

# Preliminary Study of the Therapeutic Effect of a Nitrene-Based Antioxidant Drug (HPN-07) on Acute Acoustic Trauma

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**Background & Objectives:** Acute acoustic trauma (AAT) results in oxidative stress exceeding the capacity of the antioxidant defense mechanisms in cochlea by excessive production of reactive oxygen species, reactive nitrogen species, and other free radicals. Pharmacological approaches have been developed to prevent or treat cochlear injury induced by AAT. The present study aims to investigate if another nitrogen-based antioxidant drug, HPN-07 [a derivative of 4-hydroxy phenyl-N-tert-butyl Nitrene (4-OHPBN)] can be used to treat the permanent hearing loss induced by AAT. **Method:** Eighteen female chinchillas (six for each group) were exposed to a 105 dB octave-band noise centered at 4 kHz for 6 h. HPN-07 and HPN-07 plus N-acetyl-L-cysteine (NAC) were orally administered to two experimental groups giving a first injection 4 h after noise exposure and continually injecting twice daily for the next two days. Auditory brainstem responses (ABR) before noise exposure and 21 days after noise exposure were obtained and analyzed for permanent hearing threshold shifts. **Results:** Results showed that the mean permanent hearing threshold shifts averaged at higher frequencies (2-8 kHz) for HPN-07 treated group (26 dB) and HPN-07 plus NAC treated groups (11 dB) were significantly decreased compared to the noise exposure group (control group, 36 dB). In addition, significant differences between HPN-07 treated group and HPN-07 plus NAC treated groups were also found. **Discussion & Conclusion:** HPN-07 reduced permanent hearing threshold shifts and HPN-07 plus NAC showed greater effects. These results demonstrate that HPN-07 and the combination of HPN-07 with NAC can treat acute acoustic trauma and the drug combination increase the therapeutic effect. The therapeutic effect of HPN-07 may result from its role as a free radical scavenger. (*Korean Journal of Communication Disorders* 2011;16:202-210)

**Key Words:** acute acoustic trauma (AAT), noise-induced hearing loss (NIHL), oxidative stress, antioxidant drugs, free radicals, reactive oxygen species, reactive nitrogen species, auditory brainstem response (ABR), permanent hearing threshold shifts, HPN-07

## I . Introduction

Generally, noise-induced hearing loss (NIHL) may be caused by a short exposure to an intense impulse sound from 100 to 150 dB SPL, such as explosions and blasts, called acute acoustic trauma (AAT) and continuous exposure to loud sounds at or above 85 dB SPL over extended period of time

such as noise in a woodworking shop and factory, called chronic NIHL (Rovig et al., 2004). Exposure to impulse sound and continuous noise may result in temporary hearing loss and permanent hearing loss. Temporary hearing loss may occur immediately after noise exposure and last before 21 days after noise exposure while permanent hearing loss appears after 21 days after noise exposure (Kopke et al.,

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2000; 2002). Exposure to continuous noise can damage the sensory hair cells of the cochlea more gradually than for impulse noise through mechanical and metabolic mechanisms. Hearing protection devices such as earplugs or earmuffs have been used to prevent hearing loss from both impulse and continuous noise. However, these devices could not prevent or treat AAT or chronic NIHL due to their inherent limitations resulting in permanent hearing loss (Attias et al., 1994).

Basically, AAT leads to an increase in oxidative stress playing a substantial role in the production of mechanical damage involving the organ of Corti, stria vascularis, spiral ganglion cells, basilar membrane, and inner and outer hair cell and metabolic injury involving excessive release glutamate, ischemia/reperfusion, mitochondrial injury, glutathione depletion, and ionic fluxes in mammalian cochlea (Henderson et al., 2006; Le Prell et al., 2007). Oxidative stress exceeding the capacity of the antioxidant defense mechanisms results from the overproduction of reactive oxygen species (ROS), reactive nitrogen species (RNS), and other free radicals such as hydroxyl, superoxide, lipid peroxidation, nitric oxide, and nitrotyrosine, and 4-hydroxy-2-nonenal (Evans & Halliwell, 1999; Halliwell & Gutteridge, 1999). In terms of the morphological changes induced by AAT, apoptosis known as active programmed cell death and characterized as shrunken dark cytoplasm with a pyknotic nucleus has been found as a primary cell death pathway (Cheng, Cunnungham & Rubel, 2005; Henderson et al., 2006; Wang et al., 2007). The apoptotic process has been occurred through caspase-3, -8, -9, cytochrome C, c-jun, and c-jun NH2-terminal kinase.

The major concern of our research is to develop realistic pharmacological interventions as adjuncts for preventing or treating cochlear injury caused by AAT or NIHL. Pharmacological approaches for the prevention or treatment of AAT or NIHL have been developed with oxygen-based antioxidant drugs which increase antioxidant defense mechanisms in the cochlea (Choi, 2011; Choi et al., 2008; Floyd et al., 2008; Kopke et al., 2005; 2007). Oxygen-based antioxidant drugs can inhibit the overproduction

of reactive oxygen species (ROS) induced by AAT. N-acetyl-L-cystein (NAC), a glutathione prodrug and a scavenger of ROS, effectively protected hair cells from AAT by increasing intracellular glutathione (GSH) while acetyl-L-carnitine (ALCAR), an endogenous mitochondrial membrane compound and a mitochondrial biogenesis agent, efficiently reduced mitochondrial injury caused by AAT by enhancing mitochondrial biogenesis in the face of oxidative stress and reducing ROS production.

On the other hand, noise exposure can also induce excessive production of reactive nitrogen species (RNS) such as nitric oxide (NO) (Evans & Halliwell, 1999; Henderson et al., 2006). Another pharmacological approach for the prevention or treatment of AAT or NIHL has been used with nitrogen (nitron)-based antioxidant drugs which can inhibit or reduce the production of RNS. The simplest nitron chemical structure is  $X-CH=NO-Y$  and the nitrones have been used to react with and trap and stabilize free radicals from the late 1960s (Floyd et al., 2008). Traditionally, nitrones have been originally used as potential pharmaceutical agents for stroke (Floyd et al., 2008). Nitron-based antioxidant drugs include a PBN family such as Phenyl-N-tert-butyl nitron [PBN, a nitron-based spin trapping agent], Disulfenton sodium (NXY-059, a disulfonyl derivative of the neuroprotective spin trap of PBN), and 4-hydroxy PBN [4-OHPBN, a nitron-based free radical trap and an inhibitor of inducible nitric oxide synthase and a major metabolite of PBN]. It has been reported that these drugs were effective in preventing or treating hearing loss induced by AAT. In more details, the PBN can reduce oxidative stress and function as both a neuroprotectant and a potent anti-inflammatory agent (Floyd et al., 2008; Hensley et al., 1998; Kotake, 1999; Kudora et al., 1996; Peterson et al., 2005) while NXY-059 functions as a powerful scavenger of free radicals and effectively in terminating radical chain reactions (Sydserrff et al., 2002). Comparing to those drugs, 4-OHPBN has a stronger biological effect inhibiting hepatocarcinogenesis and treating AAT than that of PBN and other derivatives (Choi et al., 2008; Floyd et al., 2008; Reinke et al., 2000).

Another nitron-based antioxidant drug, 2, 4-disulfonyl PBN (HPN-07, a derivative of 4-OHPBN) can be used to prevent or treat hearing loss induced by AAT because HPN-07 has its relative safety compared to the effects of 4-OHPBN. Therefore, the objectives of this study are to investigate if there is the therapeutic effect of HPN-07 in treating hearing loss induced by AAT and if there is a stronger synergistic effect of HPN-07 with another antioxidant drug (NAC) in treating permanent hearing loss induced by AAT because any combinations of antioxidant drugs can provide a stronger synergistic effect than single agent in preventing or treating AAT.

## II. Methods

### 1. Animals and synthesis of HPN-07

The experimental procedures used in this study were the same as reported previously by Choi et al. (2008) and reviewed and approved by the Institutional Animal Care and Use Committees of the Office of Naval Research and the Oklahoma Medical Research Foundation (OMRF).

Eighteen female adult *chinchilla laniger* (Moulton Chinchilla Ranch, Rochester, MN) weighing 500-850 grams and aging from 3 to 5 years old were used in this study because they have similar audiograms to human. Animals were housed in plastic cages in the OMRF animal facility and had free access to a standard chinchilla diet (Mazuri Chinchilla Diet, 5MO1, PMI Nutrition International Inc., Brentwood, MO) and tap water throughout the experimental periods. Chinchillas were randomized into three groups (n=6 for the control group and n=6 for each experimental group): 1) noise exposure group (control group); 2) HPN-07 treated groups (300 mg/kg); 3) HPN-07 (300 mg/kg) plus NAC (325 mg/kg) treated group (two-drug combination group). HPN-07 was synthesized using a straight forward chemical reaction, extraction, and crystallization procedure that has been done in our laboratory for over 20 years as the 4-OHPBN was synthesized (Choi et al., 2008). HPN-07 synthesized at OMRF

was first dissolved in dimethyl sulfoxide [DMSO, 0.8 ml per 100 mg, Sigma-Aldrich Inc., St. Louis, MO] at 37° C and then polyethylene glycol (PEG) 400 [0.8 ml per 100 mg, Sigma-Aldrich Inc., St. Louis] was added. Sterile saline (0.4 ml per 100 mg) was added before injection. The two drug combination group received 300 mg/kg of HPN-07 and 325 mg/kg of NAC (Hospira Inc., Lake Forest, IL). All agents were orally administered to experimental animals with the initial injection 4 hours after noise exposure and continual injections twice daily for the next two days. The control group received equal volumes of carrier solution (DMSO, PEG 400, and sterile saline, 2:2:1 ratio) orally administered at the same time points as in the experimental groups.

### 2. Noise Exposure

Two animals at a time placed in two small wire restraint cages on a wooden plate were exposed to octave-band noises (-12dB/octave) centered at 4 kHz at 105 dB SPL for 6 hours in a sound isolation booth [Industrial Acoustics Company (IAC), New York, NY]. These noises were digitally generated by a Tucker Davis Technologies (TDT, Alachua, FL) device, passed through a real time attenuator (TDT, RP2), filtered, amplified with a preamplifier (QSC audio power, Costa Mesa, CA), transduced with a high frequency acoustic driver and an acoustic speaker (JBL 2350, Northridge, CA) suspended from the ceiling of the sound booth and positioned directly above the wire cages. Before noise exposure, a sound level meter was used to calibrate the sound spectrum output of the system. During noise exposure, the noise level was continually and visually monitored with a condenser microphone (B&K 2804, Norcross, GA) coupled to the preamplifier placed between the two wire cages at the level of the animals' heads using the PULSE software system [B&K Sound & Vibration Measurement (version 10.0), Norcross, GA] including FFT Analysis Type 7770 and CPB Analysis 7771.

### 3. Auditory Brainstem Responses

Auditory brainstem responses (ABR) were used to measure hearing thresholds for the right ear of each animal within 3 days before initial noise exposure (baseline threshold), immediately after, and then 21 days after noise exposure for each frequency. All animals used in this study had normal hearing. To remove temporal effect of noise, permanent hearing threshold shifts (PTS) were obtained by subtracting the baseline threshold from the final hearing threshold measured in dB SPL (sound pressure level) at 21 days after noise exposure and analyzed for this study. Light ketamine (20 mg/kg) and xylazine (1 mg/kg) anesthesia was used for ABR recording and then small supplemental doses (1/3 of initial dose) were given if needed. For ABR recording, an active subcutaneous needle electrode, a reference electrode, and a ground electrode were placed proximal to the right ear, the left ear, and the vertex, respectively. Tone pips (5 ms duration and 1 ms Blackman rise and fall) at frequencies of 0.5, 1, 2, 4, 6, and 8 kHz as auditory stimuli were generated by a computer-aided system (Intelligent Hearing Systems, Miami, FL) coupled to high frequency transducers and transduced through the computer-controlled attenuator to a 3A insert earphone [Etymotic Research (ER)-3A, Etymotic Research Inc., Elk Grove Village, IL] placed about 5 mm from the tympanic membrane. A coupler mounted to the sound level meter approximating its placement was used to calibrate an insert earphone. The electrical responses obtained from the electrodes were amplified (x100,000), filtered (100-3,000 Hz), digitized through an A/D converter on a signal processing board, and averaged at a sample rate of 1024 for each level. Hearing were tested in 10 dB descending steps until near the threshold, and then the hearing thresholds were determined by 5 dB ascending steps. Hearing threshold of ABR was determined as the mid point between the lowest level of a clear response and the next level where no response was observed. The identity of animal groups was blinded to the two investigators performing the ABR measurements.

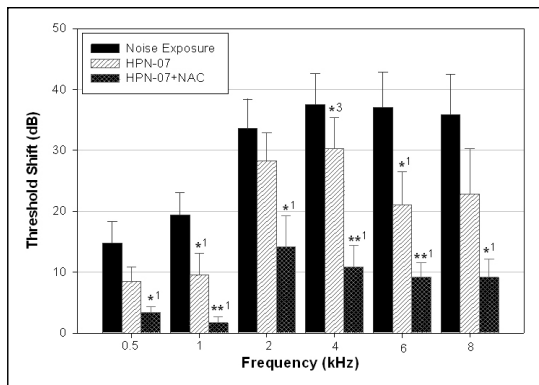
### 4. Statistical Analysis

All data are reported as mean±S.E.M. Significant differences in permanent threshold shifts of ABR among the different groups at each frequency were evaluated using one-way ANOVA (SPSS 14.0 for Windows). The Fisher's least squares difference (LSD) post hoc test was then used for comparison in different groups. A *p*-value less than 0.05 was considered to indicate a statistically significant difference.

## III. Results

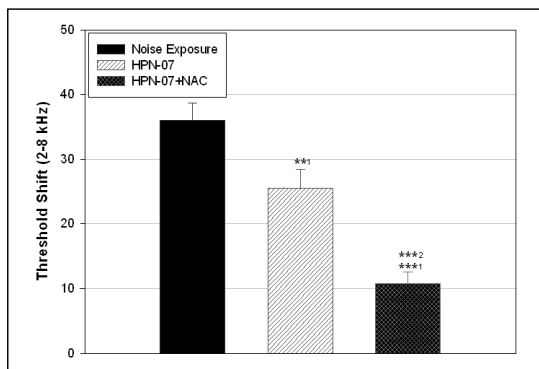
No significant differences in temporary threshold shifts measured immediately after noise exposure were found among any of the groups (data not shown). However, the permanent ABR threshold shifts (PTSs) were different among different groups at different frequencies. As shown in Fig. 1, the mean PTSs in the control group ranged from about 16 dB at low frequencies to 36 dB at high frequencies while the mean PTSs of the HPN-07 treated group were reduced across frequencies. The mean PTSs of the HPN-07 treated group were 9 dB at low frequencies and approximately 26 dB at high frequencies. Significant differences in PTSs between the control group and the HPN-07 treated group were shown at two frequencies of 1 and 6 kHz. The PTSs for the HPN-07 treated group were significantly reduced to 9 and 15 dB at 1 and 8 kHz respectively, compared to the control group. For the HPN-07 plus 4-OHPBN treated group, the mean PTSs were significantly reduced at all frequencies compared to the control group (group 1). There were no significant differences in threshold shifts between HPN-07 treated group and the two-drug combination treatment group except only 4 kHz.

<Figure - 2> compares the mean PTS averaged at higher frequencies (2-8 kHz) among three different groups. The mean permanent threshold shifts for HPN-07 treated group and the two-drug combination group were significantly decreased compared to those of the control group (group 1). The reduction



ABR permanent threshold shifts of the HPN-07 and the two-drug combination treatment groups compared with the control group. The permanent threshold shifts of HPN-07 treated group were significantly reduced at both 1 and 6 kHz at  $p < 0.05$  compared to the control group. However, the threshold shifts of the two-combination treatment group were significantly reduced at all frequencies at  $p < 0.05$ , compared to the control group. At only 4 kHz, the threshold shifts of HPN-07 was significantly decreased at  $p < 0.05$  compared to the two-drug combination treatment groups. Asterisks \* and \*\* represent statistically significant differences in ABR threshold between experimental groups and control group at  $p < 0.05$  and  $p < 0.01$ , respectively. The numbers 1, 2, and 3 represents noise exposure group (control group), HPN-07 treated group, and two drug combination group, respectively.

<Figure - 1> ABR permanent threshold shifts

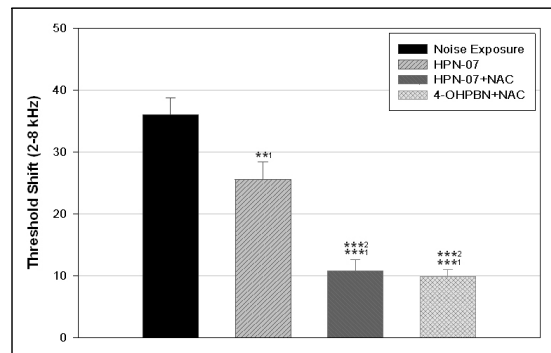


ABR permanent threshold shifts averaged at frequencies of 2-8 kHz for the noise exposure group (control group), HPN-07 treated group, and the two drug combination group. The permanent threshold shifts of HPN-07 treated group and the two drug combination group were significantly decreased compared to the control groups. In addition, the permanent threshold shifts between HPN-07 treated group and the two-drug combination group were significantly different. Asterisks \*\* and \*\*\* represent statistically significant differences in ABR threshold between experimental groups and control group at  $p < 0.01$  and  $p < 0.001$ , respectively. The numbers 1, 2, and 3 represents noise exposure group (control group), HPN-07 treated group, and the two drug combination group, respectively.

<Figure - 2> ABR permanent threshold shifts averaged at frequencies of 2-8 kHz

in the mean PTS between the noise exposure group and HPN-07 treated group was 10 dB while the reduction in the mean PTS between the noise exposure group and the two-drug combination group was 27 dB. Significant differences between HPN-07 treated group and the two-drug combination group were also found. The reduction between HPN-07 treated group and the two-drug combination group was 17 dB.

#### IV. Discussion and Conclusion



Asterisks \*\* and \*\*\* represent statistically significant differences in ABR threshold among the noise exposure group (control group) and experimental groups at  $p < 0.01$  and  $p < 0.001$ , respectively. The numbers 1, 2, 3 and 4 represents noise exposure group (control group), HPN-07 treated group, two drug combination group (HPN-07+NAC), and another two-drug combination group (4-OHPBN+NAC) respectively.

<Figure - 3> Comparison of ABR threshold shifts averaged at frequencies of 2-8 kHz for the noise exposure group and different treatment groups

The present study is the first preliminary attempt to investigate the therapeutic effect of another nitron-based antioxidant drug (HPN-07) on AAT. The use of HPN-07 generated a significant reduction in permanent hearing loss at ABR threshold shifts averaged at frequencies of 2-8 kHz. When HPN-07 was orally administered, the permanent hearing loss was reduced from 36 to 26 dB. When HPN-07 was combined with NAC, the ABR permanent hearing loss was also reduced from 36 to 11 dB. The amounts of reduction in the permanent hearing loss for HPN-07 and NAC were 69% and 58% respectively, compared to the noise exposure group and the

HPN-07 treated group. However, the therapeutic effect of the HPN-07 and NAC was similar to that of 4-OHPBN and NAC as shown in <Figure - 3> (Choi et al., 2008). These results are consistent with previous studies reporting that AAT can be reduced by 4-OHPBN alone and the combination of 4-OHPBN and NAC. Therefore, this indicates the possibility of clinical application of another nitrone-based antioxidant drug (HPN-07) in treating AAT.

The current study evaluated the effect of one of PBN-related nitrones on treatment of AAT. PBN-related nitrones have been brought to the attention of scientists because of its primary ability to trap free radicals (Floyd et al., 2008). In addition to the spin trapping ability, PBN also has its antioxidant properties, its action on important membrane enzymes (ion transport proteins), and its action as anti-inflammatory agent (Floyd et al., 2008). When PBN-related nitrones were applied to the hearing loss induced by AAT, early experiments demonstrated that several compounds such as carbon monoxide, hydrogen cyanide, and acrylonitrile can potentiate noise-induced hearing loss (Rao & Fechter, 2000; Rao et al., 2001; Fechter, Gearhart & Shirwany, 2004; Fechter, Liu & Pearce, 1997; Pouyatos et al., 2005). These toxins are involved with increase of oxidative stress in the cochlea. PBN decreased the permanent noise-induced hearing loss potentiated by these chemical compounds. However, PBN did not strong therapeutic effects in treating noise-induced hearing loss alone (Fechter et al., 2004; Rao & Fechter, 2000).

When 4-OHPBN was compared to PBN and its other derivatives such as 3-hydroxy PBN (3-OHPBN), 2-hydroxy PBN (2-OHPBN), and 2-sulfoxy PBN (2-SPBN), the effect of 4-OHPBN on inhibiting hepatocarcinogenesis was stronger than that of the parent compound (PBN) and the other derivatives (Nakae et al., 2003). This indicates that 4-OHPBN may have a stronger biological effect than PBN and its other derivatives. When 4-OHPBN was applied to treatment of AAT, similar results were observed. 4-OHPBN reduced permanent hearing loss in a dose-dependent manner and combination of 4-OHPBN with other antioxidant drugs (NAC or NAC

plus ALCAR) increased the therapeutic effectiveness and decreased the required individual medication dose (Choi et al., 2008). The present study showed the therapeutic effect of a derivative of 4-OHPBN (HPN-07) on permanent hearing loss induced by AAT. This indicates the similar effect of HPN-07 to that of 4-OHPBN. However, this study only showed preliminary data and did not prove the effect of HPN-07 in dose-dependent manners. Now, it is in preclinical process to investigate the oral dosing responses of HPN-07 on treatment of AAT.

Recently, many studies demonstrated the synergistic effects of the combination of antioxidant drugs in preventing or treating permanent hearing loss induced by AAT. Various drug combinations such as NAC and salicylate (a hydroxyl radical scavenger), salicylate and Trolox (a water soluble analog of  $\alpha$ -tocopherol), folate plus vitamin E plus ALCAR, free radical scavengers vitamins A, C, and E plus magnesium, 4-OHPBN plus NAC, 4-OHPBN plus NAC plus ALCAR have been used (Kopke et al., 2000; Dhitavat et al., 2005; Yamashita et al., 2005; Le Prell et al., 2007; Choi et al., 2008). The present study also showed the synergistic effect when HPN-07 was used in combination with another antioxidant drug (NAC). The synergistic effect of these drug combinations may result from different mechanism and site of action of individual drug. It has been known that NAC functions as a ROS scavenger and a neuroprotective agent by mainly replenishing glutathione level, reducing mitochondrial injury, and inhibiting glutamate excitotoxicity, inflammation and apoptosis (Coleman et al., 2007; Henderson et al., 2006; Kopke et al., 2000; 2007). ALCAR has been known to act as a precursor for acetyl-CoA and a mitochondrial energy substrate by mainly reducing mitochondrial injury and restoring important mitochondrial lipids (Coleman et al., 2007; Kopke et al., 2002; 2005). However, although the basic and exact mechanisms of HPN-07 associated with the treatment of AAT are still unknown, HPN-07 may function as a free radical scavenger, inhibitor of inducible nitric oxide synthase (iNOS) activation, suppressor of ROS and RNS formation, reducer of mitochondrial ROS production and inflammation,

and activator of MAP kinase cascades (Floyd et al., 2008). A variety of preclinical tests defining toxicity, mechanism and site of action, and pharmacokinetics for HPN-07 are now under way.

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# 질소에 기초한 항산화제 (HPN-07)의 급성음향외상 치료효과에 관한 예비연구

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**배경 및 목적:** 급성음향외상(Acute acoustic trauma)은 활성산소기(reactive oxygen species, ROS), 활성질소기(reactive nitrogen species, RNS), 그리고 다른 유리기(other free radicals)의 초과 생산으로 내이의 와우의 항산화방어기제 능력을 초과하는 산화적 스트레스(oxidative stress)를 만든다. 최근 급성음향외상에 발생하는 와우손상을 예방하거나 치료하는 약리학적 접근이 발전되어오고 있다. 본 논문은 질소에 기초한 항산화제인 HPN-07 [4-hydroxy Phenyl-N-tert-butyl nitron (4-OHPBN)의 파생약품]의 급성음향외상 치료효과를 조사하는데 목적을 두고 있다. **방법:** 각 집단에 여섯 마리의 친칠라를 통제군, HPN-07치료군, 그리고 HPN-07과 NAC (N-acetyl-L-cystein) 치료군으로 무작위로 할당하여 모든 집단의 동물들을 4 kHz 중심에 놓여진 옥타브당 -12 dB를 가진 옥타브밴드 소음을 105 dB의 강도로 6시간 동안 노출시켰다. 두 실험 집단의 동물들은 소음노출 후 4시간 경과 후 입으로 약물을 먹었고, 그 후 2일 동안 하루에 두 번 아침 저녁으로 계속 먹었다. 소음 노출 전과 후 21일에 측정된 청성뇌간 반응(auditory brainstem response, ABR)의 차이로 계산된 영구적인 청력역치가 얻어졌고 그것이 통계학적으로 분석되었다. **결과:** 고주파수(2-8 kHz)의 평균으로 계산된 영구적인 청력역치변화(permanent hearing threshold shifts)는 HPN-07 치료집단과 HPN-07과 NAC 치료 집단은 소음에만 노출된 통제집단보다 유의미하게 차이가 있었고 두 치료집단 사이에도 유의미한 차이가 나타났다 ( $p < 0.05$ ). 통제집단의 영구적인 청력역치변화는 36 dB인데 반해 HPN-07치료집단은 26 dB로 줄어들었고 HPN-07과 NAC 치료를 모두 받은 집단은 약 11 dB로 감소하였다. 게다가 HPN-07과 NAC 치료집단의 영구적인 청력역치변화는 4-OHPBN과 NAC 치료그룹과의 거의 비슷하였다. **논의 및 결론:** HPN-07은 영구적인 청력역치변화를 덜어뜨렸고 HPN-07과 NAC 치료집단이 가장 높은 치료효과를 보여 주었다 ( $p < 0.05$ ). 이것은 HPN-07과 NAC와의 결합은 급성음향외상을 치료하는데 효과적이며 다른 항산화제와의 결합은 그 큰 치료효과를 가져올 수 있음을 증명한다. 비록 본 연구가 정확한 활동기저를 보여주지는 않았지만 HPN-07은 유리기의 활동을 제거하는 약물로 효과적으로 사용될 수 있음을 보여주었다. 『언어청각장애연구』, 2011;16:202-210.

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