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Original Article

Valproic Acid Increases Formation of ROS and Interferes with Hearing Recovery after Noise Trauma in Guinea Pigs

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OBJECTIVE: Valproic acid (VPA) is a widely used anti-convulsant. There have been some reports that VPA causes hearing loss and tinnitus, and oxidative stress has been associated with VPA toxicity. Thus, we were interested in the effects of VPA on noise-induced hearing loss.

MATERIALS and METHODS: Animals were divided into two groups, as follows: treatment with VPA (30 mg/kg daily) and treatment with phosphate buffered saline (PBS) (control group). VPA and PBS were injected intraperitoneally once daily for 20 days (3 days before noise exposure until 2 weeks after noise exposure). Acoustic trauma was induced by continuous white noise at 110 dB SPL for 4 h/day for 3 days. After noise trauma, hearing results, anti-oxidant enzyme analysis, and histological findings were compared in both groups.

RESULTS: An auditory brainstem response threshold shift was observed in both groups 1 day after noise exposure. Two weeks following noise exposure, there was gradual recovery to normal levels in the control group; however, in the VPA group, especially with respect to 4 kHz sound, hearing loss recovered less than in the control group ($p<0.05$). Also, based on morphologic studies, more outer hair cell loss was observed (especially 10-12 mm from the apex in the 4 kHz area) in the VPA group than in the control group ($p<0.05$). Anti-oxidative enzyme mRNA expression was significantly increased in the VPA group compared to the control group ($p<0.05$).

CONCLUSION: This study demonstrates that VPA may have a harmful effect on noise-induced ototoxicity via oxidative stress in guinea pigs.

KEY WORDS: Valproic acid, hearing loss, noise, oxidative stress

INTRODUCTION

Valproic acid (VPA) is a widely used anti-convulsant that is effective for seizure disorders, such as epilepsy. It is also used as a mood stabilizer for the treatment of bipolar disorders. Even though some cases of ototoxicity using this drug have been reported [1, 2], the mechanism of the ototoxicity remains elusive. Several studies have proposed the mechanism that VPA toxicity is associated with oxidative stress [3, 4]. Also, the generation of oxygen free radicals in the cochlea after noise exposure is a potential mechanism of noise-induced hearing loss (NIHL).

It is well known that excessive occupational noise exposure can lead to NIHL. Moreover, the increase in the popularity of portable music players has increased the exposure to high sound levels, and this can cause NIHL, especially in noisy environments [5-9]. Thus, patients who have been prescribed VPA are at increased risk of exposure to hazardous noisy sounds from portable music players. However, there is no information pertaining to a relationship between the use of VPA and NIHL.

There are several underlying mechanisms of NIHL, and the most likely mechanism of noise trauma by personal music players may be explained with metabolic damage, because the intensity of portable music players is limited to below 110 dB SPL [10, 11].

Metabolic damage to cochlear cells initiates a cascade of biochemical processes in the cochlea that leads to death of hair cells. The generation of reactive oxygen species (ROS) after acoustic trauma is a well-known mechanism of NIHL [12]. High-level noise leads to significantly decreased blood flow to the stria vascularis [13]; then, resolution of the ischemic event by reperfusion leads to an elevation in ROS in the inner ear, which could elevate the levels of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, and glutathione reductase) in cochlear tissue [14-18]. Also, oxidative stress has been associated with VPA toxicity [3, 4]. Thus, we were interested in the effects of VPA on NIHL.

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Some drugs have ototoxic side effects that can be permanent or transient [19]. When these drugs are prescribed to a patient, the side effects should be considered. The aim of this study was to determine the effects of VPA on NIHL in guinea pigs.

MATERIALS and METHODS

Animals

Thirty age-matched adult albino guinea pigs (250-300 g, Damul Science, Daejeon, Korea) with normal Preyer's reflex and normal tympanic membranes were used in the current study. The animals were assigned to one of two groups. In the noise control group (n=15), 0.5 cc of phosphate-buffered saline (PBS) was administered intraperitoneally (ip) daily. In the VPA group (n=15), VPA (valproic acid sodium salt; Sigma, St. Louis, MO, USA) 30 mg/kg/day was administered ip daily. PBS and VPA were injected once daily for 20 days (from 3 days before noise exposure to 2 weeks after noise exposure). We assessed the auditory brainstem response (ABR) threshold, anti-oxidative enzyme mRNA expression, and cochlear outer hair cell (OHC) damage (Figure 1).

The study protocol and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Chonnam National University School of Medicine.

Acoustic Trauma

Guinea pigs were exposed to 110 dB SPL broadband white noise (0.2-20 kHz) for 3 days (4 hours per day). During the noise exposure, two animals were kept in a cage in a soundproof audio booth, which had a speaker in the center of the ceiling. White noise was produced by a clinical audiometer (Beltone-2000 Audiometer®; Beltone Electronics Corporation, Chicago, IL, USA), amplified by an amplifier (Inter-M R300 plus power amplifier; Canford Audio PLC, Washington, UK), and presented via a speaker. The overall noise level was monitored at the center of the cage using a sound level meter (NA-60; Rion, Tokyo, Japan).

ABR Evaluation

The ABR threshold (12 ears of 6 animals for each groups) was examined under pentobarbital sodium (ENTORBAR; HanLim Pharm Co, Seoul, Korea) (30 mg/kg) anesthesia. Auditory evoked potential equipment (Navigator SE evoked potential system; Bio-logic Systems Corp., Mundelein, IL, USA) was examined before noise exposure and 1, 3, 7, and 14 days after noise exposure using alternating click, 4 kHz, and 8 kHz tone burst sounds. Responses were recorded through subcutaneous stainless steel electrodes located at the vertex (positive) and bilateral mastoid areas (negative and ground). The sound intensity was varied in 5-dB increments. The hearing threshold was defined as the lowest intensity to produce a reliable waveform of 3-5 peaks.

RT-PCR Analysis of Anti-Oxidative Enzymes

To analyze the relative expression of anti-oxidative enzyme mRNA (glutathione peroxidase and copper superoxide dismutase), cochleae of 3 animals (n=6) for each group were obtained at 1, 3, and 6 days after the noise exposure. Each cochlea was homogenized, and the total RNA was extracted from the cochlea using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA), and 1 ug of the total RNA was reverse-transcribed using reverse transcriptase (M-MLV; Gibco BRL, Grand Island, NY, USA) for 90 min at 42°C. The PCR primers (Bioneer Co., Daejeon, Korea) used in the PCR were as follows: glutathione peroxidase (forward, 5'-GGC AAG GTG CTG CTC ATT GAG-3'; reverse, 5'-GGT CGG TCA TGA GCG CAG TG-3'; 331 base pairs [bp]); and copper superoxide dismutase (forward, 5'-ATG GCG ACG AAG GCC GTG-3'; reverse, 5'-TTG GGC GAT CCC AAT CAC AC-3'; 459 bp). The cDNA was amplified by 35 cycles of PCR (Takara Bio Inc., Shiga, Japan) using Ex-Taq polymerase (Takara Bio Inc.). RT-PCR products

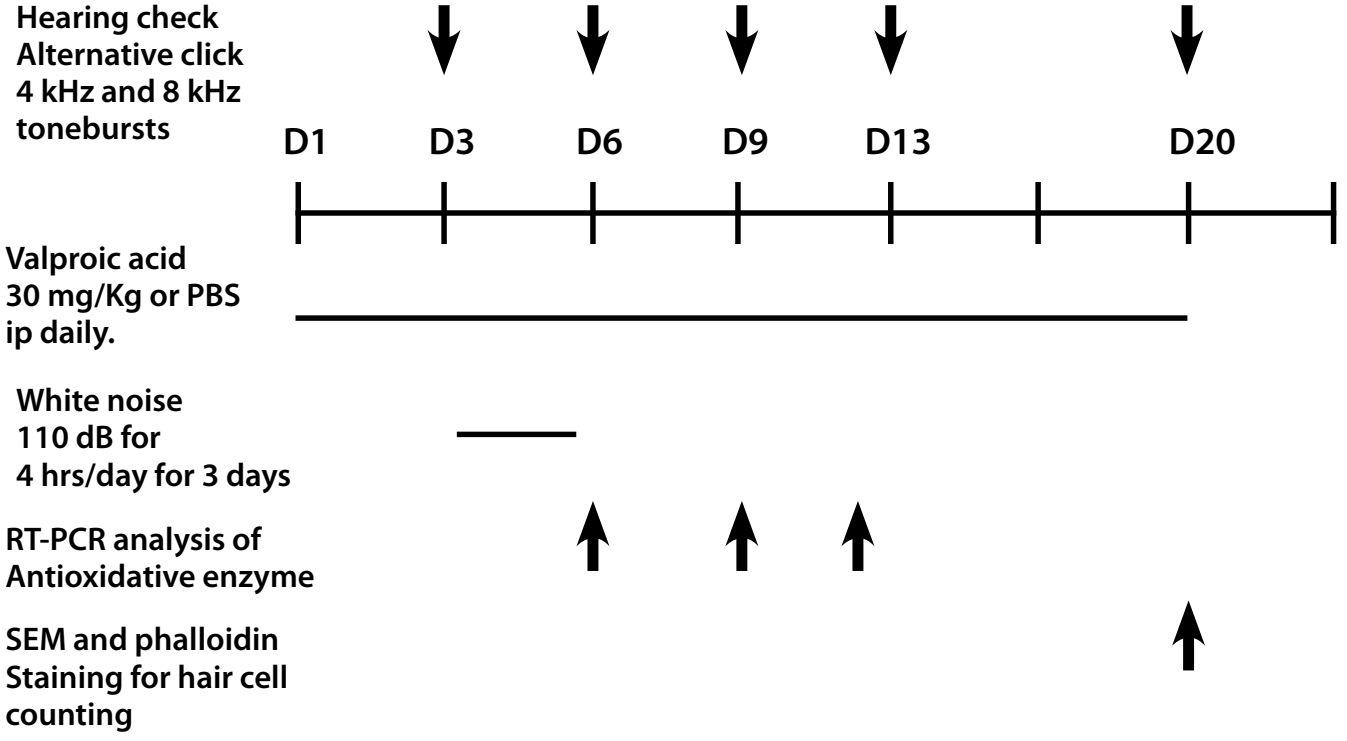

Figure 1. The scheme of animal experimental schedule (ip: intraperitoneally)

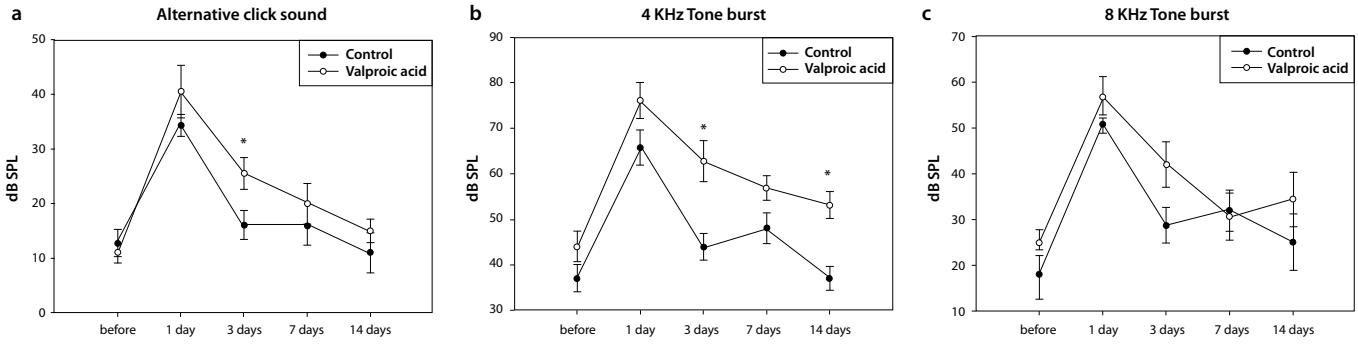

Figure 2. a-c. Auditory brainstem response (ABR) thresholds of the control group (n=12, both ears of 6 animals) and the valproic acid (VPA) group (n=12, both ears of 6 animals). Data are presented as the mean±standard error (*: p<0.05, Mann-Whitney U test)

were separated electrophoretically on 2% agarose gels (Sigma) and stained with ethidium bromide.

Histologic Analyses for Hair Cell Counting

Fourteen days after noise exposure, animals (n=6 for each group) were sacrificed with an overdose injection of pentobarbital. Cochleae of all animals were obtained. Three regions of the cochlear sensory epithelia (10-12 mm from the apex corresponding to the 4 kHz area, 13-15 mm from the apex corresponding to the 8 kHz area, and 7-10 mm from the apex corresponding to alternating click sounds in the 1-3 kHz area) [20] were used for quantitative assessments of hair cell loss.

1) Phalloidin Stain

The cochleae (n=6) of 3 animals from each group were used. Each cochlea was opened at the apex and oval window, and fixative (4% paraformaldehyde in 0.01 M PBS) was perfused into the cochleae. The organ of Corti of each inner ear was removed, permeabilized with 0.5% Triton X-100 for 20 min, and subsequently incubated with rhodamine-phalloidin (1:40 dilution; Invitrogen, Carlsbad, CA, USA) at room temperature for 20 min. After rinsing in PBS, the specimens were mounted in Vectashield (Vector Laboratories Inc., Tokyo, Japan). Surface structures were observed under confocal laser scanning microscopy (FluoView 1000; Olympus, Tokyo, Japan). The number of OHCs in each region of the cochleae was counted by two investigators and presented as a percentage of the defects.

2) Scanning Electron Microscopy (SEM)

The cochleae (n=6) of 3 animals from each group were used. After cardiac perfusion using cold PBS, the cochleae were removed from the temporal bones. Each cochlea was opened at the apex and oval window, perfused with fixative (2.5% glutaraldehyde in 0.01 M PBS), and fixed overnight. After decalcification with 10% ethylenediaminetetraacetic acid (EDTA) for 3 days at 4°C, the cochleae bony shells were meticulously removed. After dissection, cochlear tissues were incubated for 2 h in 1% osmium tetroxide cacodylate buffer, dehydrated with ethanol, dried with a critical point dryer, and coated with gold. Each specimen was viewed and photographed by means of a field emission scanning electron microscope (S-2400; Hitachi Ltd., Tokyo, Japan). The number of OHCs in each region of the cochleae was counted by two investigators and presented as a percentage of defects.

Statistical Analysis

All data values in the text and figures are means±standard error. Differences in ABR thresholds, anti-oxidant enzyme mRNA expression, and percentage of missing OHC results between the VPA and PBS control groups were analyzed by Mann-Whitney U-test and t-test using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). p< 0.05 was accepted as statistically significant.

RESULTS

ABR Thresholds

ABR thresholds before and after the noise exposure are shown in Figure 2. There was no significant difference between the control (ABR thresholds at alternative click and 4 and 8 kHz were 12.7±2.5, 37.0±3, and 18.0±5.3 dB SPL, respectively) and VPA (ABR thresholds at alternative click and 4 and 8 kHz were 11.0±1.8, 44.0±3.3, and 25.0±2.7 dB SPL, respectively) groups in the 4 kHz before the noise exposure (p>0.05). An increase in ABR thresholds at all frequencies was observed in both groups 1 day after the noise exposure in the control (ABR thresholds at alternative click and 4 and 8 kHz were 34.2±2.0, 65.7±3.8, and 50.8±2.0 dB SPL, respectively) and VPA (ABR thresholds at alternative click and 4 and 8 kHz were 40.5±4.8, 76.1±3.9, and 56.7±4.5 dB SPL, respectively) groups. Two weeks following the noise exposure, there was a gradual recovery to normal levels in the control group. However, the VPA group, especially in the 4 kHz area, had recovered less than the control group (control 37.0±2.5 dB SPL and VPA 53.1±2.9 dB SPL) (p<0.05).

RT-PCR Analysis of Anti-Oxidative Enzymes

The relative expression of anti-oxidative enzyme mRNA (glutathione peroxidase and copper superoxide dismutase) of both groups is shown in Figure 3. Anti-oxidative enzyme mRNA expression was significantly increased in the VPA group compared to the control group 1, 3, and 6 days after the noise exposure (p<0.05).

Histologic Analysis for Determining OHC Loss

In the phalloidin and SEM studies, the surface structure of the organ of Corti was composed of three rows of outer hair cells and a single row of inner hair cells. The inner hair cells in all areas were relatively well preserved 2 weeks after noise exposure. However, OHC loss was more evident 10-12 mm from the apex in the VPA group compared to the control group (Figure 4). The percentage of missing OHCs in the VPA group was significantly greater than in the control group in the 4 kHz areas.

DISCUSSION

VPA-induced hepatotoxicity and neural tube defects have been reported, and the most likely mechanism is associated with oxidative stress [3, 4].

After the noise exposure, the hearing threshold shift demonstrated in the current study was comparable to data in previous studies [21, 22]. Further-

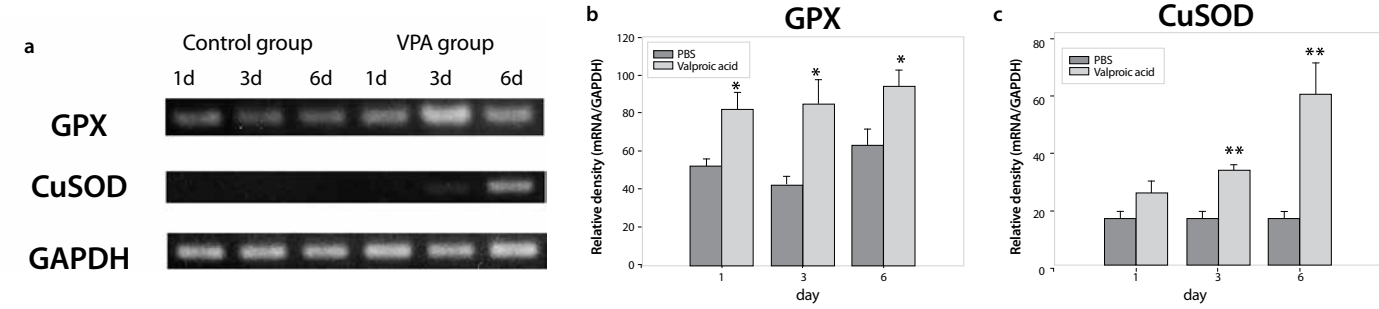

Figure 3. a-c. Anti-oxidant enzyme mRNA expression after noise exposure (a). Anti-oxidative enzyme mRNA expression was significantly increased in the VPA group than in the control group at 1, 3, and 6 days after noise exposure (b, c). GPX: glutathione peroxidase; CuSOD: copper superoxide dismutase (*, **: p<0.05, t-test)

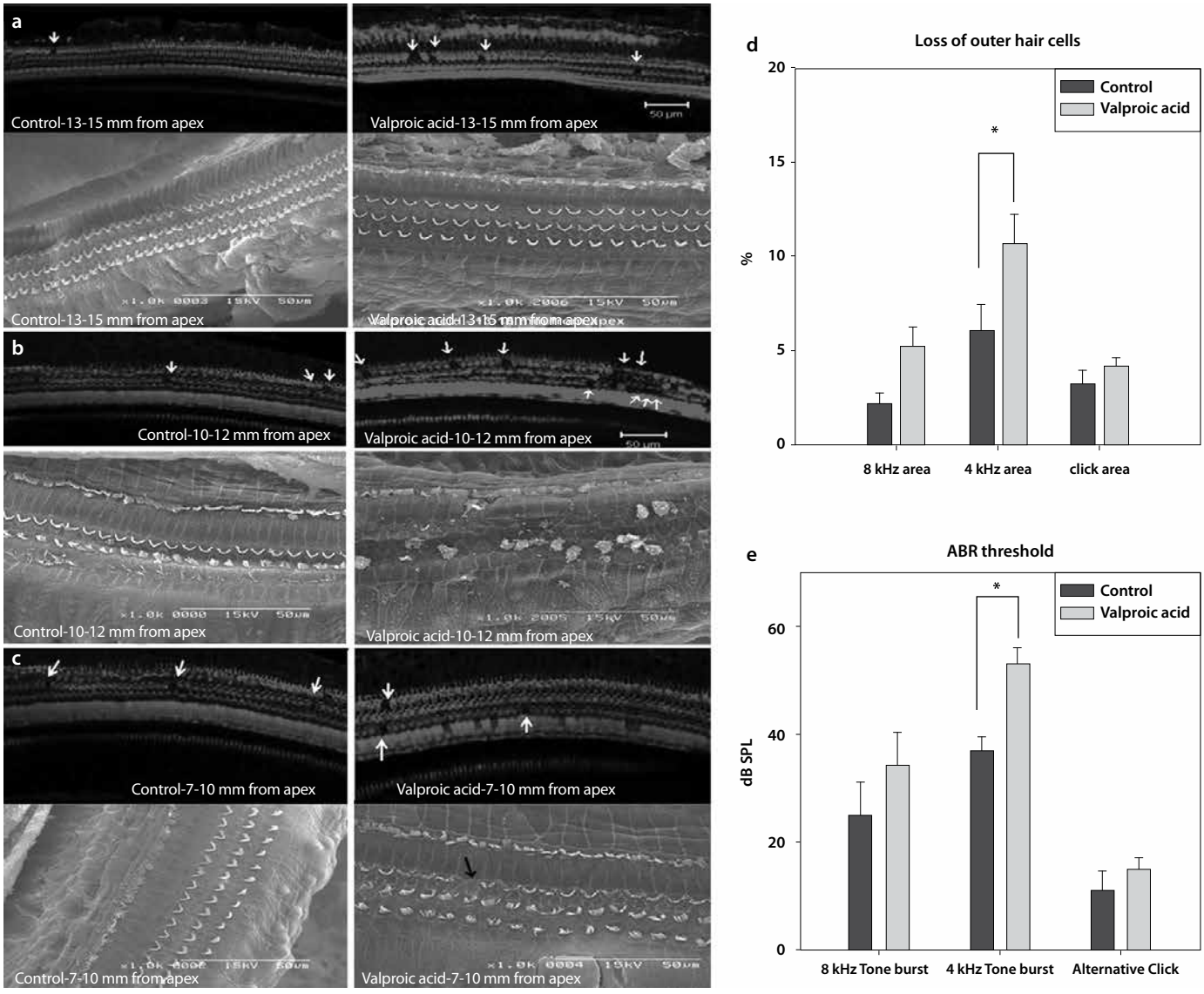

Figure 4. a-e. Morphologic changes of hair cells (a-c), outer hair cell (OHC) counts (d), and ABR threshold (e) 2 weeks after noise exposure. Arrow indicates OHC loss (8 kHz area: 13-15 mm from the apex; 4kHz area: 10-12 mm from the apex; alternating click sounds in the 1-3 kHz area: 7-10 mm from the apex, *: p<0.05, Mann-Whitney U test)

more, the present study showed the harmful effects of VPA in noise-induced threshold shifts after exposing guinea pigs to loud sounds.

The dose of VPA chosen for this study was 30 mg/kg/day for 20 days, because the daily dose of VPA in human patients is 10-50 mg/kg and the maximum dose is 60 mg/kg/day [23, 24]. In ABR studies, the hearing threshold in all animals increased following the noise exposure and gradually decreased as time passed after the cessation of noise exposure. However, this gradual recovery was limited in the 4 kHz area in the VPA group. Based on morphologic examinations, severe OHC loss was observed 10-

12 mm from the apex, corresponding to the 4 kHz area in the VPA group. There are several underlying NIHL mechanisms. The most likely mechanism for NIHL in this study, especially below 120 dB SPL noise, was metabolic damage of the organ of Corti. Various kinds of anti-oxidant enzymes have been described in mammalian cochlear tissues [14, 16-18, 25], and noise exposure increases ROS in the cochlea [26]. Also, anti-oxidant enzymes increased, followed by ROS expression in the cochlea. In the present study, we examined glutathione peroxidase and copper superoxide dismutase for revealing the effect of VPA on oxidative stress after noise exposure. Anti-oxidative enzyme mRNA expression was significantly increased in the VPA group compared to the control group; thus, more oxidative stress occurred in the VPA group with the same noise exposure. This result was comparable to the result of the ABR and histological findings. So, these results suggest that VPA could increase the formation of ROS and interfere with the hearing recovery after noise trauma in guinea pigs.

The recent dramatic increase in the popularity of the portable MPEG audio player 3 (MP3) has increased the exposure to high sound levels in youths [6]. Previous studies have revealed that an increased number of youths experience symptoms indicative of poor hearing, such as aural fullness, hyperacusis, tinnitus, or hearing threshold increases [27-29]. VPA is prevalently used in the treatment of childhood and adult seizures [30]. It is also used in the treatment of various diseases, such as bipolar disorders, migraine-type headaches, and Sydenham chorea [31-33]. Thus, many patients using VPA are at risk of being exposed to high sound levels from MP3s. The current study showed that VPA may interfere with the hearing recovery after noise trauma; therefore, when VPA is recommended to a patient, this side effect must be considered. This study demonstrated that VPA may have a harmful effect on noise-induced ototoxicity in guinea pigs.

Ethics Committe Approval: Ethics committee approval was received for this study from the Institutional Animal Care and Use Committee of the Chonnam National University School of Medicine

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Conflict of Interest: No conflict of interest was declared by the authors.

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