SPECIFIC AIMS

Hearing loss and deafness are ailments that have caused a devastating blow to the quality of life of a wide spectrum of individuals from around the world. Despite its increasing prevalence, clinicians have very few responsive therapies beyond an assessment involving a basic hearing test. Medical outlooks involving complications of the inner ear only worsen in their prognosis as time progresses, driving a shift towards prevention rather than restoration. A common form of hearing loss known as sudden sensorineural hearing loss (SSNHL), also referred to as sudden deafness, is estimated to strike approximately 65,000 people a year; however, the disease can often be undiagnosed completely [1]. Due to the increasing morbidity with age, high prevalence of misdiagnosis, and need for a rapid response to prevent further hearing loss, doctors and clinicians are in need of a reactive method to more accurately assess patients and their unique outcomes. The focus of this study is to establish a new methodology for production and utilization of hyperpolarized (HP) contrast agents in combination with functional magnetic resonance (fMRI) and real-time metabolic imaging (rMI), in order to identify bioactive molecules that may serve as indicators of different forms of hearing loss. The goal of this study is that by giving doctors and clinicians a tool that is more effective at diagnosis, they may be able to steer patients towards treatments that provide more favorable outcomes and reduced chance of further hearing loss.

It has been hypothesized that blood prestin concentration levels can be reflective of cochlear damage [2]. One study conducted by Iliadou et. al. found a direct correlation in blood prestin levels and the extent of cochlear damage in mice. Another study performed by Ardenkjaer-Larsen et. al. found that hyperpolarized molecules can produce signals with a three-fold increase in signal-to-noise ratios [3]. This phenomena was found to be transferable to ^{13}C MRI examination. We hypothesize that by combining hyperpolarizing technology and active targeting of prestin, a dual-image of the inner-ear and auditory cortex can be generated and a correlation map between metabolism and cochlear damage can be drawn.

In this study there will be two parts. The first will involve the synthesis of the targeting ligand, PrTP1, with and without radioactive ^{13}C, and verification that the signal is significant in a NMR. The second part will involve two groups of rabbits, one being a control and the other being introduced to ototoxic factors. Both groups will be examined using a fMRI, ^{13}C contrast for the ear, and blood oxygenation level dependent (BOLD) contrast for the auditory cortex. The experimental group will be examined before, and after treatment with cisplatin.

AIM 1: Assess the viability of hyperpolarized ^{13}C PrTP1 as a metabolic marker for prestin

Hypothesis: Using a classical hyperpolarizer, the signal generated by ^{13}C PrTP1 will be able to track prestin in real-time.

Approach 1: Synthesize radioactive and non-radioactive PrTP1, incubate with prestin, and verify a 10,000 to 1 increase in signal to noise.

AIM 2: Assess what parts of the inner ear's change in metabolism correlate with loss of activation in the auditory cortex.

Hypothesis 2: The region closest to the inner hair cells, rich in prestin, will correlate the most with a loss of activation of the auditory cortex.

Approach 2: ^{13}C MRI and BOLD fMRI image rabbits saturated with HP ^{13}C PrTP1 and induced deafness, and compare changes in prestin levels with loss of activation of the auditory cortex.

SIGNIFICANCE

There has been a recent paradigm shift in the medical community involving the improvement of one's quality of life rather than only quantity of life. While AI and other emerging technologies promise to solve generational diseases like Alzheimers and cancer, there is still much research and development to be done involving other debilitating, but non-lethal diseases. One of the most important contributions towards living a meaningful life is being able to accurately perceive one's world. Such a task becomes challenging when any number of the 5 senses are impaired.

About 2 to 3 per every 1000 children born in the United States will be born with detectable hearing loss in both ears [1]. Additionally, 1 in 8 people in the United States above the age of 12 have notable hearing loss in both ears [1]. The statistic for hearing loss only increases in occurrence as age increases, meaning almost everyone will experience some amount of hearing loss throughout their life. One of the most popular and expensive treatments for SSNHL include hearing aids and cochlear implants. There is still much debate in the medical community about whether severity or patient preference should be used in order to help advise patients towards specific treatments. While much research is needed to solve the over-looming problem of deafness, for those of the general populace at an increased risk of developing hearing problems, treatment of SSNHL could benefit the prevention of deafness, without the need for invasive and permanent techniques, such as cochlear implantation.

INNOVATION

Traditionally, computed tomography (CT) is used for imaging the soft tissue of the ear, while MRI is only employed for imaging its fluid filled sacs due to its low resolution. With recent advances in technology, MRI is now capable of resolutions up to 20 μm making cellular data relevant. The advances have created a need for more specific and tailored contrast agents that act on the scales of individual cells or proteins.

The metabolism of the inner ear, specifically the intratympanic membrane, has a unique mechanism. Similar to the infamous blood-brain barrier, the ear contains a blood-cochlear barrier near the labyrinth membrane. The structure of the phospholipid bi-layer only permits a select amount of molecules through. Additionally, the small fluid filled sacs surrounding the inner ear, along with the high flow through the Eustachian tubes, create an environment of high shear stress. The niche structure requires a need for both active delivery and real-time tracking. Any contrast agents not employing both will simply be washed away by the volatile nature of the organ, and prevent any imaging modalities from capturing its activity.

The study performed by Iliadou et. al. could not be ruled as irrefutably conclusive due to small sample sizes and the small temporal time scale of induced damage (both by noise and ototoxic drugs). To circumvent this pitfall, our study employs HP particles for tracking of bioactive molecules. As showcased by Ardenkjaer-Larsen et. al., the high signal-to-noise ratio ensures that despite the small time-scale, significant and differential readings can be taken [3]. Additionally, a study performed by Golman et. al. further establishes this phenomena using HP $^{13}C_1$ pyruvate [4]. The study verified that pyruvate could be used as a real-time tracker of the body's metabolic activity. Interestingly, by marking the C_1 present in the ester portion of pyruvate, the research team was not only able to track pyruvate, but also its surrogate molecules, lactate, and alanine. The chemical shifts generated by the change in proton

environments provided researchers with information about the amount and type of metabolism occurring at specific portions of the body at different times. They were able to image at a spatial resolution of 7.5 x 7.5 x 32 and 5 x 5 x 10 mm^3.

Another study conducted by Kayyali et. al. manufactured a targeting ligand known as PrTP1 which binds to the prestin of the inner hair cells [5]. The research team employed a chitosan glycerophosphate(CGP)-hydrogel system tagged with the ligand in order to ensure active delivery of their nanoparticle glucocorticoid treatment. Although our study does not employ a hydrogel, their ligand can still be used as a means to tag the protein prestin, which is found in abundance within and along the inner hair cells. Since the PrTP1 ligand is simply a reporter of the extracellular domains of OHC-specific proteins with an additional 8 amino acids connected to a terminal azide, any one of the 8 amino acids can have ^{13}C included in them in order to facilitate tracking. The concern of denaturing is not applicable either, as hyperpolarization takes place at cryogenic temperatures, and thus ensures proteins will not reach temperatures that allow for denaturing of binding sites.

Our proposed solution employs multiple engineering and chemical technologies and techniques in order to generate a high resolution image that can correlate the prevalence of biologically relevant molecules, and the deterioration of hearing in such a manner that allows doctors to provide patients with a faster and more accurate diagnosis. Additionally, neurologically relevant data can be generated that helps create a more detailed map of the specific parts of the auditory cortex and ear that relate to different hearing ability and perception.

APPROACH

Specific Aim 1: Assess the viability of hyperpolarized ^{13}C PrTP1 as a metabolic marker for prestin

A. Rationale: A previous study showcased that HP molecules have a 10,000 to 1 reduction in a signal to noise ratio, making micromolar analysis much more viable. By utilizing the high sensitivity of these molecules, we can image the volatile environment of the inner ear. Additionally, the acetyl and ester motifs present in the many amino acids comprising PrTP1 make it ideal for radio-labeling, making it even more compatible with ^{13}C MRI capabilities.

B. Study Design

B.1. Experimental Design

Two batches of PrTP1, consisting of the amino acid sequence LSTHTTESRSMVGGSCGGS[Lys(N_3)] will be synthesized. The second batch will be synthesized with a ^{13}C included in the one of the eight amino acids at the terminal end. In order to prevent any steric hindrance issues associated with binding, it is suggested to include them before the GGS sequence [4]. Following synthesis the ligands are to be incubated in FBS with prestin. Following 24 hrs of incubation at 37 degrees celsius, the samples will first be subject to gel electrophoresis to ensure binding to prestin. Following gel electrophoresis, the remainder of the sample should be dissolved and inserted into a dynamic hyperpolarizer (DNP). Immediately following hyperpolarization, samples should be analyzed using an NMR.

B.2a. Success Criteria #1

For the gel electrophoresis, success will be determined based on the size of the molecules present. Prestin has a size of approximately 81.4 kDa. In order to ensure there was not an error, the assay must be run with unconjugated prestin and incubated prestin. Any bands found beyond 81.4 kDa can be considered bound to the PrTP1 ligand.

B.2b. Success Criteria #2

For the NMR, the data between the regular ligand and the hyperpolarized ligand should be significantly less noisy. Please reference the below images for visualizations of a 10,000 to 1 reduction in signal to noise ratio and a classical DNP apparatus.

B.3 Anticipated Results

There are only bands beyond 81.4 kDa and there is a reduction of the signal to noise ratio 3-fold.

B.4 Possible Complications

One major pitfall to be aware of is that the hyperpolarization of PrTP1 causes abolishment of the ability to bind to the prestin protein.

B.5 Alternative Strategies

One alternative strategy suggests utilizing vinyl-esters for Parahydrogen-Induced Polarization via Side-Arm Hydrogenation (PHIP) in order to induce hyperpolarization in-situ. This was proven to be effective just this year by Mohiuddin et. al. [6]. The caveat of this methodology is it requires higher temperatures rather than lower ones, meaning one would have to hyperpolarize only one amino acid at a time, and then conjugate to the PrTP1 ligand, which could lead to reduce polarization by the time the contrast agent is injected.

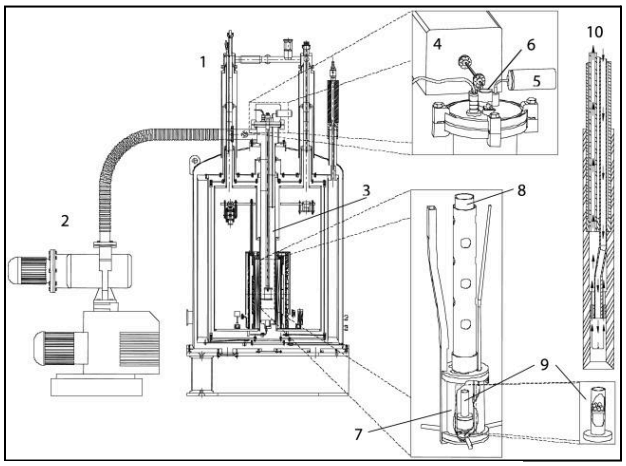

Figure 1. A classical dynamic nuclear polarizer [3].

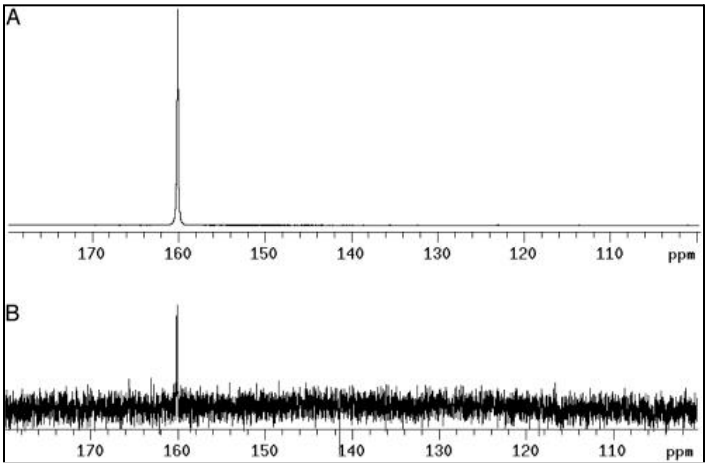

Figure 2. An example of a 10,000 to 1 reduction in signal to noise ratio, using urea. (A) Hyperpolarized urea. (B) Thermal equilibrium urea [3].

Specific Aim 2: Assess what parts of the inner ear's change in metabolism correlate with loss of activation of the auditory cortex.

Rationale: Many scientists have hypothesized that both the inner hair cells as well as prestin have an underlying mechanism that leads to hearing loss [7]. Some work at Harvard University is already being done to attempt to restore hearing using regenerative medicine, specifically focusing on the inner hair cells. By correlating the specific region of the inner ear and its change in metabolism with a deactivation of the auditory cortex, researchers can gain greater insight into what areas of the ear to target, while also providing doctors and clinicians with new methods to help diagnose patients.

Study Design:

B.1. Experimental Design

Once the first batch of PrTP1 has been verified to function as intended, a larger batch should be synthesized in order to perform a total of 40 rabbit injections twice. Each injection should be approximately 80 mM to ensure optimal contrast.

The rabbits will be split into three groups, 10 for the control, 15 for the low-dose experimental group, and 15 for the high-dose experimental group. The first trial will involve monitoring the rabbit's baseline auditory cortex activity and prestin metabolism. In order to do so the experimenter should inject the hyperpolarized ^{13}C PrTP1 within the rabbit's inner ear. Following injection the rabbit should be inserted into the MRI. The settings should be 1.5-T operating at 63.67 MHz using a ^{13}C-T_x/R_x coil.

For optimal imaging there should be monitoring for at least 60 seconds. There should be additional images taken upon injection and then following every 10 seconds. Following the analysis, the contrast images must overlay the anatomical structure, which can be gained by doing BOLD fMRI following the rMI, for yet another 60 seconds. Rabbits should be allowed to heal fully before the next step.

After the baseline has been recorded, the rabbits should be injected with 5 mg/kg and 15 mg/kg of cisplatin for the low-dose and high-dose group respectively. Following dosing, rabbits must be injected yet again with the HP ^{13}C PrTP1 and image according to the same procedure outlined above for the baseline.

B.2. Success Criteria

After completion of all rabbit injections and analysis, we aim to be able to correlate and identify an amplitude that correlates with a residual amplitude of activation based on the BOLD fMRI. If the absence of prestin is noted in a recurring region and followed by recurring deactivation of the auditory cortex, one-way ANOVA will be used in order to prove with a 95% confidence that abolishment of activation was due to cochlear damage of the given region.

B.3. Anticipated Results

We hypothesize that the absence of prestin in the inner hair cells will be indicative of cochlear degeneration or damage, and that different regions will correlate with deactivation of specific auditory cortex regions that relate to different frequencies of hearing. It is suspected that both higher and lower frequencies will be lost at higher doses, while only higher frequencies will

be lost at lower doses. As such, it is also expected that prestin will be much more prevalent in the low dose group than the high dose group.

B.4. Possible Complications

For a reliable baseline of auditory cortex activity and activity abolishment following treatment to be imaged using BOLD fMRI, the rabbits may need to be imaged in the days prior to and following treatment with cisplatin. Due to the degenerative nature of ear injury, it is possible that the rabbit's condition could worsen very quickly with time, thus causing a skew of data, making it seem as if prestin is not indicative of any specific change in auditory cortex activation or perception of different frequencies.

B.5. Alternative Strategies

The hydrogel employed by Kayyali et. al. has great utility for performing this experiment in humans with pre-existing hearing loss. HP molecules have been shown to have longer lasting states of polarization in the solid-phase. The hydrogel developed by the research group is liquid at room temperature, but becomes rigid when it is injected into the warm environment found within the inner ear. This provides the hydrogel the ability to resist the high shear stress environment produced by the Eustachian tubes. By incorporating the hydrogel along with nanoparticles loaded with HP molecules that are also tagged with PrTP1, we can create an extended release system for extensive and continuous imaging of the prestin within the inner ear of a live patient. While this data may not be relevant in small amounts, a large census could provide doctors with a better idea of what parts of the inner ear are more crucial to treat to prevent further hearing loss.

CONCLUSION

Our study offers a multimodal imaging modality for use in both humans and animals in order to gain insight into the underlying mechanisms that lead to deafness. With the combination of HP molecules and advancements in MRI technology, a very small amount of molecules can provide a large amount of clinically relevant data. With new emerging technologies such as tissue engineered medical products like hydrogels, and treatments with tailored targeting and release like nanoparticles, the possibilities for long-term imaging and real-time imaging have never been easier to access for researchers. With the new emergence of using vinyl-esters in tandem with PHIP reactions allowing hyperpolarization to occur at higher temperatures rather than cryogenic ones, it opens a new toolbox of compounds that can be modified and imaged with relative ease. Future work should consider looking into different forms of delivery or synthesis in order to extend the polarization times of the molecules, as that is the main drawback of this emerging technology.

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