A Validation Study of Auditory Function in Aminoglycoside-Furosemide Ototoxicity Mice Model: Auditroy Brainstem Response and Distortion Product Otoacoustic Emissions

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Directed by Professor Young Joon Seo

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This certifies that the masters thesis
of Joon Sik Im is approved.

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ABSTRACT

A Validation study of Auditory Function in Aminoglycoside-Furosemide ototoxicity mice model: Auditory brainstem response and Distortion Product Otoacoustic Emissions

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(Directed by Professor Young Joon Seo)

Hypothesis: Evaluation of the auditory function in ototoxic mouse model with single administration of kanamycin and furosemide.
Background: Ototoxic mouse model was produced with the administration of ototoxic drugs aminoglycoside kanamycin and loop-diuretic furosemide, thus validation of auditory function of the mouse model is much needed to determine the efficacy of the drugs.

Methods: Kanamycin sulfate 550mg/kg (VWR life sciences, PA, USA) and furosemide 130mg/kg (Lasix, Handok, Korea) were administered through subcutaneous and intraperitoneal injection respectively. Auditory brainstem response and distortion otoacoustic emission tests were performed on days

3,5,7,10,14 post administration of the ototoxic drug.

Results: Thresholds in response to the stimulus given in the auditory brainstem recordings and distortion otoacoustic emission tests were obtained. The hearing threshold shift to high stimulus intensity was observed post administration of the ototoxic drug. Latency of the ABR peak waves were recorded and analyzed, latency delay was observed as hearing threshold increases.

Conclusion: These findings will further support in the application of this animal model in various studies regarding ototoxic hearing loss.

Key words: hearing loss; auditory brainstem response; aminoglycoside; furosemide

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I. Introduction

It has long been known that the major irreversible toxicity of aminoglycosides is ototoxicity,[1,2] Aminoglycosides have variable cochleotoxicity and vestibulotoxicity.[3] Streptomycin and gentamicin are primarily vestibulotoxic, whereas amikacin, neomycin, dihydrosterptomycin, and kanamicin are primarily cochleotoxic. We introduced in the previous study that the "one-shot" injection with combinations of kanamycin and furosemide in the mouse model of ototoxicity may be a novel technique for inducing local inner ear injury[4] The mice model had the hearing loss to 70dB in auditory brainstem response(ABR) test with click sounds within 7 days after an injection.

Although auditory evoked potentials originating from the brainstem in mice are widely used due to similarity to those of humans, strains of mice have their own different responses to the ototoxicity drugs.[5] Standardisation of stimulation and recording parameters in auditory functional tests including the auditory brainstem response(ABR)/distortion product otoacoustic emission(DPOAE) has not been achieved in many species of mice, and especially in the mice model with the ototoxicity drugs.[6]

In this study a combination of aminoglycoside kanamycin and loop diuretic furosemide were used to induce ototoxic hearing loss through a single-dose regimen. We used the C57 BL/6J strain, which are frequently used as models in auditory research because they have susceptibility to aging or ototoxicity.[7] It aims to establish a database on the ototoxic hearing loss pattern in this mice model through ABR and DPOAE samples collected.

II. Materials and Method

1. Animals

20 male C57BL/6J mice, were subjected for auditory brainstem response under anesthesia. The subjects during recording time were 5 to 7 weeks' age and their weight was between 25-30 grams, they were given free access to food and water. The recordings were performed in the animal laboratory of Yonsei University Wonju College of Medicine in Wonju, Korea, in accordance with the institutional animal care and use committee(YWC-180703-1).

2. Ototoxic drug

Kanamycin sulfate 550mg/kg (VWR life sciences, PA, USA) and furosemide 130mg/kg (Lasix, Handok, Korea) were administered through subcutaneous and intraperitoneal injection respectively.[4] The mouse in the control group had received saline through subcutaneous injection followed by intraperitoneal injection.

3. ABR Procedure

Prior ABR recordings the mice were anesthetized with 100mg/kg ketamine (Yuhan, Seoul, Korea) and 10mg/kg xylazine (Rompun, Bayer, Ansan, Korea) by intraperitoneal injection. The anesthetized mice were tested in a sound attenuating chamber with built in faraday cage. Isothermal pad was used to maintain the body temperature of the test subject. The stimuli, data management and ABR collection was done by using TDT RZ6/BioSigRZ system (Tucker Davis Technologies, Alachua, FL, USA) 12mm long, gauge 27 subdermal needles electrodes (27GA 13mm, Rochester Electro-Medical, USA) were used to record the ABR (Figure 1A). One channel was recorded and the active electrodes are placed in the vertex, reference electrode placed axial to pinnae which is the same side with the stimulus delivery, and ground electrode placed in the contralateral side. The electrodes are connected with the low impedance headstage (RA4LI, TDT) which interfaces with the TDT amplifier.

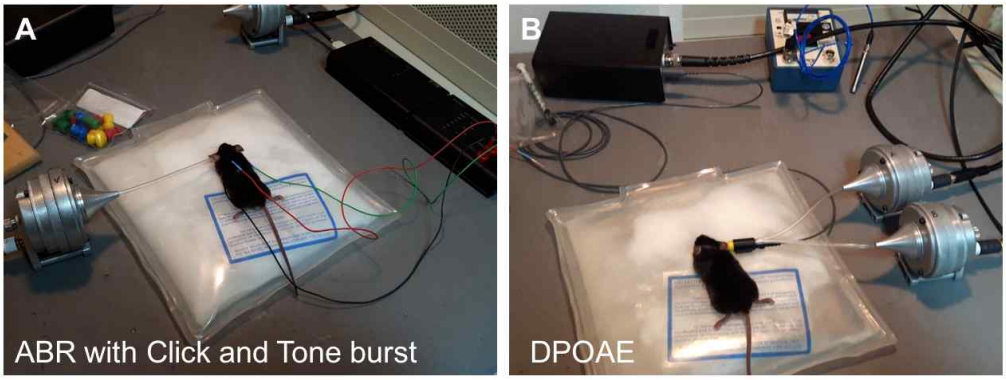

Figure 1: Experiment setting

(A) Auditory brainstem response test setting; electrodes were inserted accordingly. active electrode(red) at the vertex, reference electrode(black) at the ipsilateral ear to the stimulus and ground electrode(green) at the contralateral ear.

(B) Distortion product otoacoustic emissions test setting; pure tone stimulus from two separate sound sources were given and the response were recorded.

Acoustic stimuli were generated by auditory processor (RZ6, Tucker Davis Technologies, Alachua, FL, USA), the stimulus signals and response signal data was acquired by automated processing through BioSigRZ software installed in the PC. Stimuli were delivered in a closed field setting by a magnetic speaker (MF1, TDT, Alachua, FL, USA) with a PVC tubing with a conical cap which is inserted to the subject's ear.

4. DPOAE procedure

Mice were anesthetized prior recordings; stimuli were generated by etymotic research microphone (ER10B+) which is connected with a pair of MF1 microphones were inserted to the subject's ear canal (Figure 1B).

5. ABR and DPOAE Recording

ABR and DPOAE's were recorded from the bilateral ears, 1-day prior administration (day-1) of ototoxic drug and days 3, 5, 7, 10, 14 post administration of the ototoxic drug. Prior ABR and DPOAE recordings mice were anesthetized with mixture of ketamine 100mg/kg and 10mg/kg xylazine. The stimuli given maximum of 90dB to minimum 10dB for clicks and 8, 16, 20, 26, 32 kHz for tone bursts. 10dB steps reducing the SPL to obtain the auditory thresholds. DPOAE was measured following after ABR recordings using a pair of MF1 microphone and etymotic research microphone. The stimuli were generated by using the TDT software.

6. Statistical Analyses

Statistical analyses were processed with GraphPad Prism software. Data obtained from the peak detection were expressed as mean±SEM and values from different time point were compared using a repeated measures two-way ANOVA. In all tests, the means had p-value of ≤ 0.05.

III. Results

1. Changes of Auditory thresholds in the ototoxic mice model

Waveforms acquired from the ABR recordings from all the subject were similar. Figure 2 illustrates the typical waveforms of ABR recorded from two different types of stimulus, click and 20kHz tone burst from mouse with normal hearing before the injection of the ototoxicity drugs.

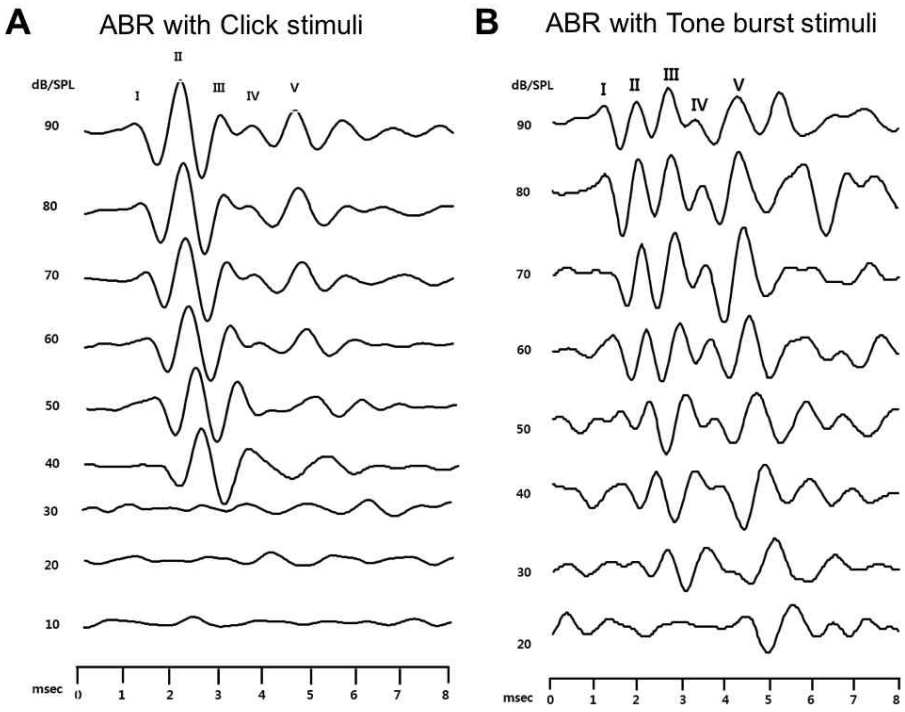

Figure 2: ABR recordings according to click (A) and tone burst 20kHz (B) in a C57BL/6 mouse with normal hearing prior ototoxic drug administration. Five peaks after the initiation of acoustic stimulus labelled with roman numbers, maximum stimuli intensity was at 90dB SPL.

Upon click stimulation the maximum stimulation intensity given was at 90 dB SPL with 10dB reducing steps to reach 10dB stimulation, five distinct positive peaks (I-V) of ABR waves were identified within 7msec after initiation of stimulus, peak II shows highest amplitude level and peak V was remained identifiable with low stimulus intensities were given. The tone burst stimulation had peak III with highest amplitude and peak V remained identifiable with low stimulus intensity. In both ABR recordings of click and tone burst stimulus, the peaks have shifted to right, which indicates that the latencies of the peak waves were delayed with decreasing stimulus delivery.

After the ototoxic drugs, Figure 3 showed the changes of auditory thresholds in click ABR, 20kHz tone burst ABR, and DPOAE according to the time up to 2 weeks. The mice model had profound hearing losses over 70dB thresholds within 7 days after the injection. In tone burst ABR, the greater hearing loss was observed significantly on the 3 day in high frequencies (20 kHz and 32kHz) than in the low frequency (8kHz). After 5 days of hearing loss, there was no differences showing frequency specificity. This phenomenon was pronounced in DPOAE test. In the 32kHz, the acute changes of auditory hearing thresholds were shown from initial day (1 day and 3 day) after the injection. The frequency specificity was also present around on the 3 day, and if the injury is severe by ototoxic drugs after 5 days, the hair cells appeared to be damaged from basal turn to apical turn.

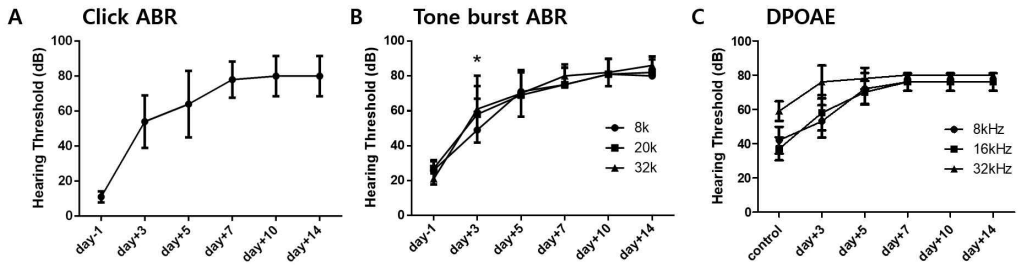

Figure 3: Changes in the hearing threshold of the C57BL/6 mouse pre and post administration of the ototoxic drug in response to click, tone burst and distortion product otoacoustic emissions. Decrease in the hearing threshold could be observed on day 3 post administration and complete hearing loss noted on days 7 and onwards, similar hearing loss pattern could be observed in the 3 different types of recordings performed.

2. Changes of Latency in Peak waves in the ototoxic mice model

The latencies on each peak wave (I-V) in click ABR were recorded in the subjects pre and post administration of the ototoxic drug kanamycin and furosemide. The latency of peak waves I-V from day prior to the administration of the ototoxic drug and days 3, 5, 7 were recorded (Table 1). Distinct losses of the hearing were observed on the 5 day, on which the hearing threshold was at 60dB. The latencies of the peak waves were analyzed in comparison with the normal latency recorded on day prior to administration of the ototoxic drug (Figure 4). The latencies of peak wave I to V has increased when decreased click stimulus was given, and latency of the ABR wave peaks recorded shows delayed latency of the peak waves on day 5 post administration of the drug when compared with the time point with normal hearing.

	dB SPL	I		II		III		IV		V	
		Lat	SD	Lat	SD	Lat	SD	Lat	SD	Lat	SD
Day -1	90	1.26	0.10	2.36	0.12	3.40	0.33	3.66	0.48	5.41	0.50
	80	1.37	0.14	2.46	0.13	3.47	0.20	3.74	0.51	5.50	0.50
	70	1.52	0.15	2.50	0.11	3.51	0.20	3.82	0.59	5.82	0.57
	60	1.62	0.16	2.59	0.13	3.64	0.26	3.95	0.60	5.90	0.63
	50	1.74	0.16	2.71	0.13	3.74	0.19	4.07	0.61	6.08	0.66
	40	1.93	0.15	2.86	0.16	3.90	0.21	4.36	0.65	6.33	0.66
	30	2.08	0.38	3.21	0.59	4.45	0.77	4.52	0.75	6.86	0.73
Day +3	90	1.56	0.19	2.63	0.18	3.63	0.29	4.74	0.39	5.63	0.43
	80	1.69	0.18	2.73	0.20	3.79	0.30	4.85	0.38	5.71	0.50
	70	1.77	0.12	2.78	0.16	3.84	0.26	4.97	0.45	5.84	0.49
	60	1.89	0.14	2.89	0.17	3.97	0.26	5.32	0.60	6.35	0.58
	50	1.96	0.26	3.08	0.21	4.15	0.31	5.65	0.64	6.62	0.58
	40	2.18	0.10	3.23	0.27	4.25	0.33	5.59	0.71	6.63	0.61
	30	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Day +5	90	1.55	0.48	2.63	0.68	3.84	0.86	4.66	0.83	5.70	0.73
	80	1.77	0.28	2.64	0.37	3.75	0.50	5.09	1.00	6.23	0.94
	70	1.52	0.49	2.57	0.45	3.65	0.47	4.57	0.47	5.58	0.46
	60	1.68	0.38	2.68	0.45	3.83	0.53	5.07	0.80	6.50	0.99
	50	1.75	0.27	2.65	0.51	3.74	0.75	5.29	1.13	6.55	1.47
	40	2.02	0.12	3.14	0.05	4.48	0.32	5.84	0.72	7.12	0.88
	30	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Day +7	90	1.82	0.24	2.87	0.21	3.95	0.25	4.80	0.31	6.02	0.36
	80	1.68	0.28	2.79	0.23	3.88	0.28	4.71	0.31	5.91	0.35
	70	1.96	0.26	2.93	0.25	4.02	0.29	5.11	0.50	6.30	0.55
	60	1.87	0.32	2.95	0.26	4.13	0.32	5.31	0.68	6.62	0.91
	50	1.69	n/a	2.67	n/a	3.74	n/a	4.77	n/a	5.80	n/a
	40	1.81	n/a	2.84	n/a	3.95	n/a	6.01	n/a	7.81	n/a
	30	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 1. Mean values and standard deviation of the latencies recorded from five ABR peaks: the latencies of the peak wave I-V have delayed with decrease in the click stimulus intensity. Post ototoxic drug administration further delay in the latencies are recorded.

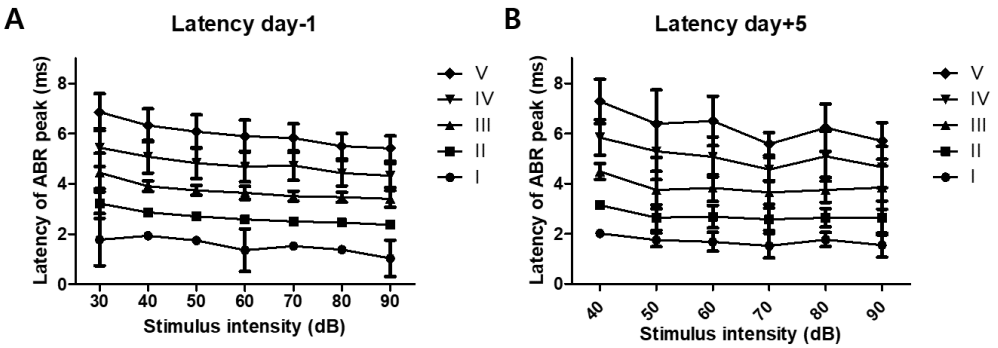

Figure 4: Latency of peak in ABR click stimulus; 1-day pre-administration of kanamycin/furosemide regimen and 5-day post administration of drug. Increased peak intensities were observed in ABR recordings 5-day post administration of ototoxic drugs.

Among five waves, we compared the amplitudes and latencies of peak II and V (Figure 5) and have noted that the latencies on wave peak II and V has increased with significance on day 5 post administration of the ototoxic drug, and there were no identifiable wave peaks at when low intensity stimulus of 30dB and below was given.

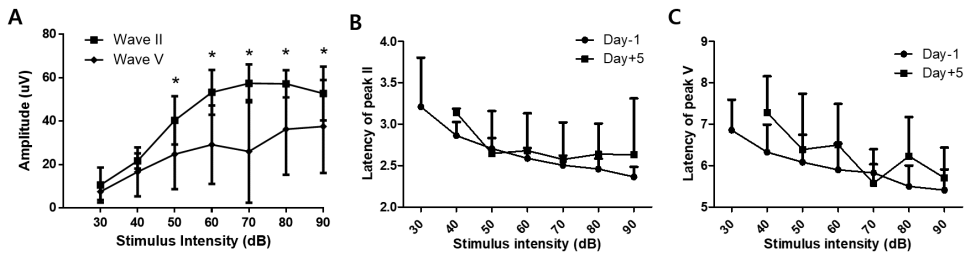

Figure 5: Latencies of 2 ABR peaks II and V. In comparison to the latencies recorded 1-day prior treatment of drugs the latencies of the ABR peaks were delayed 2-folds post administration of the ototoxic drugs

Discussion

In animal models, the hearing organ of mouse is similar to the microstructures of human hearing organ and it is an economical model for the experiment.[8] The auditory function tests like ABR and DPOAE has become a useful and practical procedure for the determination of hearing levels in animals.[9] The ABR patterns of mice typically consisted of five vertical positive waves.[10] Wave I voltage arises from the cochlea and/or compound action potential of auditory nerve. Waves from II to V reflect the evoked activity at ascending generators in the auditory midbrain and are known to originate from cochlear nuclei, contralateral superior olivary complex, lateral lemniscus and contralateral lateral inferior colliculus.[6] But there are so various differences in values of ABR parameters according to the strains of the mice.[11] Millions of mice are produced annually at the Jackson Laboratory. The Neuroscience Mutagenesis Facility at the Jackson

Laboratory has undertaken a large scale auditory screening project. Zhou et al. reported auditory brainstem responses in 10 inbred strains of mice.[11,12] Scimemi et al. reported the normative data reported in C57BL/6J mouse, which can be used as a reference for further investigations on murine models of hearing loss.[6] DPOAE should serve as a useful tool for studying the function of outer hair cells (OHCs) on the cochlea. Parham et al. reported the values of DPOAEs recorded in the young and aging C57BL/6J mouse.[13] In the mice, cochlear pathology progresses from base to apex, therefore DPOAE changes are first seen in the high-frequency region of the cochlea.[14] Though normative data in mice auditory functional tests were published, however there were no data for hearing patterns of the pathologic mice model, especially ototoxic mice model. We have suggested one shot mice model with the ototoxic drugs in the previous study, and we have performed the database in this study to establish the ototoxic hearing loss pattern in this mice model through ABR and DPOAE tests.

The threshold, amplitude, and latency analysis of the ABR provides information on the peripheral hearing status and the integrity of brainstem pathways. A click stimulus covering a wide frequency band is a commonly used stimulus to evaluate ABR. The click stimulus sound and the 4 kHz stimulus sound among the tone burst stimulus were compared in the mouse, the waveforms were similar but showed differences in latency.[15] Thus we have applied click stimuli in our study for evaluating the changes of latency. The latency in the C57BL/6J mouse is similar to that of other mice in the previous study,[11,16] and can be account as a baseline for

evaluating the rate of transmission of auditory signals modeled on mice or the function of the central nervous system. The amplitude of waves I and V in C57BL/6J mouse increased monotonically with increasing intensity, which is similar to what is commonly used in evoked potential studies.[11] Burkard et al. reported that the slope of latency–intensity functions of waves I and V were ~8 to 9μs/dB in gerbils but were ~13 to 16μs/dB in rats when examined under click stimulation conditions.[12] In this study, the slope of latency–intensity function of wave II ranged from 2.36 to 3.21μs/dB while that of wave V ranged from 5.41 to 6.86μs/dB. When considering wave I-V peak latency, the peak latency of waves decreased with increasing click intensity stimulus given.

The ototoxicity of aminoglycoside antibiotics has been well established in the mice experiments. Furosemide and other loop diuretics have well-known synergistic effects with aminoglycoside antibiotics when the 2 drugs are administered closely in time and cause profound hearing loss.[17,18] With combined dose of each drug after administration of an aminoglycoside antibiotic followed by a loop diuretic, complete OHC loss with IHC damage has been observed in our previous study.[4] This mice model would be further used in the area of ear science researches. The utilization of this mice model to validate the standard values by auditory function tests like ABR and DPOAE would be helpful for the researchers experimenting in similar field of study.

Conclusion

Establishment and validation of the hearing loss pattern in ototoxic mouse model is much needed for the researchers to determine the auditory function of the mouse model. Thus through these findings it would support researches with the utilization of one-shot mouse model.

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ABSTRACT (IN KOREAN)

아미노글리코사이드-퓨로세마이드 이독성 생쥐 모델에서의 청성뇌간반응과 변조이음향방사검사를 통한 청각기능 평가

<지도교수 서 영 준>

연세대학교 대학원 의학과

임 준 식

배경: 아미노글리코사이드 항생제인 카나마이신 투여 후 고리형 이뇨제인 퓨로세마이드 추가 투여 시 상승 효과를 통해 심한 난청을 유발할 수 있으며, 완전한 외유모세포와 내유모세포의 손실을 확인하였다. 이독성 생쥐 모델에서의 청각 기능 평가는 약물의 효용성을 결정하기 위해 중요하다.

방법: 카나마이신 550mg/kg과 퓨로세마이드 130mg/kg을 각각 피하와 복강으로 주입하였다. 약제 투여 전과 투여 3일 후, 5일 후, 7일 후, 10일 후, 14일 후 청성뇌간반응과 변조이음향방사검사를 시행하였다.

결과: 청성뇌간반응과 변조이음향방사검사를 통해 자극에 대한 반응의 역치를 측정하였다. 이독성 약제를 투여 3일 후 청각 역치 증가가 있었고, 7일 후 완전농의 소견을 보이며, 높은 자극 강도로의 청각 역치 전환이 관찰되었다. 이독성 약제 투여 후 ABR 잠복기 지연이 관찰되었고, II, V 파형의 잠복기가 이독성 약제 투여 5일 후 유의미하게 지연되었다.

결론: 본 연구는 향후 대규모 추가 연구를 통해 아미노글리코사이드-퓨로세마이드 이독성 생쥐 모델에서의 청각 기능 평가를 위한 표준값 설정, 난청 패턴의 확립 및 검증에 도움이 될 것으로 기대한다.

핵심되는 말 : 난청, 청성뇌간반응, 변조이음향방사검사, 아미노글리코사이드, 퓨로세마이드, 이독성