Award Number: W81XWH-21-1-0957

TITLE: Hair cell Regeneration and Treatment for Noise-Induced Hearing Loss in Pig Model

PRINCIPAL NVESTIGATOR: Dr. Zheng-Yi Chen

CONTRACTING ORGANIZATION: Massachusetts Eye and Ear Infirmary, Boston, MA

REPORT DATE: October 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution is unlimited.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2023	Annual	30Sep2022-29Sep2023

4. TITLE AND SUBTITLE

Hair Cell Regeneration and Treatment for Noise-Induced Hearing Loss in Pig Model

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-21-1-0957

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Zheng-Yi Chen
zheng-yi_chen@meei.harvard.edu

Dr. Ying Wang
ying.wang5.civ@health.mil

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)

Massachusetts Eye & Ear
243 Charles Street
Boston, MA 02114

WRAIR
503 Robert Grant Ave.
Silver Spring, MD 20910

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Regeneration of hair cells in fully mature mammalian inner ear is a key application for potential therapy to treat hearing loss. Adult mouse inner ear can be reprogrammed by co-activation of c-Myc and Notch1 genes, enabling adult cochlear cell types to re-enter cell cycle. Reprogrammed adult cochlear supporting cells are sensitized by transient Myc/Notch activation and respond to hair cell induction signals by overexpression of Atoh1, a hair cell fate determinant, and transdifferentiate into hair cells efficiently *in vitro* and in vivo. Our study further identified a pathway mTOR and showed its activation by gene manipulation resulted in robust attenuation of noise-induced hearing loss (NIHL). To move hair cell regeneration towards the clinic, reprogramming needs to be achieved by a clinically relevant method instead of transgene manipulation in the mouse models. A large animal pig model will be extremely valuable in developing a clinically relevant program in hair cell regeneration, due to the similarities between the pig and human inner ears in development, anatomy and physiology. In addition, we have identified a pathway with an FDA approved drug that potently attenuates NIHL. The drug could be developed into a treatment program for NIHL in the pig model and expand it to the applications in humans.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	13	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

1. Introduction

Hearing loss (HL) is one of the most common forms of sensory deficit affecting over 36 million people in the US. Noise-induced hearing loss (NIHL) affects over 9 million people whereas age-related hearing loss (ARHL) affects 30% and 50% of 60 to 75 year old, respectively. Due to the increase in the usage of wearable music devices and the aging population, significantly more people will suffer from debilitating HL. There is no pharmaceutical drug known to be efficacious in treating HL; thus, there exists an urgent medical need to develop therapies that can treat the condition and improve the outcomes of those affected by this permanent disability.

In the project, we proposed to evaluate Valproate (VPA), an FDA approved drug for treatment of NIHL and to use drug-like components to achieve reprogramming and hair cell regeneration in a large animal pig model. We have obtained compelling evidence in mice that the drug is potent to promote hair cells against noise-induced damages, to maintain the hair cell-to-neuron connections and to protect against noise-induced hearing loss. The use of drug-like molecules (small molecule compounds and siRNA) makes it possible to translate our work into clinical application, as in human the genetic manipulation we used in mice is not suited. The use of the pig as a model system is a major step forward translating our work to clinic. The pig inner rear is developmentally similar to the human inner ear, as both are fully developed at the embryonic stage. The hearing profile in the pig overlaps with human hearing range. A pig inner ear is virtually anatomically identical to a human inner ear with a similar size, making it ideal to develop surgical procedure that optimizes the route of inner ear delivery. Success of the study will help us to move the work from animal models to humans.

2. Keywords

Noise exposure, blast overpressure, hearing loss, auditory brainstem response, swine model, permanent threshold shift, temporary threshold shift, Valproate, treatment, hair cell regeneration

3. Accomplishments

- **What were the major goals for the project?**
  - Establish swine models of NIHL and blast-induced hearing loss (BIHL)
  - Hearing protection and repair by FDA-approved VPA drug in the pig
  - Regeneration of hair cells in pig model

Milestones:

Year 1: Obtain IACUC and ACURO approval of animal use protocol, establish a pig model of NIHL, establish the parameters for the induction of permanent threshold shift (PTS) and temporary threshold shift (TTS).

Year 2: Establish a pig model of blast-induced hearing loss, establish the parameters for the induction of PTS and TTS, and evaluate earing protection and repair by FDA-approved VPA drug in the pig.

Year 3: Design and screen siRNAs for pig Fir and Mxi1 genes and test their knockdown in pig fibroblast cells, reprogramming of pig inner ear in vivo by the cocktail of small compounds and siRNAs, screen and identify the AAV that transduces the pig cochlear supporting cells and construct an AAV vector in which ATOH1-HA is under the control of Sox2 promoter

- **What was accomplished under these goals?**

Bulleted list of key research accomplishments

- Continued collecting and analyzing time-cause ABR following noise exposure in micro-Yucatan pigs
- Explored the unique anatomy of pig ear and effectively performed transtympanic injection (TTI) in miniature pigs
- Presented the poster, entitled “Swine Model of Noise-Induced Hearing Loss” at MHSRS 2022
- Initiated evaluating the effectiveness of VPA through TTI prior noise exposure, aiming to provide for protection against NIHL in pigs
- Initiated investigating blast-induced hearing loss (BIHL) in pigs

Detailed research progress reports and the outcomes

1) We continued the investigation of noise-induced hearing loss in pigs in this report period. Anesthetized Micro-Yucatan pigs aged 7 to 10 weeks were placed in a sound booth for experimentation. The pigs were divided into two groups: NE1, where they were exposed to 120 dB white noise for 90 minutes, and NE2, where they experienced two noise exposures of 120 dB for 120 minutes each, with a 24-hour interval between the exposures. Each group consisted of three pigs. The noise signals were controlled by RZ6 multi I/O processor (TDT, Alachua, FL), and the speaker was positioned 10 cm above the pigs' heads. Auditory brainstem responses (ABR) were measured at various time points: 3 - 7 days before NE as a baseline, immediately after NE, and at 7-, 14-, 21-, and 28-days post-NE (Fig.1). Open-field sound simulation was employed for the delivery of sound stimuli. Sound intensity levels started at 90 dB and were reduced in 10 dB steps until no auditory response was detected. Sound frequencies at 4 kHz, 8 kHz and 32 kHz were assessed during the auditory testing.

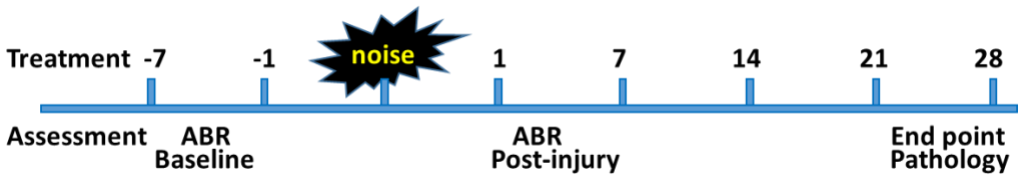

Fig.1. Schematic experiment design

(A) Auditory functional data analysis showed that ABR signals were undetectable immediately or at 1 day post exposure (not shown). As illustrated in the figures (Fig.2), ABR thresholds increased significantly over tested sound

frequency ranges from 4 - 32 kHz at 7 days after noise exposure and persisted for 28 days.

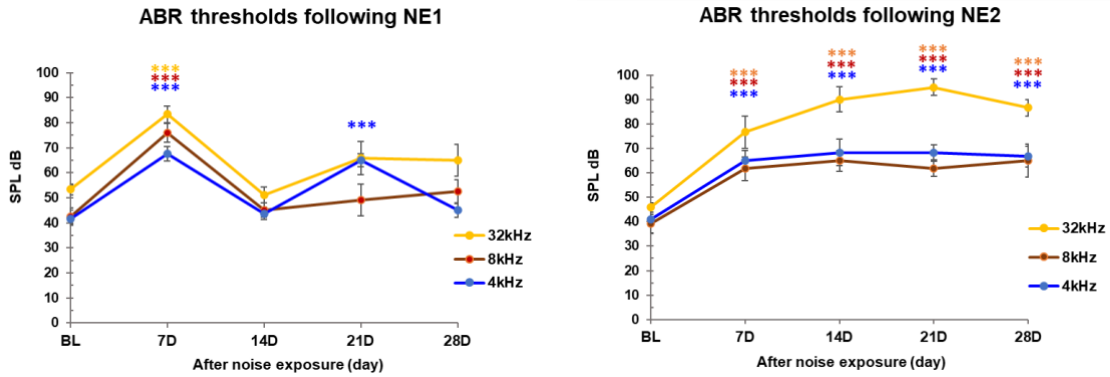

Fig. 2 Time-course of ABR thresholds following NE1 (left) and NE2 (right)

At 7 days post-exposure, shifting of ABR 25 - 40 dB was detected in NE1 and NE2 groups (Fig.3). Compared to NE1, changes of ABR threshold in NE2 raised significantly at 14-, 21- and 28-day post exposure.

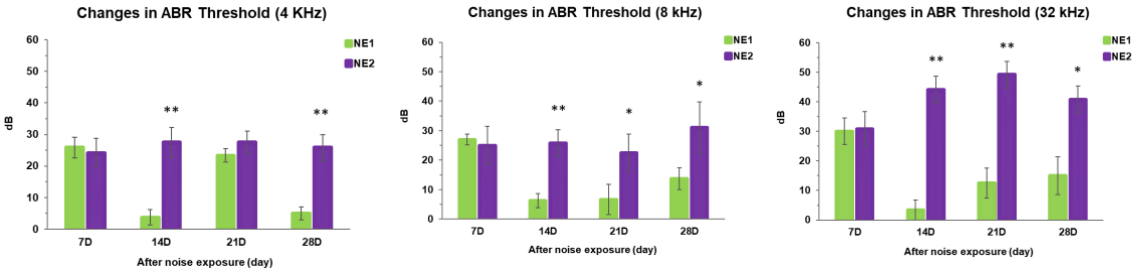

Fig. 3. Changes of ABR thresholds in groups NE1 and NE2

Compared to the baseline ABR (fig.4), NE1 induced reduction in wave-I amplitudes at 7 days post exposure only, while NE2 induced significant reduction in wave-I amplitudes in all observed time points. n = 3 pigs, 6 ears, * p<0.05. *** p<0.005.

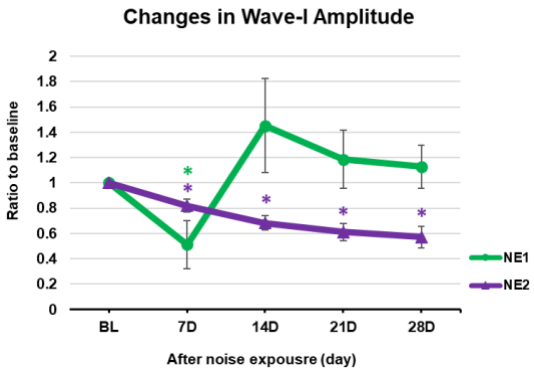

Fig. 4. Change of ABR Wave-I

(B) Pathological data analysis included
the whole-mount cochlear preparations for immunohistochemical staining with antibodies against MYO7A, Ctbp2 and NF200 at 28 days post NE. Briefly, the otic capsules were removed from the temporal bones after animal euthanasia and incubated in a solution of 0.5M EDTA for one month to decalcify the bony labyrinth. The whole-mount cochlea was dissected and divided into the apical,

middle, and basal turns. Subsequently, the number of IHCs and OHCs were quantified separately in the regions corresponding to the three cochlear turns.

To determine whether NE causes cochlear inner hair cell (IHC) and/or outer hair cell (OHC) loss, the anti-MYO7A was first used as the targeting protein. The confocal images displayed a distribution of MYO7A (green) in the basal, middle and apex cochlear turns (Fig.5a). Compared to control animals, a significant reduction of MYO7A protein level in the inner hair cells (IHC) was from the apex to the basal turn after 28 days post NE. Quantitative analysis of OHCs and IHCs (Fig.5b) reveals a decrease in MYO7A protein level due to NE, with a higher number of MYO7A-negative (unhealthy) IHCs observed in the NE2 group compared to NE1.

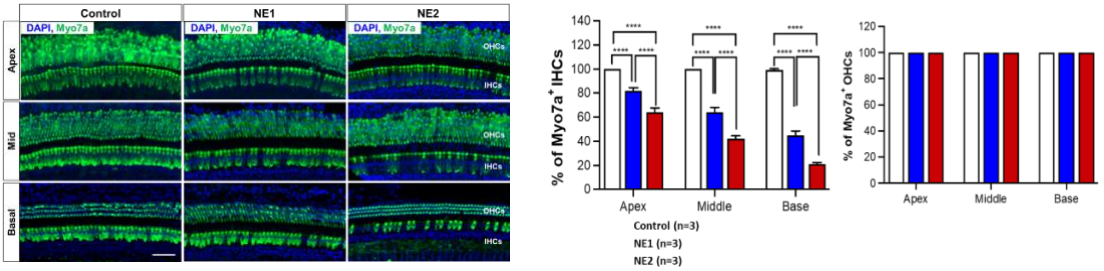

Fig.5. Cochlear Hair Cells Impairment pattern between PTS and TTS Noise Exposure. A Diagram of Experimental Design. B Immunofluorescence labeling of Myosin7a (green) and DAPI (blue). Representative confocal images of whole-mount cochlear turns displaying the distribution of MYO7A protein. C Quantitative analysis of OHCs and IHCs. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

To evaluate any potential impairment on the cochlear synapse and/or nerve due to NE, antibodies Ctbp2 were employed in the immunohistochemical staining process. Immunofluorescence labeling of MYO7A (blue) and Ctbp2 (red) presynaptic puncta in IHC synapses (Fig.6a). Quantitative analysis confirms NE-induced ribbon synapse loss in all cochlear turns (Fig.6b). NE2 leads to more severe synapse loss in MYO7A -negative IHCs compared to NE1.

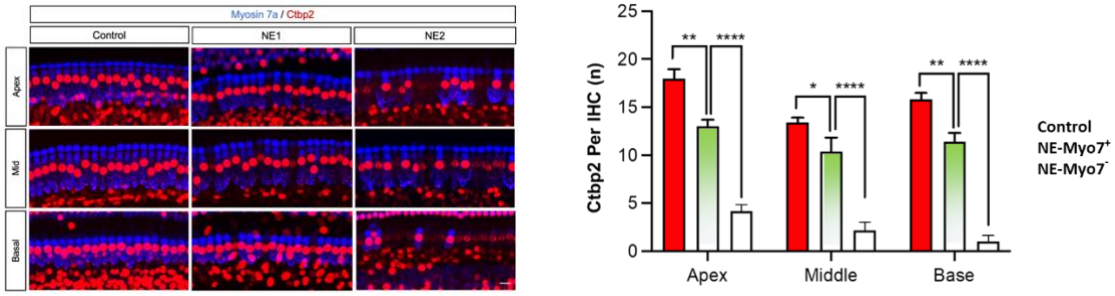

Fig.6. Representative of MYO7A (blue) and Ctbp2 (red) in IHC synapses in the whole-mount cochlear turns (a) and quantitative analysis in IHC (b)

Furthermore, IHC nuclear shrinkage is detected at 28 days post-injury (Fig.7).

Neurofilaments provide structural support to neurons. NF200, one of the subunits plays a role in maintaining the shape and structural integrity of axons. To identify

the impact of NE on synapses, NF200 protein (green) was utilized in co-immunostaining alongside with MYO7A (blue) and Ctbp2 (red). Images (fig.8) showed the detachment of synapses from neurofilaments. Data indicated a reduction in colocalization among the proteins, suggesting that synapses were impaired after exposure to noise insult.

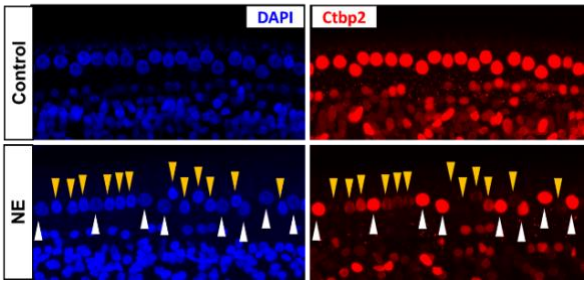

Fig. 7. Representative confocal images of IHC nuclear shrinkage (yellow arrowhead) vs. health (white arrowhead)

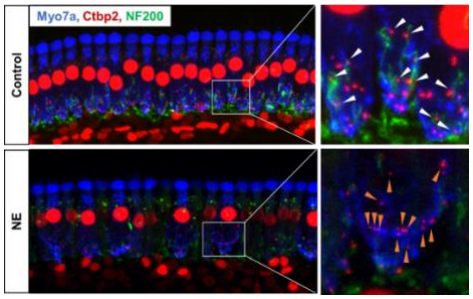

Fig. 8. Representative co-immunostaining of NF200 with Ctbp2 (red) and MYO7A (blue)

Our study demonstrates the importance of the swine model for investigating both temporary and permanent NIHL. The changes observed in the pig cochlea after noise including the reduction of MYO7A in IHCs, the survival of OHCs and the shrinkage of IHC nuclei present new information that is different from the studies in mice under similar conditions. The study raises an important issue as to which model, the mouse or the pig, is more similar to the humans. Given the inner ear anatomy, the size, the development and hearing frequency, there is a high likelihood that pigs may offer a valuable tool for studying the effects of noise exposure on hair cells and synapses, facilitating the testing of drug-mediated protection against NIHL in humans.

2) We are the first to conduct a systemic study of NIHL in pigs including drug delivery by the middle ear. As a result, we encountered significant challenges to deliver drugs to the middle ear through the tympanic membrane efficiently in pigs. We tested different parameters to develop an approach that is applicable to the pig middle ear for the delivery, the essential step before we can test the effect of VPA in the protection against NIHL and BIHL. Over the course of the year, we made the progress in the following areas:

(A) *Unique pig ear anatomy verification*: Prior to administering the drug to the middle ear of pigs, a thorough examination of the outer ear and tympanic membrane was undertaken. Our investigation involved meticulous dissection of pig cadavers, encompassing micro-Yucatan and other domestic pigs ranging in weight from 8 kg to 100 kg. Unlike rodents, the middle ear of pigs aligns nearly parallel to the outer ear canal. In rodents and humans, the tympanic membrane (TM) is situated at an angle of approximately 90 degrees to the ear canal, whereas in pigs, the TM is positioned to the side of the ear canal, approximately 2-5 mm before its termination. This implies that direct needle access to the middle ear is not possible. Nevertheless, visualization of

the TM can be achieved using a specialized otoscope capable of a 10-degree rotation.

(B) *Optimizing device selection for TTI procedure*: To facilitate both visualization and injection through the TM of a miniature pig, a specialized otoscope with specific specifications is essential. It should have a length of 8 - 16 cm, an outer diameter of 1 - 2 mm, and be equipped with a powerful light source as well as an additional working channel. Many devices and tools commonly employed in clinical setting fail to effectively view the TM of miniature pigs. For instance, the Operating Otoscope is too short for the pig ear canal, the digital Macroview Otoscope provides inadequate light for viewing the pig TM, and the Olympus Endoscope MAF-DM2 (length of 90 cm, outer diameter of 3 mm) is too large to reach the end of pig ear canal. The Sialendoscope, also called miniature telescope (Karl Storz) has been selected for the PPI procedure in miniature pigs. It is a semi rigid endoscope with an outer diameter 1.6 mm and working length of 10 cm. It contains a fiber optic light transmission for imaging and a working channel 0.85 mm which allows insertion of a 23 – 27 G needle for injection.

(C) *Middle ear injection training*: After completing training in transtympanic injection (TTI) using the Sialendoscope (Karl Storz) on cadaveric specimens, our team further improved the skills on a live pig model. To enhance opportunities while minimizing animal use, we incorporated a fluoroscope. With clear view of TM, 0.5 ml of Omnipaque containing 350mg Iodine/ml was injected into the middle ear of the micro-Yucatan pigs. A fluoroscopy was employed to pinpoint the accurate injection site. The same animal was allowed at least 2 weeks for recovery, then received the Evans Blue TTI followed by immediate euthanasia and subsequent dissection, providing conclusive evidence regarding the accuracy of dye delivery.

(D) *Evaluation of VPA in protection against NE*: The establishment of a surgical procedure for middle ear delivery allowed us to initiate the evaluation of VPA in its protective effects against NIHL. Briefly, the micro-Yucatan pig received 500 ul TTI of VPA in one ear and 500 ul TTI of saline in the other ear, 24 hours prior to the noise exposure. An injury treatment of NE2, described NIHL model above, was selected in the assessment. Auditory functions were assessed before injury, at 3- and 7- days post NE. At the conclusion of the reporting period, investigations on four pigs are still ongoing, spanning 21- and 28-days post-

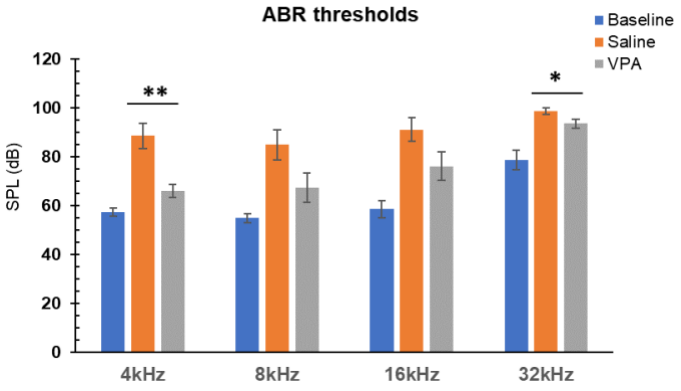

Fig. 9. ABR threshold changes in 7 days post-NE2 with or without VAP pretreatment

injury. The preliminary data summarized in figure 9 that demonstrated a significant reduction in noise-induced elevation of ABR thresholds in pig ears treated with VPA, as compared to those treated with saline.

In summary, our efforts have yielded substantial advancements in the investigation of the repercussions of noise exposure on both auditory function and cochlear hair cell pathology, along with identifying impairment in the ribbon synapses. Additionally, we have initiated the study of the potential protective effects of VPA against NE-related hearing damage in the pig models with encouraging results. The work is ongoing.

3) During this report period, we have initiated the investigation of blast-induced hearing loss (BIHL) in pigs. A few anesthetized micro-Yucatan pigs were secured in an advanced blast simulator (ABS) in a prone position with the head towards the oncoming shockwave, then exposed to a single blast overpressure at 22 psi (peak static). Preliminary data showed that blast caused elevation of ABR thresholds significantly at all observed time interval. It indicates blast-induced hearing loss may persist over a month (fig. 10). In contrast to the apex and middle turns of cochlea, the basal turn exhibits severe HCs damage (fig. 11). Compared to control pigs in the apex region, blast causes significant loss of OHC, while noise causes significant loss of IHC. Nevertheless, addition research is required to draw a definitive conclusion.

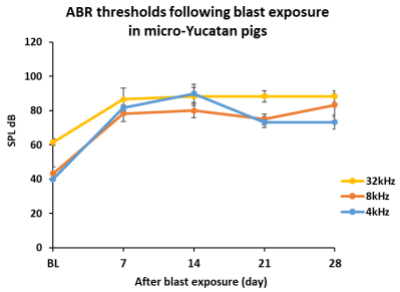

Fig. 10. Blast-induced changes in ABR thresholds

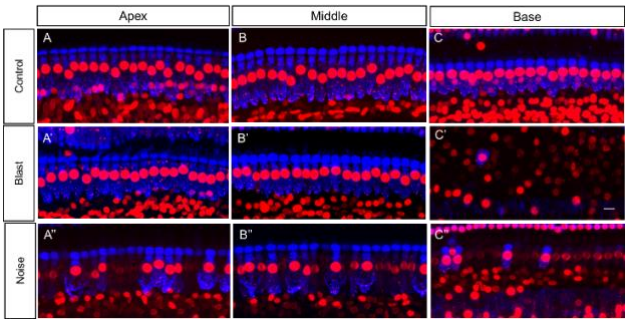

Fig. 11. Co-immunostaining of Ctbp2 (red) and MYO7A (blue)

We anticipate gathering more data will shed light on BIHL and NIHL, preserving hair cells, and safeguarding hair cell survival, as well as the integrity of synapses. We expect to provide valuable insights into the comparative effects of blast and noise exposure on auditory health, as well as the potential therapeutic benefits of VPA in the coming year.

Future research plan and focus

- Continue evaluation of VPA in protection against NIHL in micro-Yucatan pigs
- Evaluate the efficacy of VPA in mitigating BIHL in micro-Yucatan pigs
- Identify the most effective AAV for targeting supporting cells of pig
- Facilitate hair cell regeneration by delivering AAV-Atoh1s through the round window membrane of pig

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

The study is designed to test an FDA approved drug VPA in hearing protection against noise-induced (NIHL) and blast-induced hearing loss (BIHL) in pig models and to provide evidence that robust protection can be achieved. The project will further test hair cell regeneration by reprogramming and gene therapy in the pig inner ears in vivo. As the pig and human inner ears share many similarities, the success of any of the aims should have a great impact on moving the work toward clinical application. If VPA is shown to be efficacious and safe to protect NIHL and BIHL in the pigs, it makes the study in humans a logical step. If hair cells can be regenerated in the pig inner ears, it would validate the approach of reprogramming and gene therapy and lay the foundation for drug development for clinical study in humans.

What was the impact on other disciplines?

The work may have an impact beyond hearing. Blast induces damage in multiple organs, including the brain. As the VPA drug can be administrated systematically, it may offer protection in the tissues such as the brain. Further, our reprogramming and regeneration approach can be potentially extended to the regeneration of other organs and cell types, such retina and brain cells.

What was the impact on technology transfer?

The study has the potential to develop a new drug treatment for NIHL and BIHL by an FDA-approved drug. The success of the application of VPA will likely lead to a clinical trial, which should be of great interest to companies in drug development for hearing loss.

5. Changes/Problems

Changes in approach and reasons for change

There is no significant change in approach.

Actual or anticipated problems or delays and actions or plans to resolve them

It took much longer than anticipated to establish the procedure for the middle ear injection due to an unfamiliar anatomic structure that complicated the process. We had to try multiple times, including the test and procurement of a new endoscope for the procedure. We are confident that we have established a procedure for the middle ear injection, which should help greatly in the subsequent experiment.

6. Products:

Reprogramming by Drug-like Molecules Leads to Regeneration of Cochlear Hair Cells in Adult Mice. Proceedings of the National Academy of Sciences 120 (17), e2215253120

Swine Model of Noise-Induced Hearing Loss. Poster presentation at MHSRS, Florida, August 2022

7. Participants & Other Collaborating Organizations

Organization Name: Mass Eye & Ear

Name	Role	Effort	Contribution	Support
Zheng-Yi Chen	PI	2 months	design, analysis, coordination	CDMRP, NIH, Yeatts fellowship
Yi-Zhou Quan	Postdoc	6 months	study of siRNA for pig Fir and Mxi1. Construction of AAVie-Atoh1	CDMRP, NIH
Wei Wei	Postdoc	6 months	study of siRNA for pig Fir and Mxi1. Surgical procedure of middle ear delivery in pigs, and study pig ears for morphology	CDMRP, NIH

Organization Name: WRAIR

Name	Role	Effort	Contribution	Other support
Ying Wang	PI	8 months	design, planning, writing execution, analysis, coordination	MOMRP CDMRP
Yanling Wei	Research Associate	12 months	execution, analysis, management	MOMRP CDMRP
Zhilin Liao	Research Assistant	3 months	execution	MOMRP CDMRP
VSP			anesthesia and animal care	

8. Special Reporting Requirements

9. APPENDICES

The publication and poster are attached.