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Effect of freezing and embalming of human cadaveric whole head specimens on bone conduction

Lukas Graf[a], Andreas Arnold[b], Sandra Blache[c], Flurin Honegger[a], Magdalena Müller-Gerbl[c], Christof Stieger[a,*]

[a] Department of ORL, University Hospital Basel, Hebelstrasse 10, Basel CH-4031, Switzerland
[b] Department of ORL, Spital Münsingen, Inselspital Bern and University of Bern, Switzerland
[c] Department of Anatomy, University of Basel, Switzerland

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ASTM, American standard practice for describing system output in implantable middle ear hearing devices
Baha, Bone anchored hearing aid
BC, Bone conduction
CI, Confidence interval
CT, Computed tomography
LDV, Laser Doppler vibrometry
RW, Round window

ABSTRACT

Background and aims: Conserved specimens do not decay and therefore permit long-term experiments thereby overcoming limited access to fresh (frozen) temporal bones for studies on middle ear mechanics. We used a Thiel conservation method which is mainly based on a watery solution of salts. In contrast to pure Formalin, Thiel conservation aims to preserve the mechanical proprieties of human tissue. The aim of this study is to examine the effect of Thiel conservation on bone conduction in the same specimen before and after conservation.
Methods: Nine ears of five defrosted whole heads were stimulated with a direct, electrically driven, bone anchored hearing system (Baha, Baha SuperPower). The motion produced by bone conduction stimulation was measured with a single point laser Doppler vibrometer (LDV) at the promontory, the ossicular chain, and the round window through a posterior tympanotomy. After the initial experiments, the entire whole heads were placed in Thiel solution. In order to enable direct comparison between fresh frozen and Thiel specimens, our Thiel conservation did not include intravascular and intrathecal perfusion. The measurements were repeated 3 and 12 months later.

To determine the effect of freezing, defrosting, and embalming on the whole heads, CT scans were performed at different stages of the experimental procedure. Additionally, three extracted temporal bones were stimulated a Baha, motion of the promontory measured by LDV and embalmed in Thiel solution to investigate the direct impact of Thiel solution on the bone.
Results: The averaged magnitude of motion on the promontory increased in whole head specimens by a mean of 10.3 dB after 3 months of Thiel embalming and stayed stable after 12 months. A similar effect was observed for motion at the tympanic membrane (+7.2 dB), the stapes (+9.5 dB), and the round window (+4.0 dB). In contrast to the whole head specimens, the motion of the extracted temporal bones did not change after 3 months of Thiel embalming (-0.04 dB in average). CT scans of the whole heads after conservation showed a notable brain volume loss mostly >50% as well as a remarkable change in the consistency and structure of the brain. Partial changes could already be observed before the Thiel embalming but after 1–2 days of defrosting. In an additional experiment, a substitution of brain mass and weight by Thiel fluid did not lead to new deterioration in sound transmission. In contrast, a frozen (non-defrosted) whole head showed a distinctively reduced magnitude of promontory motion before defrosting.
Discussion: For our setup, the vibration of the ear due to bone conduction in the same whole head specimens significantly increased after Thiel conservation. Such an increase was not observed in extracted temporal bone specimens.

Due to brain changes in the CT scans, we investigated the consequences of the brain volume changes and structure loss on the frozen brain before defrosting. The loss of brain volume alone could not explain the increase of ear vibrations, as we did not observe a difference when the volume was replaced with

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* Corresponding author.
E-mail address: christof.stieger@usb.ch (C. Stieger).

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Thiel fluid. However, freezing and defrosting of the entire brain seems to have a major influence. Beside the destructive effect of freezing on the brain, the modified conservation method without perfusion changed the brain structure.

In conclusion, bone conduction in whole heads depends on the physical condition of the brain, rather than on the conservation.

1. Introduction

Investigations into middle ear mechanics, e.g. for the development of hearing implants, require an understanding of middle ear transmission functions. Such investigations require use of human anatomy. Therefore often fresh frozen human postmortem specimens are used (Guignard et al., 2013). Due to anatomical decay, for long term experiments, over several days or weeks, a form of conservation is necessary (Dobrev et al., 2019). A widespread anatomical conservation method is embalming in formaldehyde though this is known to harden and shrink body tissue (Burkhart et al., 2010) and greatly alter sound transduction within the middle and inner ear (Graf et al., 2021). An alternative conservation was described in 1992 by Thiel (Thiel, 1992). It is based on a solution of different salts and was originally developed to preserve the shape and color of the body. Its antibacterial, antiviral, and antifungal proprieties have been proven (Thiel, 1992), additionally it prevents unhealthy formalin content within the ambient air. Thiel conservation seems to preserve the mechanical proprieties of human tissue (Burkhart et al., 2010; Wilke et al., 2011), although there is evidence of a possible influence on the plastic energy absorption of cortical bone (Stefan et al., 2010). In a previous study, we showed that Thiel embalming causes small changes in middle ear sound transduction compared to the same fresh frozen specimens. For air conduction (AC) stimulation the deterioration was 10 dB in the mid-frequencies. Long-term results after 12 months were stable (Graf et al., 2021). Investigations of bone conduction (BC) require more elaborate techniques (Stieger et al., 2018) and Thiel conserved specimens are considered beneficial as reported by Dobrev et al. (Dobrev et al., 2017).

In contrast to AC, BC involves multiple frequency dependent transmission pathways that lead to wave motion within the inner ear, e.g., the compression of the cochlea itself, the inertia of its fluid and that of the middle and outer ear, and the pressure transmission from the skull interior to the cochlea (Stenfelt, 2011). While for AC measurements on an extracted human temporal bone specimen is accepted as a standard model, BC investigations should preferably be performed in whole head specimens (Chien et al., 2007; Quester and Schröder, 1997). BC stimulation is clinically mandatory to distinguish between sensorineural and conductive hearing loss, e.g. for pure tone audiometry. Furthermore, it can be used for speech audiometry, otoacoustic emissions, and acoustic evoked brainstem potentials (Stenfelt, 2011). Therapeutically, BC is used to overcome a dysfunctional middle ear in cases of bone conductive or combined bone and air hearing loss using bone anchored hearing aids as well as to treat single sided deafness (Dun et al., 2011).

Guignard et al. compared BC in Thiel conserved specimens to data from living subjects in the literature, fresh cadaveric bones and non-Thiel solution embalmed specimen (Guignard et al., 2013). Their results showed that Thiel conserved whole head specimens might be a useful alternative model in the study of BC stimulation. However, the authors noted that ideally such a comparison would have been performed with data of specimens which are measured fresh, subsequently embalmed according to Thiel and re-measured when conserved (Guignard et al., 2013).

Thus, the aim of the present study was to investigate the difference of BC induced motions between fresh defrosted and Thiel embalmed condition in the same specimen.

2. Material and methods

2.1. Specimens

Experiments were conducted in 10 ears of 5 human cadaveric whole head specimens (2 female, 3 male, mean age 66 +/- 12 y) and three temporal bones of 2 donors (mean age 73 +/- 9 y, 2 male). All specimens were frozen within 48 h postmortem. The use of the specimens has been approved by the local ethics committee (EKNZ BASEC 2016–00,599).

2.2. Surgical preparation of the specimens

A microscopic inspection of the external auditory canal and the tympanic membrane was conducted, and any debris was removed using suction. A conventional mastoidectomy and facial recess approach was performed preserving the pinna and the external auditory canal. The mastoid segment of the facial nerve was removed to allow a right-angled view of the round window (RW) membrane. The stapedial tendon was dissected for LDV-measurements at the posterior stapes crus. We placed a Baha Connect-implant (Cochlear, Sidney, Australia) posterior-superior to the external auditory canal with slight modifications from the surgery in living patients: The skin incision was made directly over the screw location to avoid lifting a skin flap that will not heal in cadaver specimens. The incision was continued to the periosteum and approximately 1 × 1 cm bone was exposed using a self-retaining retractor. The hole for placement of the titanium screw was drilled under irrigation using a 4 mm drill repeatedly checking the bottom of the hole for remaining bone. The implant screw and the abutment were finally mounted manually.

One ear of a whole head specimen was excluded due to a rupture of the RW membrane during preparation resulting in 9 ears (5 right, 4 left). In the three temporal bones the incus had been removed before for another study.

2.3. Measurement setup

To study BC a Baha (Baha® 5 SuperPower, Cochlear, Sidney, Australia) was attached to the Baha abutment. The measurement setup generally followed the protocol described by Graf et al. (Graf et al., 2021). In summary the magnitude of motion was detected with a single point laser Doppler vibrometer (LDV, CLV a 25–44, Polytec, Waldbronn, Germany) at the promontory, the umbo, the posterior crus of the stapes, and the RW through a posterior tympanotomy. A glass-backed coupler (Polytec, Waldbronn, Germany) with an inserted ear phone (ER2, Etymotic Research, Elk Grove Village, IL, USA) and a probe microphone (ER7C, Etymotic Research, Elk Grove Village, IL, USA) was connected to the external auditory canal. Reflecting microbeads were placed at the promontory, umbo,

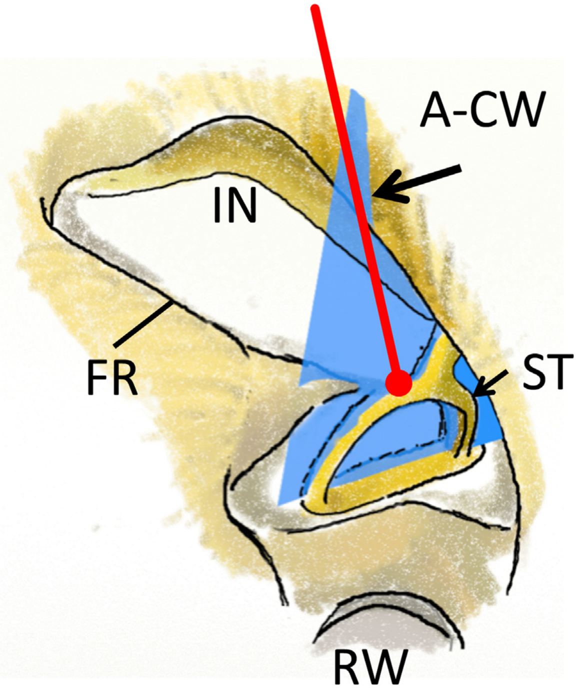

Fig. 1. Illustration of the method for a reproducible laser beam angle on a drawing of a right sided facial recess. In cranio-caudal direction the beam lays in the imaginary plane through both stapes crurae (blue area), in latero-medial direction it targets on the stapes from as lateral as possible, precisely medial to the location where it would be reflected by the bone of the anterior mastoid cavity wall (bold arrow, corresponding to a spot on the back side of the external auditory canal (EAC) wall). FR: facial recess, IN: incus, A-CW: anterior mastoid cavity wall, ST: stapes, RW: round window.

posterior crus of the stapes, and at least three positions on the RW. Measurements of RW motion were based on the RW bead with the highest magnitude in initial measurement keeping the same location for subsequent measurements. To assure a constant angle of the LDV in the same specimen for all conditions in stapes velocity measurements, the laser beam was set within an imaginary plane through both the anterior and posterior stapes crus, and targeted from as lateral as possible, directly behind the anterior mastoid cavity wall (see Fig. 1). The angle of the LDV beam for RW measurements was perpendicular to the RW membrane, and promontory measurements were from the same position as for the RW. Chien et al. (Chien et al., 2006) suggested a frequency dependent correction for a stapes measurement angle differing approximately 60° from the piston motion direction to enable a direct comparison with data taken in the piston direction. As we were only interested in differences of motion, we did no angle correction.

2.4. Stimulus generation and acquisition

We used 30 logarithmically distributed single pure tone stimuli in the frequency range of 100 to 10′000 Hz with a density of 4 stimuli per octave from the internal signal generator of the UPV system (R&S UPV, Rohde & Schwarz, Munich, Germany) which was connected in series with an attenuator (TuckerDavis Technology, Alachua, FL, USA) and a custom made amplifier. Single pure tones were applied sequentially. For each frequency 10 full cycles were averaged. The constant number of cycles resulted in frequency-dependent measurement times e.g. for 500 Hz: 10 cyles x 200 ms = 2 s (Graf et al., 2021). As a check for linearity, we verified that a 10 dB attenuated stimulus resulted in a 10 dB decrease of motion.

For tests of compliance to the ASTM standard (ASTM, 2005), voltage sinusoids with a peak amplitude of 3.16 V were delivered to the ER2 transducer. The sound pressure level was measured near the tympanic membrane with the probe microphone integrated within the ear speculum.

For BC stimulation with the Baha, the stimulation level was set to 1.7 V. The stimulus voltage and the LDV response were registered simultaneously. The LDV decoder was set to a sensitivity of 2 mm/s/V, with a highpass-filter at 100 Hz and a lowpass-filter at 20 kHz.

Fig. 2 favorably compares our AC measurements in these specimens to the ASTM standard. The acoustic transfer function was within the ASTM range in all ears for most frequencies. For frequencies between 2 and 5 kHz a peak is observed which is consistent with the first fundamental frequency in our closed A-HLV-SPEC speculum with 6.5 mm length between tip and glass cover plus the distance to the TM ($f_1 = 345$ m/s / 2×0.0065 m). The right side of Fig. 2 shows the electrical stimuli which were applied to the BC stimulation. The stimulus varied 0.36 dB over all frequencies such that we excluded a systematic error in BC stimulation (see Fig. 2).

2.5. Experimental and Thiel conservation protocol

The experimental protocol was adapted from Graf et al. (Graf et al., 2021) as follows. 4 frozen whole head specimens (7 ears) were thawed overnight (approximately 12 h, labeled "defrosted" in text and figures if not otherwise specified) before preparation in ambient air temperature and surgically prepared for measurements as described above. First, an acoustical stimulation was performed and transfer functions were compared to the ASTM standard (ASTM, 2005), followed by the electromechanical BC stimulation measuring vibration sequentially at the umbo, promontory, stapes, and round window. After the initial measurements the specimens were embalmed in Thiel solution with the following proportional composition: hot water 100 ml, boric acid 3 g, ethylene glycol 10 ml, ammonium nitrate 10 g, potassium nitrate 5 g, 4–chloro–3-methylphenol 2 ml, sodium sulfate 7 g, formalin 2 ml. The experiments were repeated with the same protocol after 3 and 12 months. The conservation method in this project only used immersion of the specimen in Thiel solution. The original protocol requires perfusion typically through the iliac artery. One specimen (one ear, ruptured RW membrane on the opposite side) was conserved with pure formalin solution after 3 months in Thiel solution. Unger et al. (Stefan et al., 2010) describe a significantly lowered Young's modulus and significantly increased plastic absorption in Thiel conserved cortical bone samples of a femur in a mechanical bending test. This does not necessarily implicate an influence of Thiel on BC-induced skull vibrations. Because of possible changes in the whole head, e.g., loss of cranial contents and changes in soft tissues and skull bones, we isolated the effect of Thiel conservation on the temporal bone using promontory vibration measurements in three extracted temporal bones before and after Thiel conservation. To study the influence of defrosting, the BC induced motion was measured after 2 h, 24 h, 2 days, and 7 days in two ears of one whole head specimen.

2.6. Computed tomography (CT)

CT scans with a 64-slice scanner of the Institute of Anatomy Basel (Somatom Emotion, Siemens Healthcare, Erlangen, Germany) were acquired to examine the intracranial contents. The main postmortem scan parameters were a slice thickness of 0.75 mm, with a stimulation of 130 kV and 270 mAs. Raw data were reconstructed with a recon increment of 0.3 mm using a smooth kernel (H30s),

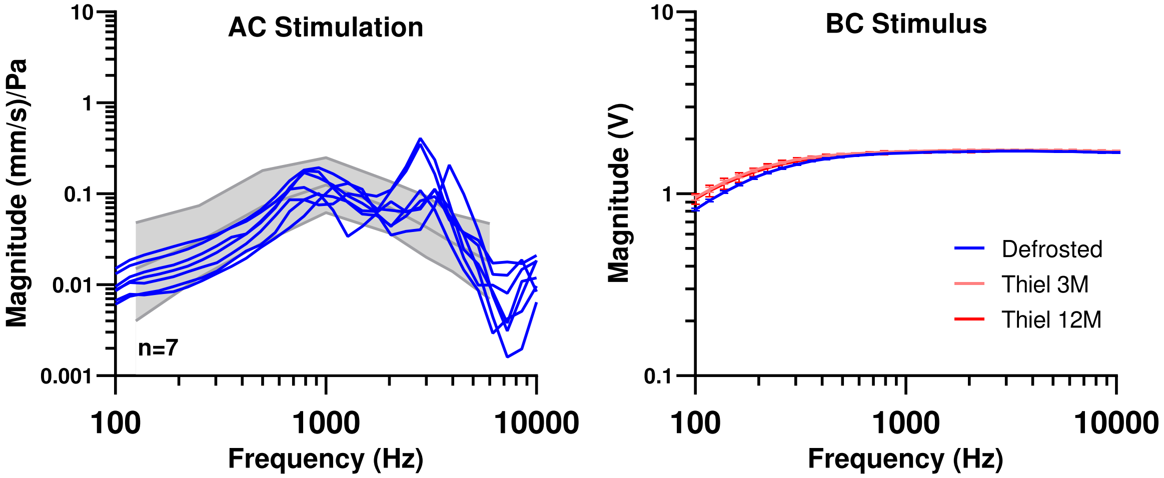

Fig. 2. Left: AC stimulated middle-ear transfer functions in our specimens compared to the ASTM standard (gray). Right: Mean electrical stimulus applied to the Baha during BC stimulation for all experiments: defrosted ($n = 7$), Thiel 3 months ($n = 7$), Thiel 12 months ($n = 6$), error bars depict 95% CI.

hard kernel (H70s), and ultra-hard kernel (U90s) for 3D-analysis. The size of the reconstruction field of view was 250 mm.

2.7. Data analysis and statistics

Data were compiled with Microsoft® Excel® 2016 (Microsoft Corporation, Redmond, WA, USA). For statistics and figures we used GraphPad Prism® (Version 8.4.3, GraphPad Software, San Diego, CA, USA). For AC stimulation, the vibration transfer function of the stapes and RW membrane with respect to sound stimulation (mm/s/Pa) were computed including both magnitude and phase. Phases were unwrapped individually. We performed non-parametric one sample Wilcoxon-sign-rank statistical tests. Post-hoc corrections for repeated significance testing were not performed.

Slight frequency changes for individual specimens such as for stiffening might be masked when all samples are averaged. We have chosen a cross correlation approach to detect slight frequency shifts due to changes in stiffness that may directly affect resonance frequency, and shift the frequency-dependence of the vibratory response. Instead of calculating the cross correlation in the time domain to detect a time delay, we used the cross correlation in the frequency domain to detect potential frequency shifts of resonances. As these resonances might be small compare to the overall vibration, individual data between 221 Hz and 5300 Hz were normalized and used for further analysis. First, we calculated the difference in magnitude to the averaged data of the corresponding conservation status for each ear. The cross correlation of defrosted versus 3 month of Thiel conservation as well as between 3 months versus 12 months of Thiel conservation was then calculated. The cross correlation analysis included ten frequency shifts. Each shift was a quarter octave corresponding to the logarithmic frequency distribution of the data acquisition (see Section 2.4). For systemic resonance shifts pre and post conservation we would expect a high correlation value at a certain relative frequency shift, e.g., one or two quarters of an octave. In case of pure stochastic variations of the normalized individual measurements, low correlation values for all frequency shifts are expected. In clinical audiology, such as in otoacoustic emissions, correlation values lower than 60% are typically estimated as noise or artifact (Hoth and Baljić, 2017).

3. Results

3.1. Magnitudes

Fig. 3 shows a global overview of the BC stimulation induced magnitudes at all measurement sites and conservation conditions. The averaged magnitude of motion across all frequencies was higher after Thiel conservation. After 3 months the average increase for the promontory was 10.3 dB, for the umbo 7.2 dB, for the stapes 9.5 dB and for the RW 4.8 dB. After an additional 9 months of Thiel embalming the magnitude changed by an additional −2.2 dB at the promontory, +0.7 dB at the umbo, −1.2 dB at the stapes and +1.3 dB at the RW. A constant increase across all frequencies is seen for the promontory and the stapes, whereas the changes were larger at lower frequencies at the umbo and the RW.

Fig. 4 shows magnitude ratios for three months Thiel embalming versus defrosted condition for all measurement sites. One sample Wilcoxon-sign-rank test showed a significant increase (marked by the filled cycles) of magnitude at the promontory after Thiel embalming over all frequencies except at 788 Hz, 1080 Hz, and 2400 Hz and at the stapes except at 489 Hz, 2400 Hz, 2810 Hz, and 8530 Hz. Another 9 month of Thiel conservation (Fig. 3) did not show an additional significant change except for one frequency (1490 Hz) on the promontory.

At the tympanic membrane after 3 months of Thiel conservation most frequencies below 2 kHz show a significant increase (mean 8.2 dB), while for higher frequencies the increase (mean 5.3 dB) is not significant for most of the tested frequencies. No significant further change in magnitude for additional 9 months of Thiel embalming was found.

The round window shows a significant increase of magnitude for most of the frequencies below 700 Hz (mean +6.1 dB) and no significant change above 700 Hz (+3.8 dB) after 3 months of Thiel embalming. In general, for all measurement sites, there is almost no significant difference between Thiel embalming for 3 months and 12 months with an averaged decrease of 0.35 dB over all specimens and conditions.

Fig. 5 shows the averaged cross correlations for all measurement locations after 3 and 12 months of Thiel embalming for 10 quarter octave shifts. Individual curves are provided in supplemen-

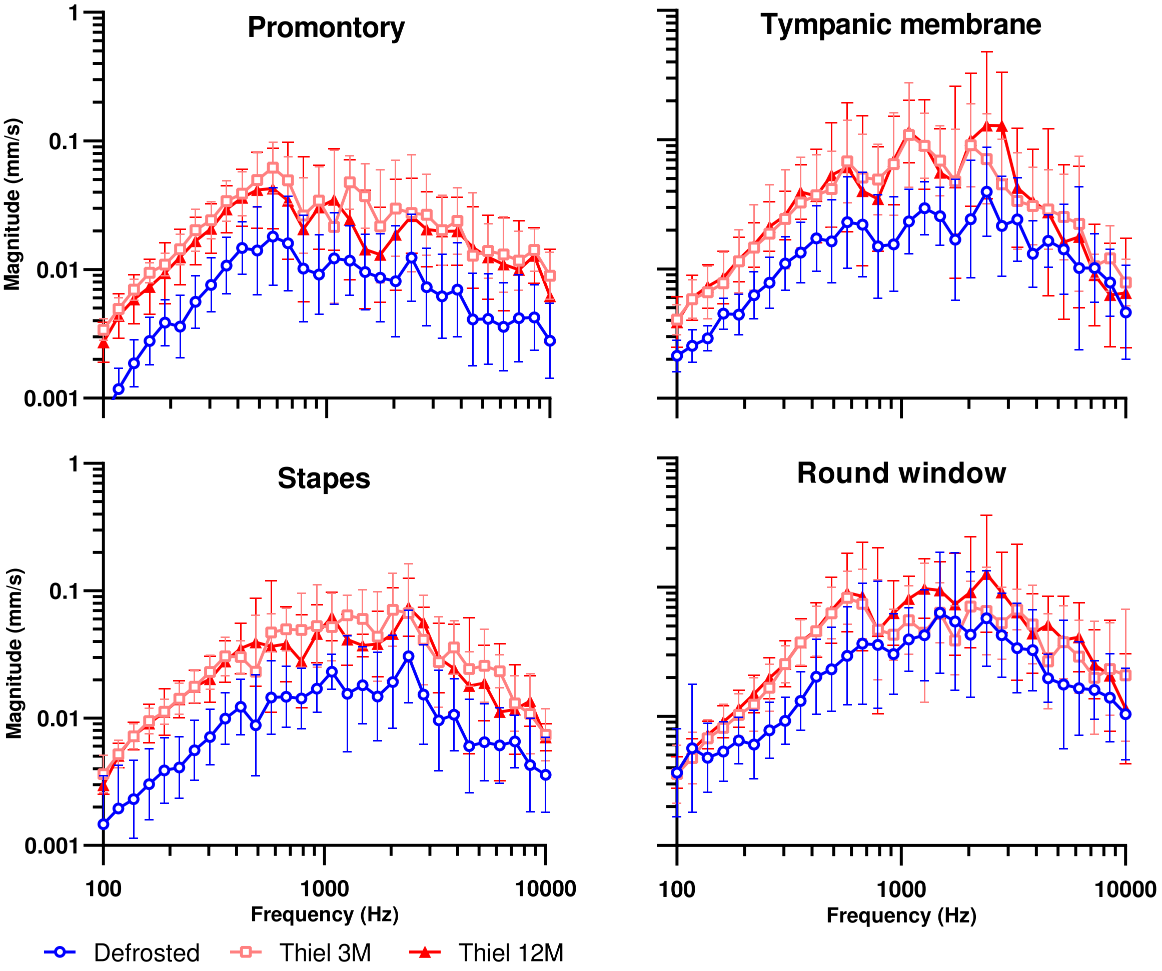

Fig. 3. Velocity of four measurement sites during BC stimulation. Mean magnitude of motion for defrosted ($n = 7$) and after 3 ($n = 7$) and 12 ($n = 6$) months of Thiel embalming measured at the promontory, tympanic membrane, stapes and round window. Error bars depict 95% CI.

tary figure 1. For all measurement positions the maximal cross correlation value is seen at zero quarter octave shift. Correlation levels greater than 60% were only found in few individual measurements.

In addition to the absolute induced motions (Fig. 3), the relative motion of the stapes versus the promontory, umbo and RW are provided in the supplementary material. They show comparable magnitude and phase shifts to Stenfelt et al. (Stenfelt et al., 2002) and no systematic differences between all conditions in this study.

3.2. Phases

Fig. 6 plots the phase difference between stapes and round window for AC (Graf et al., 2021) (left panel) and BC in this study (right panel). For AC stimulation there is a systematic 180° phase shift between stapes and round window motion in frequencies below 1000 Hz with higher variation up to 8000 Hz, as is consistent with the two-window hypothesis for forward stimulation (Stieger et al., 2013). In BC stimulation, in-phase motion of the stapes and RW is observed with low variation for frequencies up to 400 Hz, but not at higher frequencies, as the vibration pattern of the cochlear windows differ between AC and BC stimulation (Stenfelt, 2011). The limitations of our single point measurement of RW motion also contribute to the observed high-frequency differences between AC- and BC-induced window motion phases. Phase shifts between defrosted and Thiel embalmed specimen in different stages at low frequencies do not show a systematic difference.

3.3. Formalin

Fig. 7 shows the effect of additional formalin conservation in one specimen. For AC stimulation (upper plots) it was shown that formalin embalming following three months of Thiel extensively decreased sound transmission to the stapes and almost nullified vibration at the round window (Graf et al., 2021). Due to magnitudes below the noise levels at the RW for AC stimulation it was not possible to plot RW-phases below 1000 Hz, hence it was impossible to compare phase differences and make a statement about a fluid-filled cochlea after formalin embalming. However, in BC stimulation (lower plots) formalin embalming has only a minor effect on the magnitude of vibration of the tympanic membrane, stapes and RW.

4. Discussion

4.1. Defrosted versus Thiel embalmed whole head specimens

In this study we found significant differences of middle ear motion between defrosted and Thiel embalmed conditions for BC simulation in the same specimen. The umbo, promontory and stapes motion are increased by 10 dB in magnitude after Thiel conservation at most frequencies.

Such an increase was unexpected, as previous investigations using AC stimulation within the same specimens after Thiel embalming showed only a minor influence on the sound transmis-

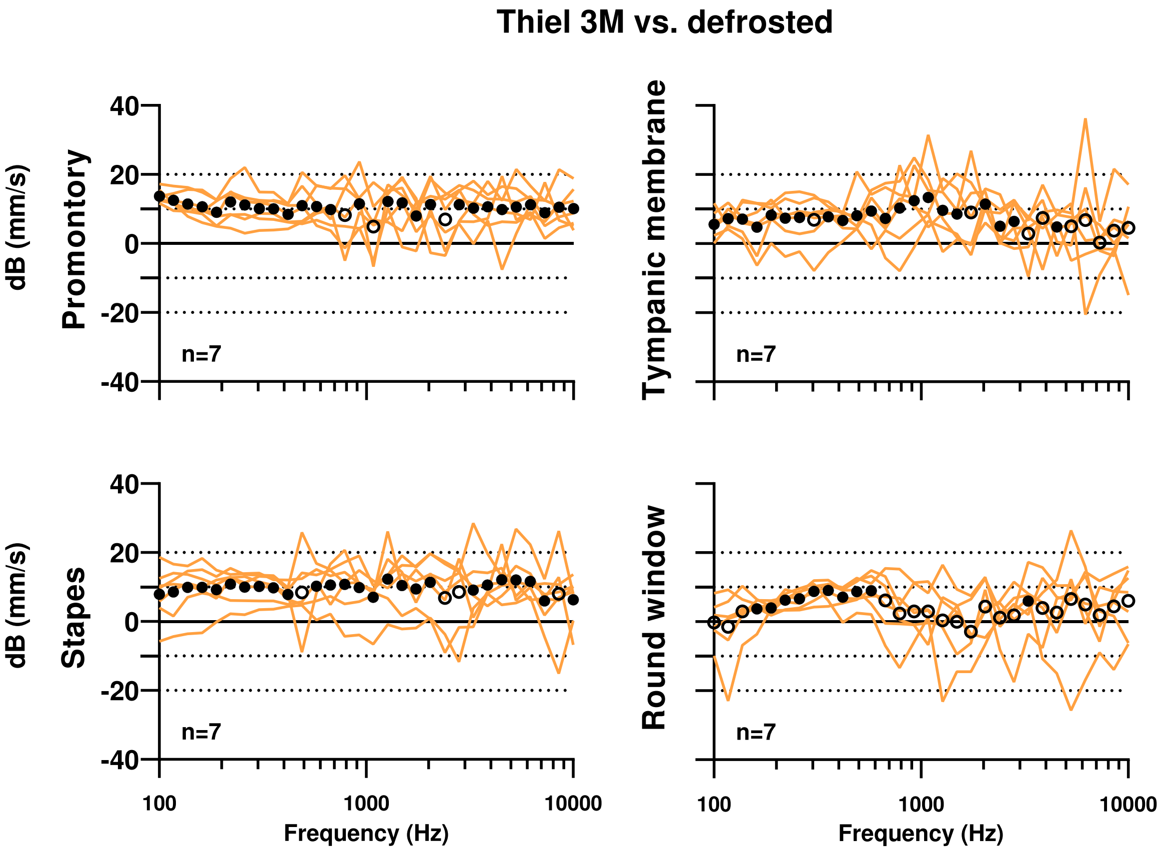

Fig. 4. Differences before and after 3 months of Thiel conservation for BC stimulation. Ratio of magnitude difference between defrosted and Thiel conservation for promontory, tympanic membrane, stapes, and round window. Averaged magnitude: dotted, significant differences ($p<0.05$): full black bullets, individual specimens: solid lines.

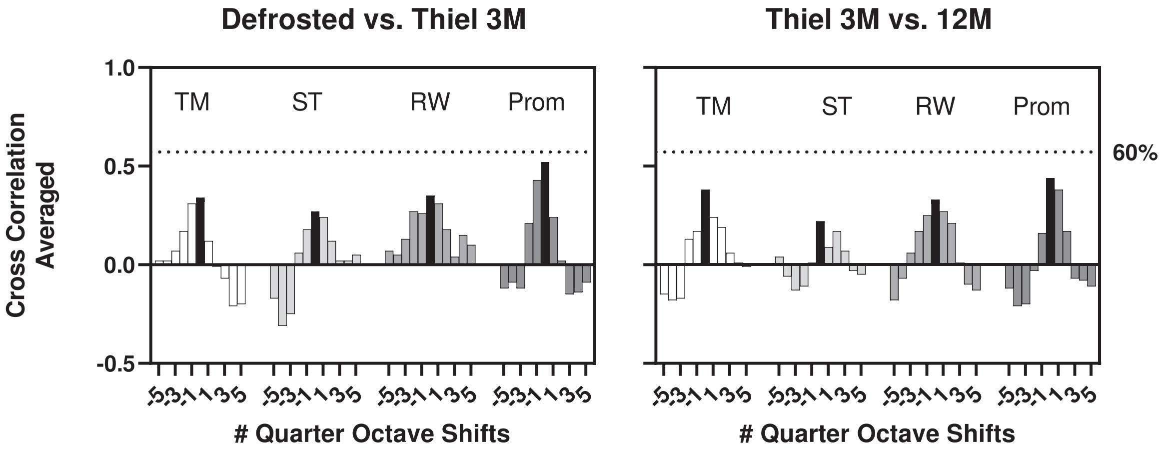

Fig. 5. Cross correlations analysis of all measurement locations and conditions. Dotted lines depict 60% level.

sion within the middle ear (Graf et al., 2021). A decreased stapes motion of up to 10 dB is limited to a mid-frequency range, which we attributed to a possible stiffening of the middle ear tendons and ligaments due to the 1.5% Formalin content in the Thiel fluid (Thiel, 1992).

In this study we used cross-correlation analyses to detect stiffening induced changes in ossicular motion, and only found correlation levels greater than 60% with an unambiguous peak in some individuals (see supplemental figure 1). Additionaly, we observed the highest aveaged cross corelation value at zero frequency shift for all locations and conservation status. Therefore, these results do not support a Thiel-related change in stiffness in BC induced vibration patterns.

The frequency-independent 10 dB magnitude increase (Fig. 3) at the umbo, promontory and stapes after Thiel embalming is consistent with a systematic error, we found no evidence of such an error. We can exclude a systematic electrical stimulation error, as the recorded voltage at the Baha differs less than 0.5 dB for each embalming condition (Fig. 2). Furthermore, a 10 dB attenuated stimulus resulted in an appropriately lowered motion magnitude for each condition, consistent with measurement and stimulus linearity (data not shown).

Prior to the BC experiments, we confirmed the integrity of the middle ear in each specimen before and after Thiel conservation by comparing the AC stimulated stapes motion to the ASTM standard ((Graf et al., 2021), and Fig. 2). Additionally, we confirmed a 180°

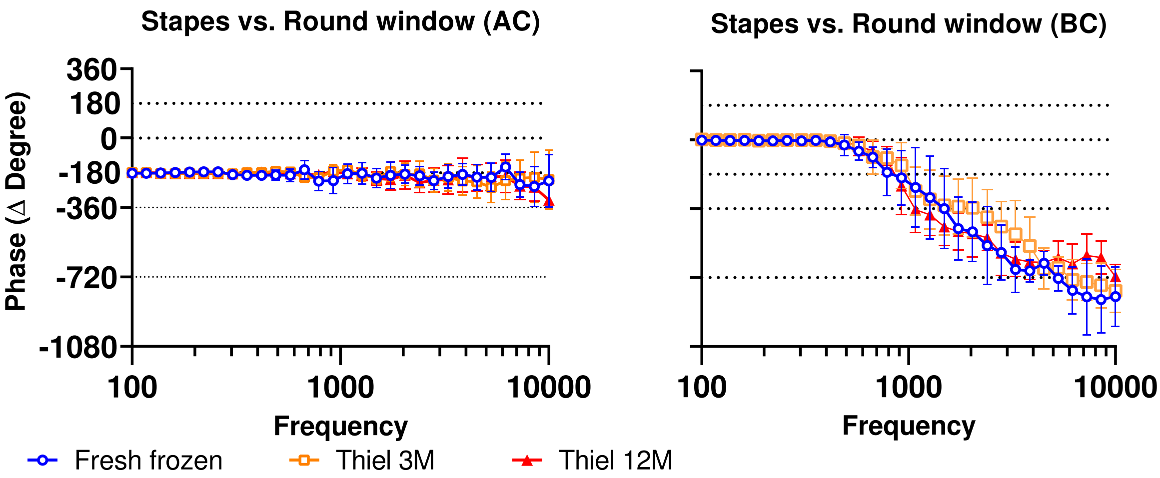

Fig. 6. Phase differences between stapes and round window with AC (left) and BC stimulation (right) stimulation for all preservation conditions. AC: defrosted (n = 10,) Thiel 3 months (n = 9), Thiel 12 months (n = 9). BC: defrosted (n = 7), Thiel 3 months (n = 7), Thiel 12 months (n = 6).

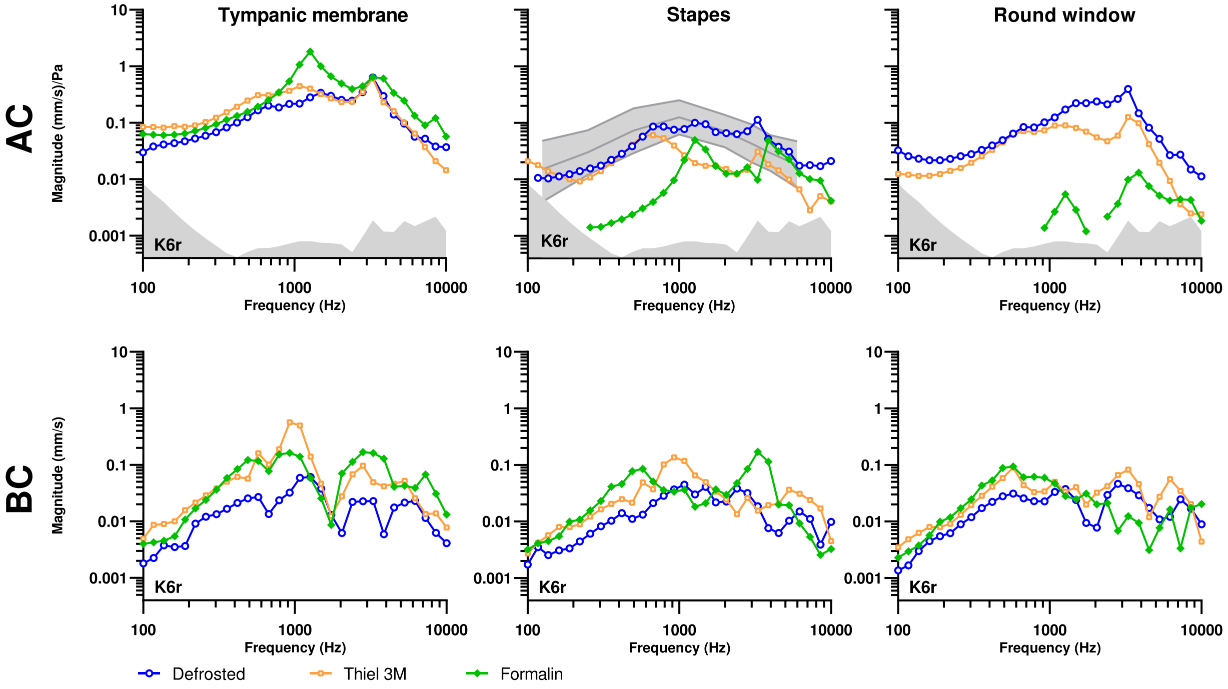

Fig. 7. Transfer function on tympanic membrane, stapes and round window of one ear, which was first measured defrosted (blue circles), then conserved in Thiel for 3 months (orange squares) and then was conserved in formalin (green filled diamonds) for AC (upper row (Graf et al., 2021)) and BC (lower row). Gray range in stapes with air conduction stimulation depicts the ASTM standard. Dark gray basal area in upper row: promontory motion at AC stimulation to define the artifact/noise level.

phase difference between the stapes and round window motion for AC stimulation at low frequencies (Fig. 6, left) which indicates that the gross cochlear mechanics for forward stimulation is not altered both before and after Thiel embalmment (Stieger et al., 2013).

Guignard et al. (Guignard et al., 2013) showed that the sound transmission in BC stimulated Thiel conserved whole head specimens is comparable to literature data of live subjects (0–10 dB difference at the umbo) (Röösli et al., 2012) and fresh cadaveric specimen (0–4 dB difference at promontory) (Eeg-Olofsson et al., 2008). These similarities are contrary to the consistent 10 dB in the BC-induced motions we report after exposure to Thiel solution (Fig. 3, 4). There is a major methodological difference between Guignard et al. and the present study. Guignard used whole heads from bodies that were embalmed according to the original Thiel embalming protocol (Thiel, 1992), where the whole body was perfused with Thiel fluid through the iliac artery in addition to the immersion bath. Furthermore, the brain and central nervous system were perfused with a modified solution through the Lamina cribrosa and a lumbar puncture (Thiel, 2002). This procedure would have required us to perform the first measurements in a fresh whole-body cadaver prior to the conservation in the anatomic institute, which was not possible due to logistical reasons. To investigate the effect of these differences we conducted several additional investigations.

4.2. CT scans

CT scans were obtained in our local anatomic institute to quantify the intracranial content of the specimens at different stages of the experiment. In the initial frozen condition, the brain filled the entire neurocranium and the ventricular cerebrospinal fluid system is differentiated (Fig. 8, top left). Similar radiological findings were seen in an independent head scan series of Thiel embalmed whole

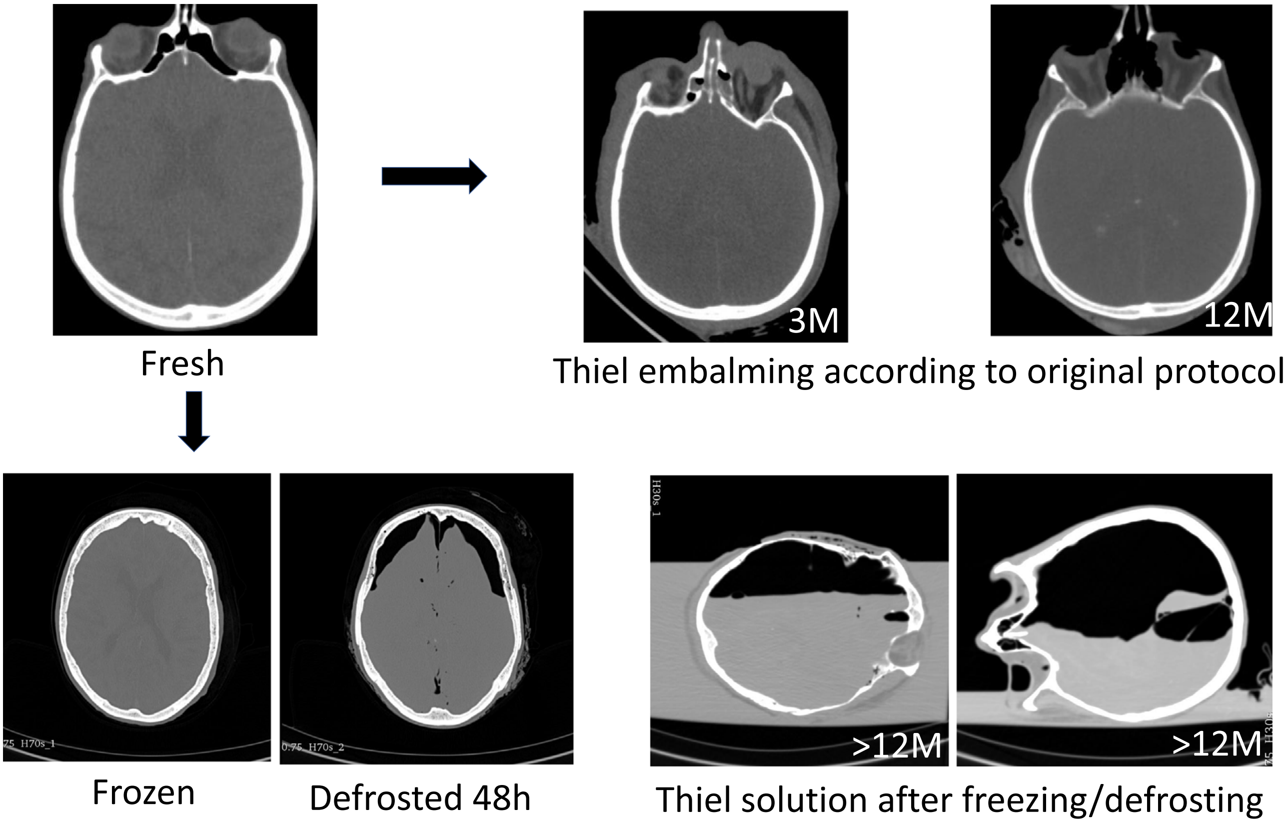

Fig. 8. CT scans of cadaveric heads. Upper row left: immediately postmortem. Upper row right: head CT scans of whole body specimens conserved according to the original Thiel protocol after 3 and 12 months. Lower row left pictures: CT scans of a whole head specimen frozen and 48 h defrosted as in our experimental setup. Lower row right pictures: CT scans of whole heads taken within storage containers with different levels of fluid after more than 12 months of embalming with initial freezing/defrosting according to our experimental protocol.

bodies according to the original protocol (Fig. 8, two top right images) where the skull remains completely filled with brain mass over 12 months.

As a modification to Guignard (Guignard et al., 2013) and the original Thiel embalming protocol, in our study the whole head specimens were initially frozen and defrosted before the measurements for the purpose to compare defrosted to the Thiel embalmed condition, which was the actual aim of our study. There is evidence that freezing and defrosting changes the volume and structure of the brain (Sugimoto et al., 2016), and such changes may alter the response to skull vibrations. CT scans showed changes of brain structure and intracranial distribution after a defrosting time of 48 h and prior to Thiel exposure (Fig. 8 lower left plots). The lower right plots of Fig. 8 show CT scans of whole heads of the current study, taken >12 months after the initial measurement within a container. The specimens have been fully immersed in Thiel solution during the 12 months in a tank, and after the very last measurement they were stored in separate containers. Two to three hours before each measurement the heads were taken out of the Thiel solution to allow fluid to drip from the ear and avoid the later accumulation of fluid within the middle and external ear during the measurements (Stieger et al., 2012). Because the head was detached from the body and the brain changes before and after embalming it is probable that environment air went inside the cranial cavity through the foramen magnum or untight vessels. Sugimoto et al. (Sugimoto et al., 2016) describe major intracranial accumulation of gas due to putrefaction within a few days in whole body cadavers without any extrinsic injury. We did not conduct a chemical distinction between gas and ambient air.

4.3. Replacement of brain volume

We evaluated the effect of the changed brain structure after embalming by substituting the air-filled part of the cranial cavity with Thiel fluid in one whole head specimen with predominant intracranial gas content 18 months after initial embalmment. The promontory vibration was measured in one ear of this partially air-filled whole head (similar to Fig. 8 lower row, right), afterwards all skull openings were sealed and the neurocranium was filled with approximately 1.8 liters of Thiel fluid through a drilled hole at the most elevated point of the skull in measurement position. To ensure the head stayed nearly completely full of fluid, we confirmed that not more than a few milliliters had to be refilled after the measurement for 2–3 min. The BC transfer functions before and after fluid substitution are plotted in Fig. 9 (left).

The Thiel fluid-filling was associated with a minimal increase in the magnitude of promontory motion around 1000 Hz but was comparable to the previous measurements after 3 and 18 months of embalming (Fig. 9, left). Therefore, fluid-filling did not reverse the 10 dB magnitude increase between the defrosted condition and the embalming. A finite element method (FEM) study of the consequences of a liquid filled brain and an extracted “air-brain” describes a minor increase of the liquid-filled brain of maximally 5 dB on the ipsilateral promontory motion in predominantly low frequencies, an "air-brain" had a negligible effect (Prodanovic and Stenfelt, 2021). Although a 5 dB decrease after fluid-filling could not clearly be seen in this single specimen, this suggests that the pure weight of the intracranial content cannot explain our observations. Possible alterations of the brain’s consistency and adher-

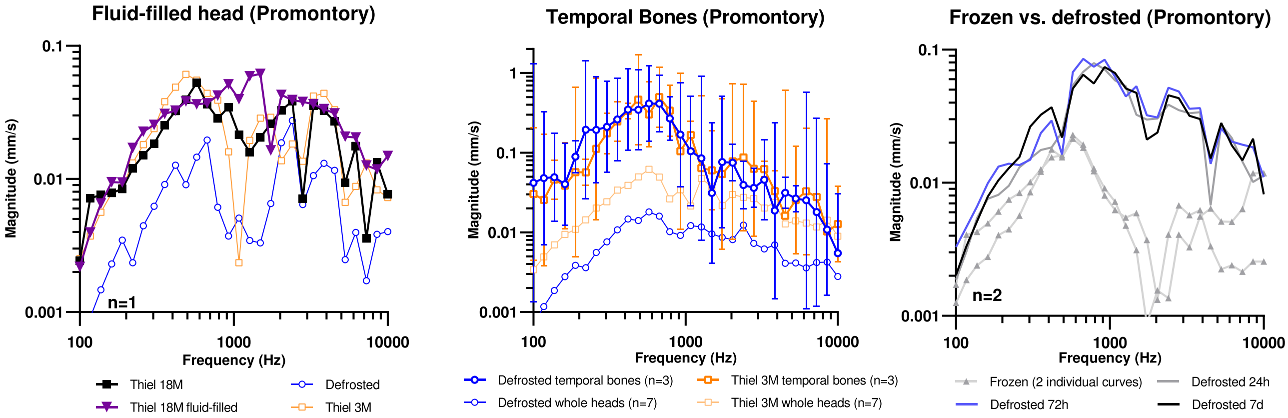

Fig. 9. Promontory motion during BC stimulation for different investigations. Left: motion in different conditions of one ear of a whole-head specimen. Replacement of lost brain volume by fluid had no major influence on sound transmission. Middle: Effect of Thiel embalming on bone conduction stimulation in $n = 3$ extracted temporal bone specimens, promontory. Right: Measurement of one additional whole head ($n = 2$ ears) short-term fresh frozen: 2 h after defrosting (individual curves plotted), and after 24 h, 48 h, 168 h (=7d) (all mean curves of $n = 2$).

ence to the skull might contribute to the vibration changes we see post-exposure to Thiel (see Section 4.5).

4.4. Temporal bone specimens

To assess if the middle ear mechanics and the temporal bone itself contribute to the observed 10 dB increase vibration measurements during BC stimulation after Thiel embalming, we analyzed the effect of Thiel embalming in extracted temporal bones in the same specimen. Therefore, the promontory motion of $n = 3$ Baha-stimulated extracted temporal bones was measured before and after embalming in the same manner as the whole heads. The magnitude of promontory motion in the temporal bones was higher than in whole heads over all frequencies, which is expected because of the clearly lower mass of the temporal bones (average 0.25 kg) compared to the entire whole heads (approximately 5 kg, (Panjabi et al., 1998)). For frequencies below 1000 Hz the promontory motion in temporal bones are 20–30 dB higher that in whole heads. The low frequency change can be explained directly with the estimated weight difference of 26 dB, as BC motion in whole heads is mass-spring system-like below 1000 Hz (Dobrev et al., 2017; Stenfelt, 2011), whereas it gets more complicated in frequencies above 1000 Hz. After 3 months of fixation in a Thiel bath there was no significant change of the average magnitude measured in the temporal bone (Fig. 9, middle panel). We found an increased weight in the extracted temporal bones after Thiel conservation (237 g to 268 g, +14%). 14% corresponds to 1.5 dB increase in weight. Such a small increase can hardly be seen (Fig. 9) when inverse linear effect between motion and weight is expected. Therefore, neither the change in mass nor the middle ear mechanics of the temporal bone seem to be main reasons for the 10 dB difference before and after Thiel conservation in defrosted whole heads.

4.5. Initial measurement prior to conservation

Thiel conserved specimens show good reproducibility for LDV measurement using BC stimulation between 3 and 12 months, and in a single specimen up to 18 months. Long-term reproducibility was also shown for AC stimulation (Graf et al., 2021). As quality indicators for good AC measurements (i.e. 180° phase shift between RW and stapes motion and comparison of AC stimulation transfer function to the ASTM standard) have been positive, we directly performed the BC stimulation after defrosting. To avoid decay, we started conservation as soon as possible after the initial measurements. In one whole head specimen, we investigated the effect of defrosting on the promontory motion after 2, 24, 48, and 168 h conservation at ambient room temperature without subsequent Thiel (Fig. 9, right plot). After two hours the promontory motion over all frequencies was on average 13 dB lower when compared to 24, 48 h and 7 days. In the frequencies above 570 Hz the difference was up to 20 dB.

We used the ASTM standard and the 180° window motion difference as indicators of normal responses to AC stimuli in our protocol, and these tests were positive between 2 and 24 h of defrosting. This was the case in the period between 2 and 24 h of defrosting. We assume the reduced motion during BC stimulation before Thiel embalming to be due to the fact that parts of the brain were still frozen.

Hence, the problem of defrosted whole head specimen seems to be that sound transmission characteristics in BC stimulation are significantly lower in case of too early measurement (approximately 12 h) and thereafter the brain mass is shrinking. A proof of quality by measuring middle ear transfer function (ASTM) or 180° phase shift between stapes and round window is no reliable indicator. In contrast, a Thiel embalmed cadaver head stays constant over long term.

4.6. Summary

The additional investigations for an explanation of a frequency-independent 10 dB BC motion increase after Thiel embalming supports the notion that possible changes of the skull bone's elastic and plastic characteristic (Stefan et al., 2010) do not have a major influence on BC. Instead, the 10 dB BC motion increase is rather caused by alterations inside the cranial cavity. We discovered a progressing brain damage that can be explained by freezing and defrosting (Sugimoto et al., 2016) but the brain weight- and volume loss does not seem to explain the BC motion changes as shown in Section 4.3 and FEM simulations (Prodanovic and Stenfelt, 2021). We found evidence of a major increase in BC induced motion when entirely frozen specimens are completely defrosted (Section 4.5), hence 12 h of defrosting used in our experimental protocol (Section 2.5) is likely insufficient. Further systematic investigations or simulations would be required to determine if a frozen intracranial content leads to a mechanically firm connection to the skull.

Conclusion

Similar to AC induced motion, BC induced motion in Thiel conserved specimens provide reproducible results over long period with comparable results to defrosted human cadaver specimens. However, while the ASTM standard and half cycle shift between stapes and round window serves as quality control for AC, for BC stimulation is has to be ensured that the specimen is completely defrosted.

Author statement

Only human cadaveric specimens were used for this experiment. The use of the specimens has been approved by the local ethics committee (EKNZ BASEC 2016-00,599)

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Lukas Graf: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Andreas Arnold:** Validation, Visualization, Writing – original draft, Writing – review & editing. **Sandra Blache:** Formal analysis, Investigation, Methodology. **Flurin Honegger:** Data curation, Formal analysis, Software, Writing – review & editing. **Magdalena Müller-Gerbl:** Conceptualization, Methodology, Resources. **Christof Stieger:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.heares.2023.108700.

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