Changes in cochlear blood flow in mice due to loud sound exposure measured with Doppler optical microangiography and laser Doppler flowmetry

Roberto Reif1, Zhongwei Zhi1, Suzan Dziennis1, Alfred L. Nuttall2, Ruikang K. Wang1

1University of Washington, Department of Bioengineering, Seattle, WA 98195, USA; 2Oregon Hearing Research Center, School of Medicine, Oregon Health and Science University, Portland, OR 97239, USA

Corresponding to: Ruikang K. Wang. University of Washington, Department of Bioengineering, 3720 15th Avenue N.E., Seattle, WA 98195, USA. Email: wangrk@uw.edu.

Abstract: In this work we determined the contributions of loud sound exposure (LSE) on cochlear blood flow (CoBF) in an in vivo anesthetized mouse model. A broadband noise system (20 kHz bandwidth) with an intensity of 119 dB SPL, was used for a period of one hour to produce a loud sound stimulus. Two techniques were used to study the changes in blood flow, a Doppler optical microangiography (DOMAG) system; which can measure the blood flow within individual cochlear vessels, and a laser Doppler flowmetry (LDF) system; which averages the blood flow within a volume (a hemisphere of ~1.5 mm radius) of tissue. Both systems determined that the blood flow within the cochlea is reduced due to the LSE stimulation.

Keywords: Cochlear blood flow (CoBF); hypoxia; Doppler optical microangiography (DOMAG)

Submitted Oct 01, 2013. Accepted for publication Oct 08, 2013. doi: 10.3978/j.issn.2223-4292.2013.10.02

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Introduction

Adequate cochlear blood supply is crucial for supplying the high cellular metabolic demand required for proper cochlear function. Cochlear ischemia has been related to several hearing disorders, which include noise-induced hearing loss, age related hearing loss, sudden sensorineural hearing loss, tinnitus and Ménière’s disease (1-9). The study of the cochlear microcirculation can be noteworthy for determining the causes and possible treatments of hearing loss. Understanding the underlying mechanisms of cochlear microcirculation is vital for the management of these pathologies (10).

The ability to study cochlear blood flow (CoBF) in vivo, has been challenging due to the invasiveness of accessing the inner ear and the cochlea, and the limited number of tools available. Histology has provided morphological information that captures a single time-point measurement about the cochlea vasculature; however, its results are difficult to interpret due to its invasiveness and ex vivo analysis (11). Other single time-point methods include the microsphere method, which has been used to study CoBF (12-14); however, it requires a large number of captured spheres for statistical purposes. Corrosion cast provides three-dimensional images of the vasculature; however, it is an invasive ex vivo technique that provides vessel morphology but lacks blood flow information (15). Laser Doppler flowmetry (LDF) is a real-time method that can measure relative changes in blood flow within a volume of tissue (a hemisphere of ~1.5 mm radius), which depends on the source detector separation and the wavelength of light used by the system (16,17). LDF can measure the changes in flow (number of red blood cells in a given volume times the mean velocity); however, its spatial resolution is limited. LDF has been previously and extensively used to measure the changes in CoBF during the occlusion of the stapedial artery and anterior inferior cerebellar artery in rats (18), topical application of vasodilators in rats and guinea pigs (19), loud sound exposure (LSE) in guinea
pigs (20,21), and hypoxia in mice (22). Laser speckle contrast imaging, which is a two dimensional (no depth information) real-time method that obtains relative values of the changes in blood flow within a volume of tissue, has been used to study the cochlea due to a systemic hypoxia (23). Doppler optical microangiography (DOMAG) is an imaging modality based on optical coherence tomography (OCT) (24-30). DOMAG is another real-time method which differs from LDF in that it enables the non-invasive measurement of the three dimensional microstructural and microvascular composition of the biological tissues. OCT and DOMAG have previously been used to image the cochlea in vivo (31-33), and used for studies of systemic hypoxia (22,23). However, the study of the relationship between LSE and vascular changes has not yet been investigated with DOMAG.

Currently, there is some ambiguity over the effects of LSE on CoBF. For example, histological studies indicate a decrease in circulation based on the alteration of its vessels, such as endothelial wall swelling, constriction of capillaries and unusual spacing of red blood cells (11,34-36). Using various sound parameters, studies with LDF have also indicated a decrease in CoBF (20,37). On the other hand, other studies have indicated an increase (12,38-40), or no change (13,14,41) in CoBF.

In this study we used DOMAG and LDF to examine the changes in CoBF in anesthetized mice, subjected to a LSE (119 dB SPL) stimulus which consists of white noise (20 kHz bandwidth) for a one hour period.

**Materials and methods**

The experiments were performed based on the National Institute of Health guidelines for research animal care and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Washington. Male mice C57BL/6J (Charles River Laboratories, Hollister, CA) approximately 10 to 14 weeks old of age with body weight from 25 to 28 g were used in all experiments.

**Surgical exposure of the mouse cochlea**

Mice were anesthetized with 1.5% isoflurane in oxygen-enriched air (20% oxygen/80% medical air). The body temperature was maintained between 36.5-37.5 °C by using a feedback rectal probe and a heating pad (Harvard Apparatus, location). The head of the mouse was immobilized onto an imaging platform to minimize movement such that the right cochlea was exposed ventrally through the neck, as previously described (22,23,42). An incision was made down the midline of the neck and the left submandibular gland and posterior belly of the digastric muscle were removed by cautery. The external carotid artery was ligated inferior to the bifurcation. The positions of the hypoglossal and facial nerves and the sternocleidomastoid muscles were used to identify the location of the boney bulla that contains the cochlea. A small hole was made on the bulla to expose the cochlea, while maintaining the tympanic membrane and vessels intact. As a result only part of the cochlea was properly exposed to image with the DOMAG system, as observed in Figure 1A, while the view of the rest of the cochlea was blocked by the tympanic membrane.

**DOMAG**

A spectral domain OCT system was used as presented in Figure 1B (43). A broadband superluminescent diode with a central wavelength of 1,310 nm and a spectral bandwidth of 110 nm was used to achieve a theoretical axial resolution of ~7.2 µm in air. The light was split into two beams using a 2×2 optical coupler. The light of one beam was reflected by a mirror (reference arm), and the light from the other beam was reflected by the cochlea (sample arm). In the sample arm, the light was coupled into an optical system, which contained a collimator, a pair of galvo mirrors, and an objective lens (focal length 30 mm), which was used to focus the probing light on the region of interest, and provided a lateral resolution of ~12 µm. The spectrometer contained an InGaAs line scan camera (SUI, Goodrich Corp.) capable of capturing data at a ~92 kHz A-line scan rate. The spectrometer has a spectral resolution of 0.141 nm, providing a measured imaging depth of ~3.0 mm in air. The system dynamic range was measured at ~105 dB at the depth position of 0.5 mm with an incident optical power of 2.5 mW in the sample arm.

The methods used to capture and process the data were based on the DOMAG technique (44). This method enables the extraction of the three-dimensional microvascular images. Briefly, the X-Y galvanometer where used to scan the OCT beam across the sample, with one scanner moving the beam in the X direction, also known as the fast scan or B-scan, and another scanner moving the beam in the Y direction, also known as the slow scan or C-scan. In this study, the camera had an A-line scan rate of 5 kHz. Each B-scan contained 2,000 A-lines that span 1.5 mm. In each
experiment we acquired a three-dimensional data set which consisted of 240 discrete locations in the slow scan where 5 B-frames were collected at each location and averaged together. The slow scan had a length of 1.5 mm. The frame rate was set to two frames per second. The data cube of the three-dimensional image was composed of 1,024×2,000×240 (Z-X-Y) voxels, and the total acquisition time was ten minutes (240 locations ×5 frames per location/2 frames per second).

We collected a three dimensional data set at five time points throughout the experiment. LSE was applied for 60 minutes. The first data point was collected before the LSE stimulus, then three data points were collected at 15, 30 and 45 minutes after the initiation of the stimulus, and finally a data point was collected immediately after the noise stimulus was terminated. The time of these data points will be referred to as 0, 15, 30, 45 and 60 minutes, respectively. It is important to note that the collection of a data point took ten minutes. Also, on a separate animal we acquired data with and without the presence of the LSE, and determined that the LSE does not add artifacts to the measurements; therefore, we were confident we could acquire the data while the LSE stimulus was present.

DOMAG is a method that has been used to calculate the axial blood flow velocity inside vessels. DOMAG has previously been validated by using tissue calibration phantoms, and it has been used for several in vivo studies (44).

The red blood cell velocity in the axial direction (direction of the incident OCT beam) is derived by the phase difference between adjacent lines. The relationship between the phase difference ($\Delta \phi$) and the axial velocity ($V_z$) is given by:

$$V_z = \frac{\Delta \phi \cdot \lambda_0}{4 \cdot \pi \cdot n \cdot \Delta t}$$

where $\lambda_0$ is the central wavelength of the light source (1,310 nm), $n$ is the refractive index of the tissue (~1.35) and $\Delta t$ is the time interval between adjacent lines (1/5 kHz =200 µs). The maximum axial velocity measured by our system parameters was ±1.2 mm/s.

The absolute blood flow rate is an important parameter that can be quantified within blood vessels. An approach to obtain the absolute blood flow velocity consists on obtaining the absolute velocity of blood flow which requires the knowledge of the Doppler angle ($\theta$) (45), where $V = \text{abs}(V_z / \cos \theta)$. The Doppler angle can be calculated by the three-dimensional data set; however it is a cumbersome procedure. A recent approach has been used to determine the absolute blood flow without requiring the knowledge of the Doppler angle (46,47); therefore, simplifying the calculation. The method consists of obtaining a three dimensional map of $V_z (x,y,z)$, from which an en face plane can visualize the vessels, as presented in Figure 2. The absolute blood flow velocity is obtained by integrating the axial flow components over the vessel cross-section on the
en face plane by using:

\[ F = \int_{xy-plane} \int V_z(x,y) \, dx \, dy \]

To determine the flow within a vessel, the area of the vessel was manually selected. To minimize the error in the vessel area, each vessel was segmented three times and averaged together, and the value of the area and flow was averaged together.

Figure 2C shows the scalas within the cochlea and the large radiating blood vessels that originate in the apical turn close to the helicotrema. The colors of the vessels indicate the flow direction (red towards the incident OCT beam, and blue away from the incident OCT beam). The radiating vessels resemble the images depicted by Iwagaki et al. who used a resin cast technique (48).

LDF

A LDF system (Periflux PF 2B, Perimed), was used to estimate the changes in flow within a large volume of the cochlea. The tip of the LDF was placed on the cochlea. Given the size of the probe, which evaluates the flow within a hemisphere of ~1.5 mm radius, it was difficult to localize the measurement to only the apical or basal turn. Therefore, we speculate that the measurements cover a volume that contains the whole cochlea (both the apical and basal turns). The sound was turned off during the time when measurements were obtained to avoid the presence of noise artifacts (21). Measurements were obtained every five minutes throughout an hour.

LSE

A loud sound speaker system was home built, which produced a broadband white noise (up to 20 kHz), with an intensity of 119 dB SPL. The exposure stimulus was generated by a custom-made white noise source, and delivered through a cone fitted to a plastic tube in the top of the sound exposure box. The tip of the tube was placed within 1 mm of the ear canal. The LSE system and an example of the time and frequency domain of the white noise are presented in Figure 3.

A total of 12 mice were used. There were six mice (three control and three exposed to noise) that were imaged by the DOMAG system. Similarly, a separate cohort of six mice (three control and three exposed to noise) that were measured by the LDF system.

Statistical analysis

Statistical analysis was carried out using Matlab. The differences in flow between the control and noise group using DOMAG were analyzed by a student’s t-test, where it is assumed that the sample populations are normally distributed. The criterion for statistical significance was P<0.05.

Results

Using the DOMAG system we were able to obtain both
structural and microvascular images of the cochlea. For the DOMAG analysis, five of the radiating vessels (as shown in Figure 2B,C) were selected from each cochlea; therefore, there were 15 vessels in the control group and 15 vessels in the noise group. Figure 4A presents the mean and standard deviation of the percentage change in flow of the 15 vessels in each group, referenced to the zero time point.

For the LDF measurements, the data was acquired from three cochleas in each group (control and noise). The mean and standard deviation of the percentage change in flow, referenced to the zero time point, is presented in Figure 4B.

Discussion

This study demonstrates that LSE decreases the blood flow within the cochlea throughout one hour stimulus. Two methods (DOMAG and LDF) were used to validate this hypothesis. It is important to mention that although both methods can determine the changes in CoBF, they probe different tissue volumes. Therefore, the change in blood flow measured by each technique contains a different set of blood vessels. DOMAG can estimate the blood flow within single large vessels in the cochlea; however, LDF can estimate the change in blood flow within a volume of tissue (a hemisphere of ~1.5 mm radius), which contains large vessels and capillaries. Figure 2C shows the three dimensional image of the large radiating vessels that expand from the apical turn. The mouse cochlea has 1.75 turns (49).

DOMAG was used to estimate the blood flow inside individual vessels. An en face analysis was used which allows for the total blood flow estimation without the knowledge of the Doppler angle, which can be challenging to calculate. This method uses Equation 1 and 2. Figure 4A presents the changes in flow for the vessels analyzed using the DOMAG method. In general we note that after 60 minutes there is a blood flow reduction in both groups; however, the noise...
group has a drastic reduction (~30%) compared to the control counterpart (~10%). The difference between both groups is statistically significant. We speculate that the slight decrease in flow in the control group may be due to the long period of time that the mice were under anesthesia, which could affect part of the blood flow. Another noticeable characteristic is the overlap of the standard deviations observed between the two groups. This indicates that not all vessels within a mouse have the same response to the LSE.

Figure 4B depicts the changes in blood flow averaged throughout the cochlea using LDF. This technique has previously been used in the cochlea of guinea pigs (20), and it has demonstrated that noise contributes to a reduction in CoBF. Our results demonstrate that there is approximately a 10% reduction in blood flow throughout one hour of LSE in the noise group compared to an approximately 0% change in the control group. Given that the size of the mouse cochlea is small, the LDF averages the vessels in both the apical and basal turns. The noise bandwidth was limited to 20 kHz (mostly affecting the apical turn), while the mouse hearing can go up to 75 kHz (50). Therefore, the LSE stimulus only affects a sub-set of the whole cochlea which was averaged together by the LDF, contributing to the smaller reduction in flow compared to the DOMAG method.

Although both DOMAG and LDF indicate a reduction in blood flow due to LSE, the magnitude of change after 60 minutes was different, 30% and 10%, respectively. Also, LDF shows a change within the first five minutes which remains constant through time, while DOMAG presents a strong change at about 30 minutes. The difference in the results may be due to the difference in the vessels analyzed with both techniques.

Conclusions

In this study, we have validated that the cochlea has a reduction in blood flow due to LSE. Both DOMAG and LDF determined that there is a reduction in CoBF of ~30% and ~10%, respectively. The difference between the values determined by these techniques can be explained by the differences in the vessels analyzed by each method. DOMAG analyzes the blood flow in a few selected large vessels in the apical turn, while LDF averages all the vessels and capillaries within a volume of tissue containing the whole cochlea. Further studies should be made to better understand the phenomenon of the change in CoBF due to LSE. Understanding the underlying causes in the reduction of blood flow can aid in improving the diagnosis and treatment for several hearing disorders, such as the ones caused by LSE.

Acknowledgements

This work was supported in part by research grants from the National Institutes of Health (R01DC01201 and R01DC000105). We would like to thank Edward Porsov for the technical help and support received throughout this work. The content is solely the responsibility of the authors and does not necessarily represent the official views of the grant-giving bodies.

Disclosure: The authors declare no conflict of interest.

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