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Original Research

Establishment of a cochlear injury model using bone-conducted ultrasound irradiation in guinea pigs and investigation on peripheral coding and recognition of ultrasonic signals

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Abstract: The cochlea of guinea pigs was irradiated with different frequencies of bone-conducted ultrasound (BCU) at a specific dose to induce cochlear hair cell-specific injuries, in order to establish frequency-related cochlear hair cell-specific injury models. Cochlear near-field potentials were then evoked using BCU of different frequencies and intensities to explore the peripheral coding and recognition of BCU by the cochlea. The inner ears of guinea pigs were irradiated by 30 kHz at 100 db and 80 kHz at100 db BCU for 6h to create frequency-related, ultrasound-specific cochlear injury models. Then, 30 kHz and 80 kHz BCU of different intensities were used to evoke auditory brainstem response (ABR) thresholds, compound action potential (CAP) thresholds, and action potential (AP) intensity-amplitude input-output curves in the normal control group and the ultrasonic cochlear injury group. This allowed us to explore the coding and recognition of BCU frequencies and intensities by cochlear hair cells. Immunofluorescence assay of outer hair cell (OHC) Prestin and inner hair cell (IHC) Otofelin was performed to verify the injury models. Irradiation of guinea pig inner ears by 30 kHz and 80 kHz BCU at a specific dose induced hair cell injuries at different sites. Irradiation with low frequency BCU mainly induced OHC injury, whereas irradiation with high frequency BCU induced IHC injury; moreover, IHC injury was more serious than OHC injury. The 30 kHz-evoked ABR threshold was significantly higher in the 30 kHz ultrasonic cochlear injury group compared to the normal control group. The 30 kHz-evoked ABR threshold was significantly higher in the 30 kHz ultrasonic cochlear injury group compared to the 80 kHz ultrasonic cochlear injury group. The difference in the 80 kHz-evoked ABR thresholds were not significant between the 30 kHz and 80 kHz ultrasonic cochlear injury groups. The click- and 30 kHz-evoked AP intensity-amplitude curves for the 30 kHz ultrasonic cochlear injury group indicate that the AP amplitude evoked at the same intensity was higher in the 30 kHz-evoked group than the click-evoked group. The spatial positions of cochlear hair cells in guinea pigs had a coding function for the frequencies of low-frequency ultrasound. OHCs have an amplification effect on the coding of low-frequency ultrasonic intensities. The peripheral perception of high frequency BCU may not require the participation of cochlear hair cells.

Key words: Ultrasound; Cochlea; Outer hair cell; Inner hair cell; Auditory brainstem response; Electro-cochleography.

Introduction

The potential applications of ultrasonic hearing aids in hearing rehabilitation have received widespread attention. However, there is still considerable controversy over the peripheral perception and recognition of ultrasonic hearing. Moreover, the existing hypotheses for the peripheral perception and recognition of ultrasonic hearing have not yet been verified. Therefore, the speech recognition rates of ultrasonic hearing aids are relatively low and are far below the level required for applications. Some scholars have used bone-conducted ultrasonic signals to induce near-field cochlear potentials in guinea pigs and found that the peripheral ultrasonic receptors were located in the cochlea, and that ultrasound was perceived by hair cells in the cochlea (1). Lenhardt et al. first reported the use of bone-conducted ultrasonic hearing aids to perform ultrasonic speech tests in severe sensorineural hearing loss (normal saccular function), and found that the patients' speech recognition rates were higher than random recognition rates. Hence, they inferred that the peripheral ultrasonic receptors are located in the saccule (2). Some scholars have also suggested that ultrasonic signals directly stimulate the spiral ganglion or brainstem cochlear nuclei(3). Hence, they proposed that the site of peripheral ultrasonic perception is in the spiral ganglion or brainstem cochlear nuclei (4).

Existing studies have only demonstrated that the cochlea is one of the peripheral receptors of ultrasonic signals, and it is unclear whether the vestibuleis also an ultrasonic receptor. Furthermore, the encoding mechanisms of ultrasonic frequency and intensity by the cochlea have not yet been clarified. Specifically, we do not understand the effects of hair cell positions, and the roles of inner hair cells (IHCs) and outer hair cells (OHCs) on the coding of ultrasonic frequency and intensity. In addition, there is still a lack of direct evidence on the

cellular and molecular levels, and existing conclusions are only speculative hypotheses. As the frequency range of acoustic signals in guinea pigs is far higher than that of humans, they are the ideal model for research on ultrasonic hearing (5-9). The present experiment irradiated the cochlea of guinea pigs with specific doses of low frequency and high-frequency bone-conducted ultrasound (BCU), and successfully created a frequency-related cochlear hair cell-specific injury model. Different ultrasonic frequencies and intensities were used to induce auditory brainstem response (ABR) threshold, compound action potential (CAP) threshold, and action potential (AP) intensity-amplitude input-output curves in normal guinea pigs and ultrasonic cochlear injury model, in order to examine the coding and recognition of BCU frequency and intensity by cochlear hair cells.

Materials and Methods

Experimental animals and grouping

Thirty healthy, 3-month-old Hartley guinea pigs, weighing 250-300 g, with sensitive auricular reflex were randomly allocated to the normal control group, 30 kHz BCU-irradiated cochlear injury group, and 80 kHz BCU-irradiated cochlear injury group (20 ears each). All animals were housed in micro-isolator cages with free access to food and water according to the Guide for the Care and Use of Laboratory Animals. In particular, any effort was put to avoid unnecessary pain of the animals. Ethical approval was given by the medical ethics committee of Huazhong University of Science and Technology.

Preparation of animal models

Preparation of cochlear ultrasonic injury model: The retroauricular fur from an area of about 2 cm in diameter was shaved in the experimental guinea pigs. The animals were anesthetized using intraperitoneal injection of 1% pentobarbital sodium, and secured to a thermostatic operating table. The tracheal tube was connected to an animal ventilator (Midmark), with the respiratory rate maintained at 40-60 breaths/minute, and the oxygen saturation above 90%. The posterior mastoid region of the left ear was coated in paraffin, and an ultrasonic probe (3 mm in diameter) was fixed to the bone surface to irradiate the cochlea at 30 kHz and 80 kHz with an output of 100 db for 6 h.

Detection of BCU-evoked auditory brainstem response (ABR) thresholds and electro-cochleographic compound action potential (CAP) in normal control group and ultrasonic cochlear injury group

Detection of ABR thresholds: Detection was performed using the System 3 auditory evoked potential workstation (Tucker-Davis Technologies, USA). The Pentusa Base Station was used as the acquisition module; the RX6 Multifunction Processor was used as the sound module, whereby frequencies exceeding 32 kHz were connected to the ultrasonic transmitter using an external synchronizer; the RA16PA was used as the preamplifier; and the PA5 was used as the attenuator. The experimental guinea pigs were anesthetized using intraperitoneal injection of 1% pentobarbital sodium. The periauricular fur was shaved, and the animals were transferred to a thermostatic chamber (temperature: 38.5°C) and secured. Then, the animals were transferred to a quiet, acoustically and electromagnetically shielded room. The acquisition electrode was placed on the top of the head, the reference electrode was placed on the ipsilateral retroauricular mastoid of the measured ear, and the ground electrode was placed on the nasion. The acquisition amplification was 1000k, number of sweeps was 500, and testing began from 90 db or 100 db. The frequencies were evoked using click, 30 kHz, and 80 kHz. Click was introduced from the left via air conduction using headphones; 30 kHz and 80 kHz were introduced via bone conduction through the left mastoid process. ABR threshold was determined using wave V. CAP detection: The cochlea of the same experimental samples was exposed, and the animals were transferred to a quiet, acoustically and electromagnetically shielded room. The silver-wire electrode was placed on the round window, and the reference electrode was placed at the auricular skin wound. CAP was detected using the same parameters as ABR threshold.

Protein immunofluorescence assay of OHC Prestin and IHC Otofelin in normal control group and animal model

The guinea pigs were sacrificed immediately after detection of BCU-evoked ABR thresholds and electrocochleography. The auditory bulla was removed and prepared using 4% paraformaldehyde perfusion fixation. After 8h of fixation, the samples were dissected under a microscope to retrieve the basilar membrane, which was mounted on a slide for protein immunofluorescence assay of Prestin and Otofelin in cochlear hair cells. Reagents and instrument for Prestin immunofluorescence assay: primary antibody (rabbit anti-Prestin 1:200, Santa Cruz, SC-30163), mouse anti-Otoferlin (1:200, Abcam, AB53233); secondary antibody (Alexa Fluor 568 conjugated goat anti-rabbit IgG, 1:1000, Molecular probe, A-11011); confocal microscope (Zesis LSM800).

Statistical analysis

Data were expressed as mean ±SEM. Comparison between two groups was performed using the Student *t*-test. Significance testing of difference between in means was performed using the Sigma State software, with $P<0.05$ as the significance level.

Results

Detection of BCU-evoked ABR threshold and electro-cochleographic CAP in normal control group and ultrasonic cochlear injury group

In the normal control group, click-, 30 kHz-, and 80 kHz-evoked ipsilateral ABR thresholds were 30.00±0.21, 55.78±4.43, and 68.15±5.33, respectively; and the CAP thresholds were 30.12±0.27, 54.28±3.43, and 67.75±4.23, respectively. The ABR and CAP thresholds evoked by different frequencies were significantly different, $P<0.05$.

In the 30 kHzBCU cochlear injury group, click-, 30 kHz-, and 80 kHz-evoked ipsilateral ABR thresholds were 30.67±0.41, 69.17±5.63, and 67.85±4.73, respectively; and the CAP thresholds were 31.09±0.21,

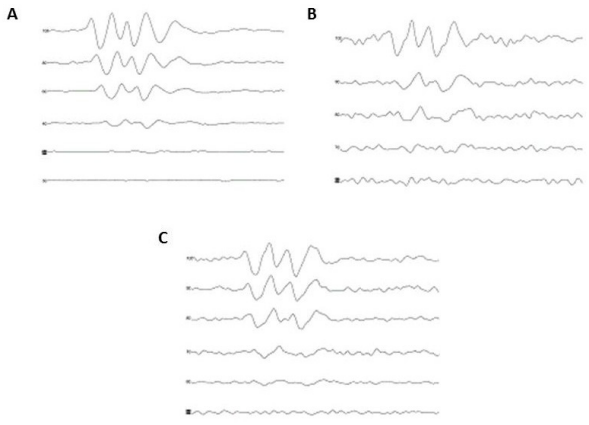

Figure 1. A. Click-evoked ABR threshold of 30 kHz ultrasonic cochlear injury group; B. 30 kHz-evoked ABR threshold of 30 kHz ultrasonic cochlear injury group; C. 80 kHz-evoked ABR threshold of 30 kHz ultrasonic cochlear injury group.

68.68±4.45, and 68.15±5.11, respectively. The ABR and CAP thresholds evoked by different frequencies were significantly different, P<0.05. Comparison between the 30 kHz BCU cochlear injury group and the normal control group indicated that ABR threshold and CAP threshold evoked by click and 80 kHz were not significantly different, P>0.05 (Figure 1A).Comparison between the 30 kHz BCU cochlear injury group and the normal control group indicated that the 30 kHz-evoked ABR threshold and CAP threshold were significantly higher in the 30 kHz BCU cochlear injury group, and the difference was significant, P<0.05 (Figure 1B, Figure 1C).

In the 80 kHz BCU cochlear injury group, click-, 30 kHz-, and 80 kHz-evoked ipsilateral ABR thresholds were 31.11±0.36, 56.19±4.47, and 68.95±6.23, respectively; click- and 30 kHz-evoked CAP thresholds were 30.79±0.42 and 55.17±4.15, respectively. The ABR thresholds evoked by different frequencies were significantly different, P<0.05. Comparison between the 80 kHz BCU cochlear injury group and the normal control group indicated that ABR thresholds evoked by click,30 kHz, and 80 kHz were not significantly different, P>0.05 (Figure 2A-C). CAP could not be evoked by 80 kHz in the 80 kHz BCU cochlear injury group. Comparison between the 30 kHz and 80 kHz BCU cochlear injury groups indicated that the 30 kHz-evoked ABR threshold

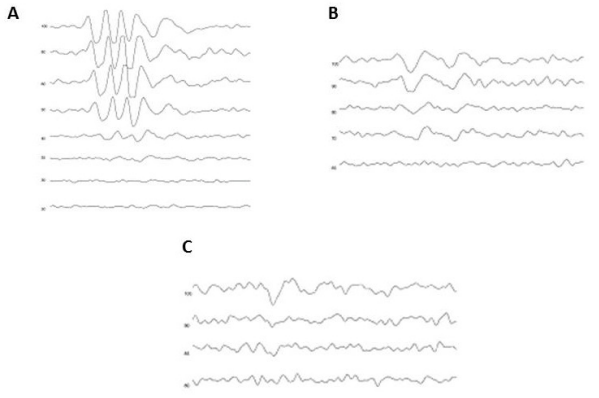

Figure 2. A. Click-evoked ABR threshold of 80 kHz ultrasonic cochlear injury group;B. 30 kHz-evoked ABR threshold of 80 kHz ultrasonic cochlear injury group; C. 80 kHz-evoked ABR threshold of 80 kHz ultrasonic cochlear injury group.

was significantly higher in the 30 kHz BCU cochlear injury group, and the difference was significant, P<0.05.

The click and 30 kHz-evoked AP intensity-amplitude input-output curves evoked in the 30 kHz BCU cochlear injury group indicated that the AP amplitude evoked at the same intensity was higher in the 30 kHz-evoked

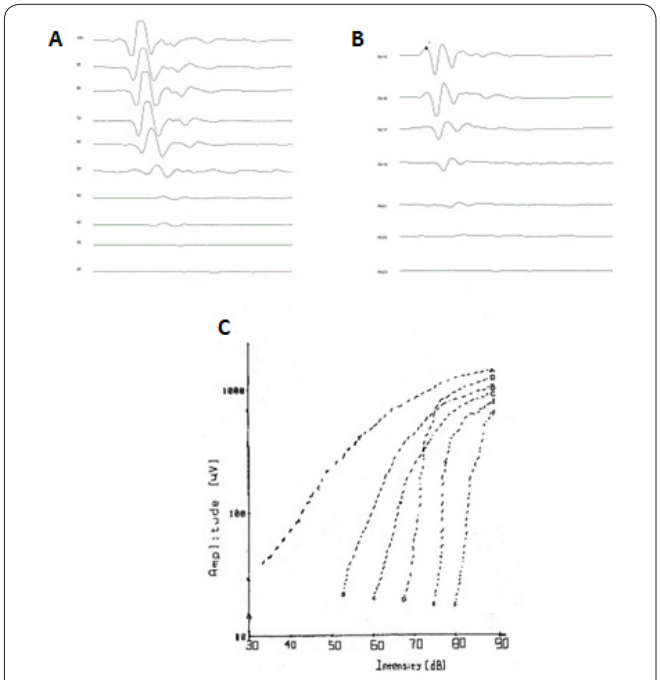

Figure 3. A. Click-evoked CAP threshold of 30 kHz ultrasonic cochlear injury groupclick; B. 30kHZ-evoked CAP threshold of 30 kHz ultrasonic cochlear injury group click; C. Click- and 30 kHz-evoked CAP intensity-amplitude curves in the 30 kHz ultrasonic cochlear injury group (A is left click induction; B, C, D, E, and F are 30 kHz left BCU induction).

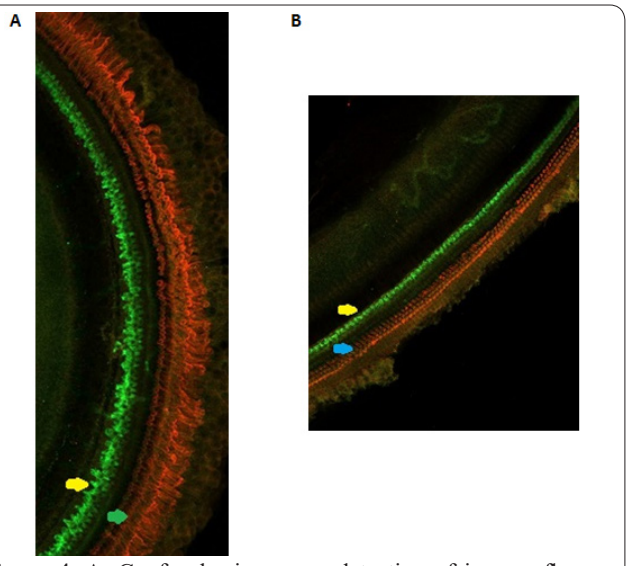

Figure 4. A. Confocal microscopy detection of immunofluorescence forcochlear Prestin (red) + Otofelin (green) within a range of 400um±5um at 95mm±10um from the bottom edge of the basilar membrane in the 30 kHz ultrasonic cochlear injury group. OHC injury; comparison of IHC and normal control did not reveal significant changes (Red→ indicates IHC; yellow → indicates OHC), ×200 magnification; B. Confocal microscopy detection of immunofluorescence forcochlear Prestin (red) + Otofelin (green) within a range of 400um±5um at 95mm±10um from the bottom edge of the basilar membrane in the 30 kHz ultrasonic cochlear injury group. OHC injury; comparison of IHC and normal control did not reveal significant changes (Red→ indicates IHC; yellow → indicates OHC), ×100 magnification.

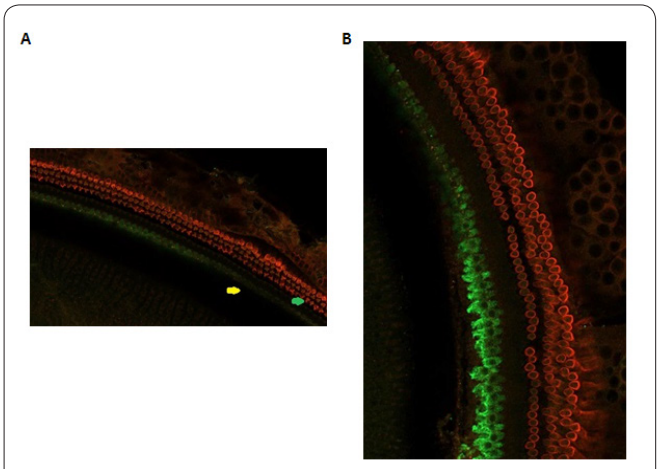

Figure 5. A. Confocal microscopy detection of immunofluorescence forcochlear Prestin (red) + Otofelin (green) within a range of 300um±5um at 45mm±10um from the bottom edge of the basilar membrane in the 80 kHz ultrasonic cochlear injury group. Weakened immunofluorescence in both IHC and OHC, with a more significant decrease in IHC (Yellow → indicates IHC; green→ indicates OHC), ×200 magnification; B. Confocal microscopy detection of immunofluorescence forcochlear Prestin (red) + Otofelin (green) within a range of 300um±5um at 45mm±10um from the bottom edge of the basilar membrane in the 80 kHz ultrasonic cochlear injury group. Weakened immunofluorescence in both IHC and OHC, with a more significant decrease in IHC (Yellow→ indicates IHC; green → indicates OHC), ×400 magnification.

group than the click-evoked group (Figure 3A-C,).

Protein immunofluorescence assay of cochlear hair cell Prestin and Otofelin in normal control group and ultrasonic cochlear injury group

The immunofluorescence of OHC Prestin within a range of 400um±5um at 95mm±10um from the bottom edge of the basilar membrane was significantly lower in the 30 kHz ultrasonic cochlear injury group when compared to the normal control group in the corresponding site; there were no significant changes in the immunofluorescence of IHC Otofelin compared to the normal group in the corresponding site (Figure 4A-B).

The immunofluorescence of OHC Prestin and IHC Otofelin within a range of 300um±5um at 45mm±10um from the bottom edge of the basilar membrane was significantly lower in the 80 kHz ultrasonic cochlear injury group when compared to the normal control group in the corresponding site (Figure 5A). The decrease in immunofluorescence was more significant in for IHC Otofelin than that for OHN Prestin (Figure 5A-B).

Discussion

Ultrasonic speech and ultrasonic hearing have gained widespread attention from researchers due to their promising applications of in hearing rehabilitation and special environments (e.g. underwater) (5,7-10). Currently, the application of ultrasonic hearing aids has entered the clinical trial phase. However, the ultrasonic speech recognition rates of profoundly deaf participants are still relatively low, and this is mostly related to the unclear mechanisms of peripheral and central perceptions of ultrasound.

In the research on ultrasonic hearing, studies related to ultrasonic perception and perceptual mechanisms are still controversial. Kenji Ohyma et al. reported the electro-cochleograms recorded in guinea pigs during stimulation by BCU signals (98.8k Hz and 143.5kHz), and found that the L-part of the CAP stimulation intensity-latency input-output curve had disappeared(1) .They also inferred that the peripheral ultrasonic receptors were located in the cochlea, and that ultrasound is perceived by cochlear hair cells. Lenhardt et al. first reported about the use of bone-conducted ultrasonic hearing aids and found that the speech recognition rates of patients with severe sensorineural hearing loss (normal saccular function) were up to 40%, which inferred that the peripheral ultrasonic receptors were located in the saccule (2). Nishimura T et al. reported that BCU signals in humans could be masked by high-frequency air-conducted speech frequency sounds, indicating that the peripheral perceptual organ of ultrasound was located in the cochlea (11). Some scholars have suggested that ultrasonic signals can directly stimulate the spiral ganglion or brainstem, thereby inducing ultrasonic hearing (12) . Torbatian Z et al. performed Doppler ultrasound at the round window and were the first to observe the vibrational signals of the cochlear basilar membrane evoked by BCU (12) . Scholars have also used masked speech-modulated BCU to test the speech recognition rates of volunteers (13-14) .They found that the test results between modulated and non-modulated ultrasonic speech signals were different for profoundly deaf patients, but were not different for participants with normal hearing, suggesting that the direct stimulation of ultrasonic signals played a crucial role in the signal perception of profoundly deaf patients (4).

At present, studies have only verified that the cochlea is one of the peripheral receptors of ultrasonic signals, and it is unclear whether the vestibule is also an ultrasonic receptor. Furthermore, the coding mechanisms of ultrasonic frequency and intensity by the cochlea have not yet been clarified. Specifically, we do not understand the effects of basilar membrane site, and the roles of IHCs and OHCs on the coding of ultrasonic frequency and intensity. In addition, there is still a lack of direct evidence on the cellular and molecular levels, and existing conclusions are only speculative hypotheses.

Due to the high level of similarity in the structure and physiology of human and guinea pig cochlea, we selected guinea pigs as our ideal research model. In a previous study, we exposed normal guinea pigs to specific doses of BCU signals with different frequencies, and observed that different BCU frequencies can lead to the decreased succinate dehydrogenase activity in hair cells at different sites (15) . As Prestin is mainly distributed in OHCs and Otofelin is mainly distributed in IHCs, we irradiated the cochlea of guinea pigs for 6 h using low-frequency BCU (30 kHz, 100 db) and high-frequency BCU (80 kHz, 100 db), followed by Prestin immunofluorescence labeling + Otofelin immunofluorescence detection in cochlear hair cells. This allowed us to observe the effects of irradiating the cochlear hair cells of guinea pigs with different frequencies of BCU at a specific dose.

In the present study, we found that a specific dose of irradiation with low-frequency ultrasound mainly induced OHC injury at the corresponding site, whereas

irradiation with high-frequency ultrasound induced IHC injury. Moreover, IHC injury was more serious than OHC injury. Thus, we have successfully created a cochlear injury model related to ultrasonic frequency. The ABR and CAP thresholds of the experimental guinea pigs were evoked using click, 30 kHz, and 80 kHz ultrasound. The 30 kHz-evoked ABR threshold was significantly higher in the 30 kHz ultrasonic cochlear injury group compared to the normal control group and the 80 kHz ultrasonic cochlear injury group. The difference in the 80 kHz-evoked ABR thresholds were not significant between the 30 kHz and 80 kHz ultrasonic cochlear injury groups. These results indicate that low-frequency ultrasound is perceived by cochlear hair cells, and the coding of frequency is determined by the spatial position of hair cells, whereas high-frequency ultrasound may not require the participation of cochlear hair cells. The click- and 30 kHz-evoked AP intensity-amplitude curves for the 30 kHz ultrasonic cochlear injury group indicate that the AP amplitude evoked at the same intensity was higher in the 30 kHz-evoked group than the click-evoked group. These results indicate that OHCs exert an amplification effect in the perception of low-frequency ultrasound. 80 kHz BCU could evoke ABR thresholds in the 80 kHz ultrasonic cochlear injury group, but could not evoke CAP. Moreover, the histological results of the 80 kHz ultrasonic cochlear injury group showed IHC and OHC injuries in specific sites. This result implies that the perception of high frequency BCU may not require the perceptual coding of hair cells. In this experiment, we encountered difficulties in the direct acquisition of changes in evoked potentials at the lesion site; hence, electrophysiological parameters were based on cochlear near-field evoked potentials. Further detection of changes in evoked potentials at the lesion site will enhance the persuasiveness of our experiment.

In conclusion, we established a cochlear injury model using bone-conducted ultrasound irradiation in guinea pigs and investigated peripheral coding and recognition of ultrasonic signals. Results showed that the spatial positions of cochlear hair cells in guinea pigs had a coding function for the frequencies of low-frequency ultrasound and outer hair cells had an amplification effect on the coding of low-frequency ultrasonic intensities. What's more, the peripheral perception of high frequency BCU might not require the participation of cochlear hair cells. These results may give deeper understanding for cochlear injury.

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Interest conflict

All of the authors have no conflict of interest in this research.

Author's contribution

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

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