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A new method with an explant culture of the utricle for assessing the influence of exposure to low frequency noise on the vestibule

Nobutaka Ohgami[1,2], Tingchao He[1,2], Reina Negishi-Oshino[1,2], Yishuo Gu[1,2], Li Xiang[1] and Masashi Kato[1,2]

[1]Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, Nagoya, Aichi, 466-8550 Japan. [2]Voluntary Body for International Health Care in Universities, Nagoya, Japan.

Running title: *Ex vivo* assessment of health risks by LFN exposure

Correspondence:

Masashi Kato M.D. Ph.D.,

Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan.

Phone: +81-52-744-2122. Fax: +81-52-744-2124.

E-mail: katomasa@med.nagoya-u.ac.jp

Abstract

Health risks attributed to low frequency noise (LFN) exposure are serious global issue. Therefore, the development of a method for a prevention based upon risk assessments for LFN is important. Previously *in vivo* exposure of mice to LFN at 100 Hz, 95 dB for 1 hr produced imbalance with breakage of the otoconial membrane, which covers hair cells, and impaired activity of hair cells in the vestibule. However, methods for inhibition of LFN-mediated imbalance have not been developed, since at present there are no techniques available with *in vitro* or *ex vivo* assessments to evaluate LFN-mediated imbalance by direct administration of preventive chemicals into the vestibule. Our findings demonstrate the usefulness of an explant culture of the utricle with a fluorescent styryl dye, FM1-43FX. In addition, examination of the morphology of the otoconial membrane with explant cultures of utricles was conducted to determine the risk of LFN. *Ex vivo* exposure of the utricle to LFN at 100 Hz, 95 dB for 1 hr induced breaks in the otoconial membrane as well as decreased uptake of FM1-43FX in hair cells. Taken together, the results of this study provide a novel technique for assessing the risk of LFN exposure using an *ex vivo* experiment.

Keywords: low frequency noise, risk assessment, vestibule, otoconial membrane, balance.

Introduction

Low frequency noise (LFN) is noise with frequencies below 100 Hz and sound levels of 60-110 dB. LFN, which is generated by various electrical instruments including air conditioners, ventilation fans and freezers, traffic (Barbaresco et al., 2019; Tzivian et al., 2016) and industrial instruments (Berglund and Hassmén 1996), reported to produce cognitive dysfunctions, stress and hearing loss (Carlson and Neitzel, 2018). Therefore, it is an important issue to clarify the target tissues of LFN exposure in order to develop a method for prevention based upon evaluation of the health risks. However, there is limited information regarding a technique for evaluating LFN-mediated adverse health risks.

The vestibule, which consists of hair cells covered by the otoconial membrane with otoconia in the saccule and utricle in the inner ears, is a sensory organ for balance. In previous studies, the influence of LFN exposure on vestibular function in humans was noted (Evans and Tempest, 1972; Takigawa et al., 1988; Harrison, 2015). In our experimental studies, *in vivo* exposure to LFN was found to impair balance in mice, while there was no marked influence on hearing (Tamura et al., 2012; Ohgami et al., 2017; Ninomiya et al., 2018). Chen et al (2020) presented *in vitro* a cell line for screening of preventive drugs for noise-induced hearing loss. However, preventive methods for LFN-mediated imbalance have not been developed thus far. At present there is no apparent technique with *in vitro* or *ex vivo* assessments to effectively evaluate LFN-mediated imbalance by direct administration of preventive chemicals in the vestibule.

In our recent study, *in vivo* exposure of mice to LFN at 100 Hz, 95 dB for 1 hr initiated imbalance with breakage of the otoconial membrane and impaired activity of

hair cells in the vestibule (Negishi-Oshino et al., 2019), suggesting that the otoconial membrane is one of the target tissues for LFN exposure (Figure S1). The otoconial membrane was found to be crucial for the activity of vestibular hair cells and balance (Lundberg et al., 2015). Therefore, it is important to establish a method for *ex vivo* assessment of LFN-mediated imbalance with utricles containing not only vestibular hair cells but also otoconial membrane. Previously Bartolami et al (2011) demonstrated using explant cultures of utricles from mice, the otoconial membrane was clearly observed under a stereomicroscope. Kawashima et al (2011) determined the uptake of a fluorescent dye, FM1-43, by non-selective mechanotransduction channels in vestibular hair cellswith an explant culture of the utricle. The aim of this study was to assessed the influence of LFN exposure for 1 hr on the vestibule by a new method combining morphologic examination of the otoconial membrane, determination of the activity of hair cells using FM1-43FX and measurement of hair bundles stained with phalloidin in explant cultures of utricles from mice.

Materials and Methods

The detailed methods are provided in supplementary information and Figure S2. Briefly, after separating utricles from male and female ICR wild-type mice (Japan SLC, Hamamatsu, Japan) at postnatal days 7-9 (3-4 g in body weight), *ex vivo* exposure to LFN with a frequency of 100 Hz at 75 dB, 85 dB and 95 dB (Figure 1C-E) for 1 hr was performed with explant culture of utricles in the experimental setting illustrated in Figure 1A. This study was approved by the Institutional Animal Care and Use Committee in Nagoya University (approval number: 20238) and followed the Japanese Government Regulations for Animal Experiments.

Results and Discussion

Ex vivo exposure to LFN at 95 dB produced breakage of the otoconial membrane (arrows in Figure 1F) and significantly decreased the area covered by the otoconial membrane, whereas these alterations did not occur in the control, 75 or 85 dB groups (Figure 1F, G). The fluorescent intensity of FM1-43FX incorporated by hair cells after *ex vivo* exposure to LFN at 95 dB was significantly less than that in control, 75 or 85 dB (Figure 2A, C). After removal of the otoconial membrane, phalloidin staining was conducted (Figure 2B). The total numbers of hair bundles were comparable in the three groups (Figure 2B, D). Thus, data demonstrated that *ex vivo* exposure of utricles to LFN decreased the uptake of FM1-43FX with damaged otoconial membranes, but not the number of hair bundles, that correspond to the affected site shown in our *in vivo* study (Negishi-Oshino et al., 2019).

Recently Negishi-Oshino et al (2019) reported, breakage of the otoconial membrane attributed to imbalance was rescued by an increase of HSP70 expression in the otoconial membrane in *HSP70*-transgenic mice exposed to LFN for 1 hr. May et al (2013) found that explant culture of an utricle heated at 43˚C for 30 min was shown to induce the expression and secretion of Hsp70 in inner ear supporting cells. In this study, pre-heating of an explant culture of the utricle at 43˚C for 30 min was performed. LFN-mediated breakage of the otoconia membrane was rescued in the pre-heated explant culture of the utricle (Figure S3). Thus, data indicated the usefulness of an experimental system with *ex vivo* exposure to LFN for assessing prevention of LFN-mediated vestibular impairment.

In conclusion, this study provides a novel technique for assessing the risk of LFN exposure using an *ex vivo* experiment. Future study is needed to determine preventive drugs for LFN-mediated imbalance by *ex vivo* assessment.

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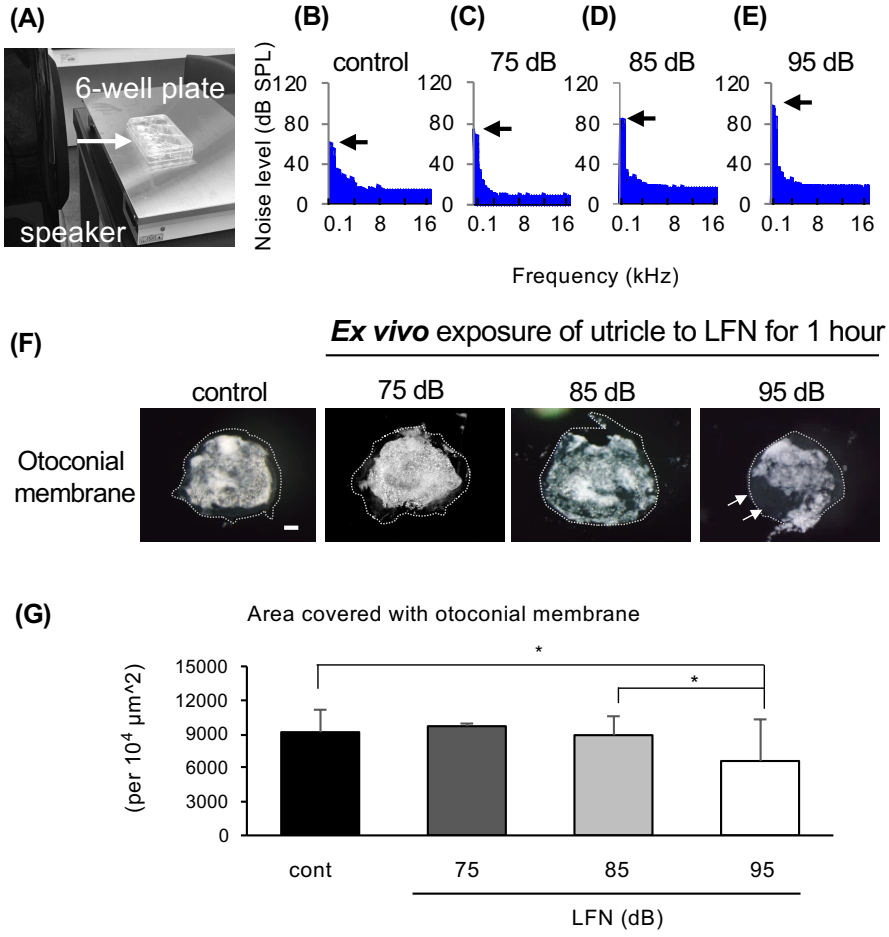

Figure 1. *Ex vivo* exposure to LFN at 95 dB produced breakage of the otoconial membrane. (A) Experimental setting. We used the same conditions as those used for *in vivo* LFN exposure in our recent study (Negishi-Oshino et al., 2019). (B-E) Sound patterns of low frequency noise (LFN; 100 Hz). Control (no exposure) (B), LFN at 75 dB (C), LFN at 85 dB (D) and LFN at 95 dB (E) are shown. Background level of noise at 100 Hz in the control was 55 dB. Peak levels of sound with a frequency of 100 Hz are indicated by arrows. (F) After *ex vivo* exposure of the utricle to LFN for 1 hour at 100 Hz, 75 dB (second panel from the left), at 85 dB (third panel from the left), at 95 dB (fourth panel from the left) and without exposure (control, first panel from the left), the otoconial membrane with a "cloud-like shape" in utricles observed under a stereoscopic microscope are shown. Dotted lines show the edges of utricles and arrows show a damaged area not covered with the otoconial membrane. Scale bar: 50 µm. (G) Area covered with the otoconial membrane in utricles (per 10,000 µm^2, mean ± SD, black bar: control, n = 10; dark gray bar: LFN at 75 dB, n = 3; gray bar: LFN at 85 dB, n = 5; white bar: LFN at 95 dB, n = 5). Significant differences (*$p < 0.05$) among the three groups were determined by Tukey's *post-hoc* multiple comparison tests.

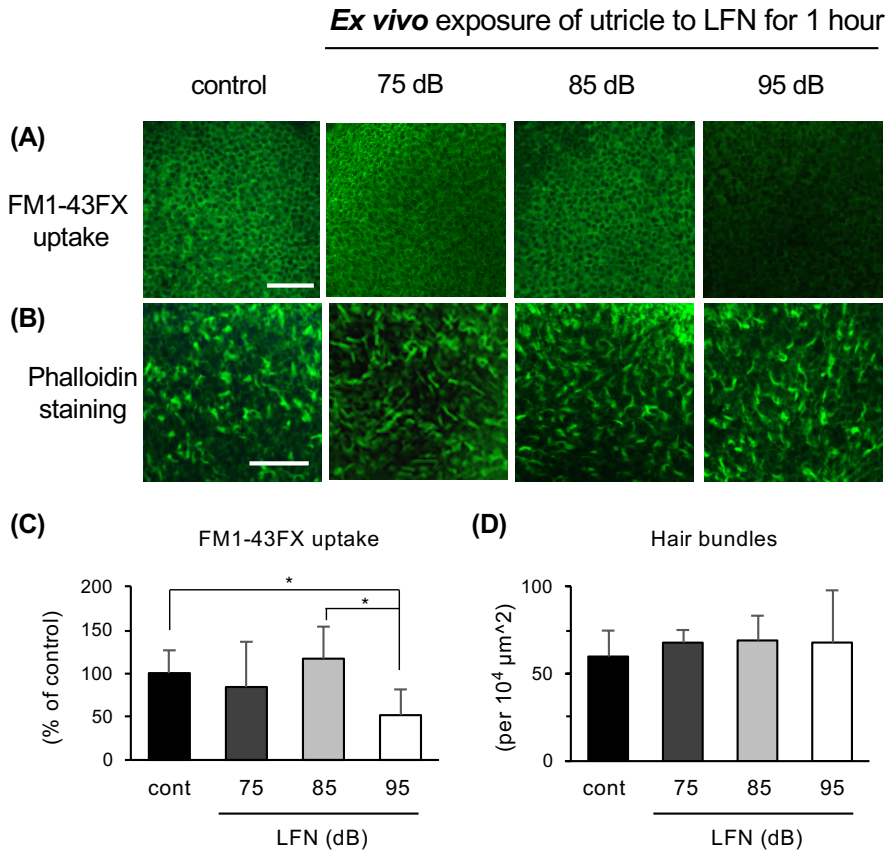

Figure 2. *Ex vivo* exposure of the utricle to LFN at 95 dB decreased uptake of FM1-43FX in hair cells. (A-D) After *ex vivo* exposure of the utricle to LFN for 1 hour at 100 Hz, 75 dB (second panels from the left), at 85 dB (third panels from the left), at 95 dB (fourth panels from the left) and without exposure (control, first panels from the left), (A) uptake of FM1-43FX by vestibular hair cells and (B) hair bundles stained by fluorescein-phalloidin are shown. Scale bars: 50 µm. (C) Fluorescence intensity of FM1-43FX incorporated by the utricles (% of control, mean ± SD) and (D) number of hair bundles (per 10^4 µm^2, mean ± SD) were determined [black bar: control, n = 10 (C), n = 3 (D); dark gray bar: LFN at 75 dB, n = 3 (C, D); gray bar: LFN at 85 dB, n = 5 (C), n = 3 (D); white bar: LFN at 95 dB, n = 5 (C), n = 6 (D)]. Significant differences (*$p < 0.05$) among the three groups were determined by Tukey's *post-hoc* multiple comparison tests.