± 0.00
c2 (K)	-7.2	-7.2	-7.2	-7.2	-7.2	-7.2	-7.2	-7.20 ± 0.00

Table 2C

The shift factors, c1 and c2 of WLF equation, and maximum frequency of chinchilla SAL 8D samples.

Specimen	8D-1	8D-2	8D-3	8D-4	8D-5	8D-6	8D-7	Mean $\pm$ SD
α_{25}	9.6	10.3	8.9	10.8	8.9	9.1	8.9	9.5 $\pm$ 0.8
α_5	113	125	106	120	141	119	127	122 $\pm$ 11
$\ln(\alpha_{25})$	2.3	2.3	2.2	2.4	2.2	2.2	2.2	2.2 $\pm$ 0.1
$\ln(\alpha_5)$	4.7	4.8	4.7	4.8	4.9	4.8	4.8	4.8 $\pm$ 0.1
Maximum Frequency (kHz)	4.52	5.00	4.24	4.80	5.64	4.76	5.08	4.86 $\pm$ 0.45
c1	3.6	3.7	3.6	3.6	3.6	3.6	3.6	3.60 $\pm$ 0.01
c2 (K)	-7.3	-7.3	-7.3	-7.3	-7.3	-7.2	-7.2	-7.30 $\pm$ 0.01