

# 변조이음향방사를 중심으로 본 결합된 항산화제(4-OHPBN plus NAC)의 급성음향외상 치료효과

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## ABSTRACT

Therapeutic effect of combined antioxidant drugs (4-OHPBN plus NAC) on acute acoustic trauma in terms of distortion product otoacoustic emission

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Acute acoustic trauma (AAT) induces hearing loss through the primary hair cell death pathway called apoptosis. The hair cell death occurs by over-production of reactive oxygen species, reactive nitrogen species, and other free radicals, which result from oxidative stress destroying the antioxidant defense mechanisms in the cochlea. Pharmacological approaches have been developed to prevent or treat cochlear damage induced by AAT. The objective of this study is to investigate the therapeutic effect of the combined antioxidant drugs (4-OHPBN plus NAC) in treating hearing loss induced by AAT when these drugs are orally administered. Thirty female chinchillas (six for each group) were exposed to a 105 dB SPL octave-band noise centered at 4 kHz for 6 hours. A combination of 4-hydroxy phenyl-N-tert-butyl nitro (4-OHPBN) plus N-acetyl-L-cysteine (NAC) were orally administered to all experimental groups giving a first injection 4 hour after noise exposure and continually injecting twice daily for the next two days. Cubic distortion product otoacoustic emissions (DPOAE) before noise exposure and 21 days after noise exposure were obtained and statistically analyzed. The cubic DPOAE amplitudes were significantly different among different groups at different frequencies. The oral administration of 4-OHPBN plus NAC significantly increased permanent DPOAE amplitude. The increase was partially correlated with the dose of 4-OHPBN plus NAC. These results demonstrate that the combination of 4-OHPBN plus NAC can treat acute acoustic trauma although these drugs are orally administered. At the high dose of 4-OHPBN (75 mg/kg) plus NAC (200 mg/kg), the cubic DPOAE amplitude was reduced compared to those of 4-OHPBN (50 mg/kg) plus NAC (100 mg/kg). This reduction indicates that the only highest dose of 4-OHPBN may be ototoxic.

**KEY WORDS :** Acute acoustic trauma (AAT), Antioxidant drugs, Distortion product otoacoustic emission (DPOAE), NAC, Oral administration, Oxidative stress, 4-OHPBN.

## INTRODUCTION

Basically, acute acoustic trauma (AAT) leads to oxidative stress exceeding the antioxidant defense capacity in the cochlea through the over-production of reactive oxygen spe-

cies (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 addition, AAT may morphologically damage the cochlea through both mechanical injuries involving the organ of Corti, stria vascularis, spiral ganglion cells, basilar membrane, and inner and outer hair cell and metabolic injuries involving excessive release glutamate, ischemia/reperfusion, mitochondrial injury, glutathione depletion, and ionic fluxes in mammalian cochlea (Choi, 2010; Choi, 2011). The primary cell death path-

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way induced by AAT is known as apoptosis, which is active programmed cell death and characterized as shrunken dark cytoplasm with a pyknotic nucleus and occurred through caspase-3, -8, -9, cytochrome C, c-jun, and c-jun NH2-terminal kinase (Cheng et al., 2005; Henderson et al., 2006; Wang et al., 2007).

Pharmacological approaches for the prevention or treatment of AAT or noise-induced hearing loss (NIHL) have been developed with a variety of antioxidant drugs which improve antioxidant defense mechanisms in the cochlea (Choi et al., 2008; Flyod et al., 2008; Kopke et al., 2005; Kopke et al., 2007). N-acetyl-L-cysteine (NAC), a glutathione prodrug and a scavenger of ROS, has been used and effectively protected hair cells from AAT by increasing intracellular glutathione (GSH). Another pharmacological approach has been used for the prevention or treatment of AAT or NIHL. 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 free radical trap and an inhibitor of inducible nitric oxide synthase and a major metabolite of PBN] have been used for the same objective (Flyod et al., 2008). 4-OHPBN among the PBN family showed the strongest effect in preventing and treating hearing loss induced by AAT. This indicates that the intraperitoneal (IP) administration of 4-OHPBN alone and in combination with other antioxidant drugs (NAC or ALCAR (Acetyl-L-carnitine)) has stronger biological effect treating AAT than that of PBN and other derivatives (Choi et al., 2008; Floyd et al., 2008; Reinke et al., 2000).

For measuring the effectiveness of the 4-OHPBN alone and in combination with other antioxidant drugs, our previous study used IP injection. However, the most-commonly used injection method to deliver drugs in human is oral administration. Since our research goal is to develop realistic pharmacological interventions for AAT, it is necessary to use the oral administration as an injection method for evaluating the effect of injection method in investigating the effect of 4-OHPBN plus NAC on AAT. Therefore, the objectives of this study are to investigate the therapeutic effect of the combined antioxidant drugs (4-OHPBN plus NAC) in treating hearing loss induced by AAT when these drugs are orally administered. The cubic distortion product otoacoustic

emissions (DPOAE) on 25 days after noise exposure were assessed in this study.

## Materials and Methods

### 1. Animals and synthesis of 4-OHPBN

The experimental procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committees of the Office of Naval Research and the Oklahoma Medical Research Foundation (OMRF) and similar to those previously described except distortion product otoacoustic emission (DPOAE) (Choi et al., 2008).

Thirty 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 of the similar audiograms to human. Animals housed in plastic cages in the OMRF animal facility had free access to a standard chinchilla diet (Mazuri Chinchilla Diet, 5MO1, PM1 Nutrition International Inc., Brentwood, MO) and tap water throughout the experimental periods. Chinchillas were randomized into five groups (n=6 for the control group and n=6 for each of four experimental groups): noise + saline exposure group (control group); 1<sup>st</sup> experimental group treated with 4-OHPBN (10 mg/kg) plus NAC (20 mg/kg); 2<sup>nd</sup> experimental group treated with 4-OHPBN (20 mg/kg) plus NAC (50 mg/kg); 3<sup>rd</sup> experimental group treated with 4-OHPBN (50 mg/kg) plus NAC (100 mg/kg); 4<sup>th</sup> experimental group treated with 4-OHPBN (75 mg/kg) plus NAC (200 mg/kg). The relationship between the specific groups and the treatment was shown in <Table 1>.

**Table 1.** Control group and different experimental groups with different dosage of 4-OHPBN plus NAC

Groups	Treatment
Group1 (control group)	Noise+saline
Group2	Noise+4-OHPBN
(1 <sup>st</sup> experimental group)	(10 mg/kg) plus NAC (20 mg/kg)
Group3	Noise+4-OHPBN
(2 <sup>nd</sup> experimental group)	(20 mg/kg) plus NAC (50 mg/kg)
Group4	Noise+4-OHPBN
(3 <sup>rd</sup> experimental group)	(50 mg/kg) plus NAC (100 mg/kg)
Group5	Noise+4-OHPBN
(4 <sup>th</sup> experimental group)	(75 mg/kg) plus NAC (200 mg/kg)

4-OHPBN was synthesized using a straight forward chemical reaction, extraction, and crystallization procedure that has been done in our laboratory for over 20 years (Choi et al., 2008). 4-OHPBN 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 400 [PEG 400, 0.8 ml per 100 mg, Sigma-Aldrich Inc., St. Louis, MO] was added. Sterile saline (0.4 ml per 100 mg) was added before injection. A combination of 4-OHPBN and NAC (Hospira Inc., Lake Forest, IL) was orally administered to the experimental animals. Experimental animals received 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) which were 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 an octave-band noise 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 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, the sound spectrum output of the system was calibrated with a sound level meter. During noise exposure, a condenser microphone (B&K 2804, Norcross, GA) was used to visually and continually monitor the noise level, which was 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. Distortion Product Otoacoustic Emission

Hearing of all animals used in this study was evaluated before measuring DPOAE. Hearing thresholds were measured

with auditory brainstem responses (ABR) and 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. Light ketamine (20 mg/kg) and xylazine (1 mg/kg) anesthesia was used for ABR and DPOAE recording and then small supplemental doses (1/3 of initial dose) were given if needed. DPOAE was obtained with SmartDPOAE (Intelligent Hearing System, Miami, FL) using probe-tip microphone (10B+) in the ear canal to provide a good seal and reduce external noise. Cubic DPOAE ( $2f_1-f_2$ ), the largest amplitude of distortion product responses was acquired with two primary tones ( $f_1$  and  $f_2$ ,  $f_1 < f_2$ ) of one-second duration,  $f_2/f_1$  ratio=1.2, and identical intensities ( $L_1=L_2$ ) ranged from 20 to 75 dB SPL in 5 dB increments over the frequency range of 1-8 kHz as previously reported (Choi & Oghalai, 2008). In addition, the SmartDPOAE parameters for DPOAE acquisition were set as follows: 32 sweeps per frequency and 8 sweeps per block. As the test is being completed, the SmartDPOAE system provided the averaged responses to each sweep in a DP Gram (Distortion Product audiogram) displaying the intensity of the response at each frequency point and the noise level around it. A signal-to-noise ratio of +10 was used as a passing criterion. When the criterion was satisfied, the data was used for analysis. Finally, cubic DPOAE amplitudes for both ears of each animal were measured before initial noise exposure (baseline) and 21 days after noise exposure for each frequency.

## 4. Statistical Analysis

All data are reported as mean  $\pm$  standard error of mean (S.E.M). Significant differences in DPOAE amplitude 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, if needed. A  $p$ -value less than 0.05 was considered to indicate a statistically significant difference.

## Results

Before initial noise exposure, we confirmed that there were no significant differences in cubic DPOAE amplitudes

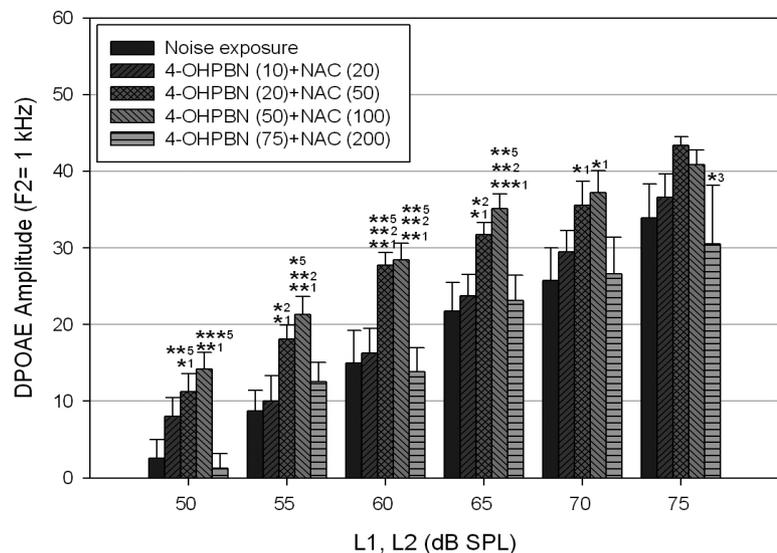
among any of the groups. However, the DPOAE amplitude measured at 21 days after noise exposure were significantly different among different groups at different frequencies.

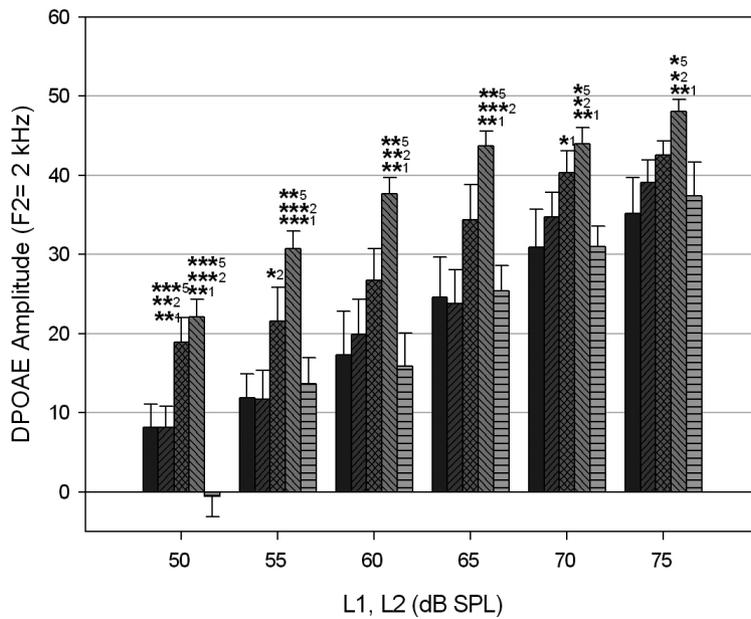
<Fig. 1> shows the cubic DPOAE amplitudes at  $f_2=1$  kHz at different groups. There were significant differences in the mean DPOAE amplitudes among different groups. At the level of 50 dB SPL, the 2<sup>nd</sup> experimental group (11 dB) showed significant differences in DPOAE amplitudes compared to the control group (3 dB) and the 4<sup>th</sup> experimental group (1 dB), respectively. The 3<sup>rd</sup> experimental group (14 dB) showed significant differences in DPOAE amplitudes compared to the control group and the 4<sup>th</sup> experimental group, respectively. At the level of 55 dB SPL, the 2<sup>nd</sup> experimental group (17 dB) showed significant differences in DPOAE amplitudes compared to the control group (8 dB) and the 4<sup>th</sup> experimental group (13 dB), respectively. The 3<sup>rd</sup> experimental group (22 dB) showed significant differences in DPOAE amplitudes compared to the control group, the 1<sup>st</sup> experimental group (10 dB) and the 4<sup>th</sup> experimental group, respectively. At the level of 60 dB SPL, the 2<sup>nd</sup> experimental group (27 dB) showed significant differences in DPOAE amplitudes compared to the control group (15 dB), the 1<sup>st</sup> experimental group (16 dB) and the 4<sup>th</sup> experimental group (14 dB), respectively. The 3<sup>rd</sup> experimental group (28 dB) showed significant differences in DPOAE amplitudes compared to the control group, the 1<sup>st</sup> experimental group and the 4<sup>th</sup> experimental group, respectively. At the level of 65 dB SPL, the 2<sup>nd</sup> experimental group (32 dB) showed significant differences in DPOAE amplitudes compared to the

control group (22 dB) and the 1<sup>st</sup> experimental group (23 dB) respectively. The 3<sup>rd</sup> experimental group (35 dB) showed significant differences in DPOAE amplitudes compared to the control group, the 1<sup>st</sup> experimental group, and the 4<sup>th</sup> experimental group (23 dB), respectively. At the level of 70 dB SPL, the 2<sup>nd</sup> experimental group (35 dB) showed significant differences in DPOAE amplitudes compared to the control group (25 dB). The 3<sup>rd</sup> experimental group (36 dB) showed significant differences in DPOAE amplitudes compared to the control group. At the level of 75 dB SPL, the 2<sup>nd</sup> experimental group (43 dB) showed significant differences in DPOAE amplitudes compared to the 4<sup>th</sup> experimental group (31 dB).

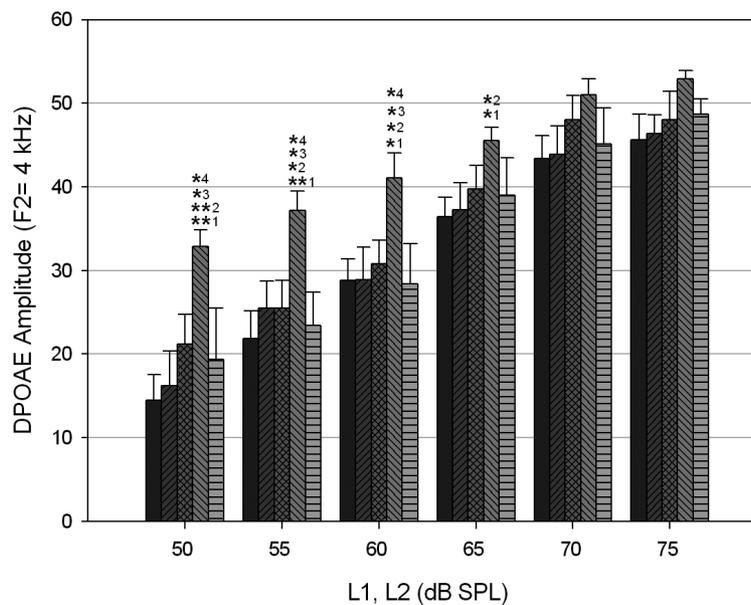
<Fig. 2> shows the cubic DPOAE amplitudes at  $f_2=2$  kHz at different groups. There were also significant differences in the mean DPOAE amplitudes among different groups. At the level of 50 dB SPL, the 2<sup>nd</sup> experimental group (19 dB) showed significant differences in DPOAE amplitudes compared to the control group (8 dB), the 1<sup>st</sup> experimental group (8 dB), and the 4<sup>th</sup> experimental group (-1 dB), respectively. The 3<sup>rd</sup> experimental group (22 dB) showed significant differences in DPOAE amplitudes compared to the control group, the 1<sup>st</sup> experimental group, and the 4<sup>th</sup> experimental group, respectively. At the level of 55 dB SPL, the 2<sup>nd</sup> experimental group (22 dB) showed significant differences in DPOAE amplitudes compared to the 1<sup>st</sup> experimental group (13 dB). The 3<sup>rd</sup> experimental group (31 dB) showed significant differences in DPOAE amplitudes compared to the control group (13 dB), the 1<sup>st</sup> experimental

**Fig. 1.** DPOAE amplitude at  $F_2=1$  kHz for control and different 4-OHPBN and NAC dosage groups. Distortion product otoacoustic emission (DPOAE) was obtained with  $F_2/F_1$  ratio (1.2) and the same amplitude of  $L_1$  and  $L_2$ . Asterisks indicate \*  $p < .05$ , \*\*  $p < .01$ , and \*\*\*  $p < .001$ , respectively. The number after the asterisk represents the groups compared for statistical analysis as shown in <Table 1>.





**Fig. 2.** DPOAE amplitude at F2=2 kHz for control and different 4-OHPBN and NAC dosage groups.

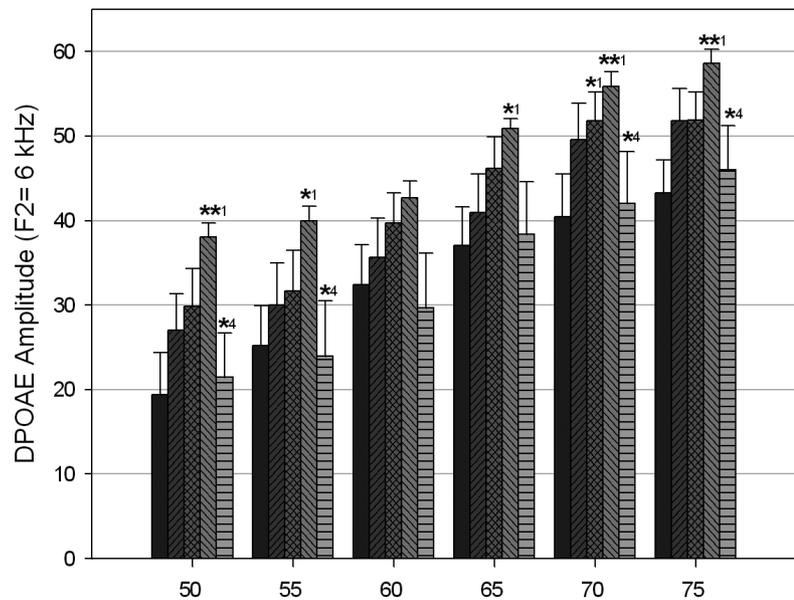


**Fig. 3.** DPOAE amplitude at F2=4 kHz for control and different 4-OHPBN and NAC dosage groups.

group (13 dB), and the 4<sup>th</sup> experimental group (14 dB), respectively. At the level of 60 dB SPL, the 3<sup>rd</sup> experimental group (37 dB) showed significant differences in DPOAE amplitudes compared to the control group (17 dB), the 1<sup>st</sup> experimental group (20 dB), and the 4<sup>th</sup> experimental group (16 dB), respectively. At the level of 65 dB SPL, the 3<sup>rd</sup> experimental group (43 dB) showed significant differences in DPOAE amplitudes compared to the control group (24 dB), the 1<sup>st</sup> experimental group (23 dB), and the 4<sup>th</sup> experimental group (25 dB) respectively. At the level of 70 dB SPL, the 2<sup>nd</sup> experimental group (40 dB) showed significant differences in DPOAE amplitudes compared to the

control group (31 dB). The 3<sup>rd</sup> experimental group (43 dB) showed significant differences in DPOAE amplitudes compared to the control group, the 1<sup>st</sup> experimental group (35 dB), and the 4<sup>th</sup> experimental group (31 dB) respectively. At the level of 75 dB SPL, the 3<sup>rd</sup> experimental group (48 dB) showed significant differences in DPOAE amplitudes compared to the control group (35 dB), the 1<sup>st</sup> experimental group (39 dB), and the 4<sup>th</sup> experimental group (37 dB).

<Fig. 3> shows the cubic DPOAE amplitudes at f2=4 kHz at different groups. There were also significant differences in the mean DPOAE amplitudes among different groups. At the level of 50 dB SPL, the 3<sup>rd</sup> experimental



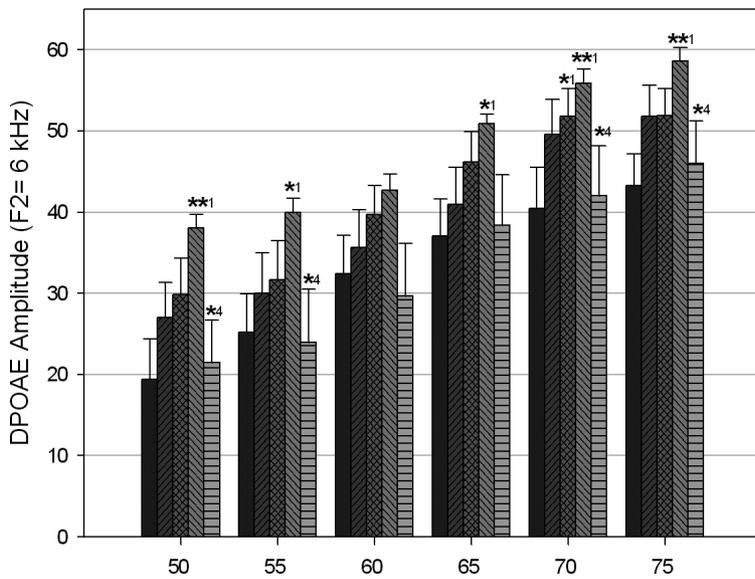
**Fig. 4.** DPOAE amplitude at F2=6 kHz for control and different 4-OHPBN and NAC dosage groups.

group (33 dB) showed significant differences in DPOAE amplitudes compared to the control group (15 dB), the 1<sup>st</sup> experimental group (16 dB), the 2<sup>nd</sup> experimental group (21 dB), and the 4<sup>th</sup> experimental group (19 dB), respectively. At the level of 55 dB SPL, the 4<sup>th</sup> experimental group (37 dB) showed significant differences in DPOAE amplitudes compared to the control group (22 dB), the 1<sup>st</sup> experimental group (25 dB), the 2<sup>nd</sup> experimental group (25 dB), and the 4<sup>th</sup> experimental group (24 dB), respectively. At the level of 60 dB SPL, the 3<sup>rd</sup> experimental group (42 dB) showed significant differences in DPOAE amplitudes compared to the control group (28 dB), the 1<sup>st</sup> experimental group (28 dB), the 2<sup>nd</sup> experimental group (31 dB), and the 4<sup>th</sup> experimental group (28 dB), respectively. At the level of 65 dB SPL, the 3<sup>rd</sup> experimental group (46 dB) showed significant differences in DPOAE amplitudes compared to the control group (36 dB) and the 1<sup>st</sup> experimental group (37 dB), respectively. At the level of 70 and 75 dB SPL, there were no significant differences in the mean DPOAE amplitudes among different groups.

<Fig. 4> shows the cubic DPOAE amplitudes at f2=6 kHz at different groups. There were also significant differences in the mean DPOAE amplitudes among different groups. At the level of 50 dB SPL, the 3<sup>rd</sup> experimental group (38 dB) showed significant differences in DPOAE amplitudes compared to the control group (19 dB) while the 4<sup>th</sup> experimental group (22 dB) showed significant differences in DPOAE amplitudes compared to the 3<sup>rd</sup> experimental

group. At the level of 55 dB SPL, the 3<sup>rd</sup> experimental group (40 dB) showed significant differences in DPOAE amplitudes compared to the control group (25 dB) while the 4<sup>th</sup> experimental group (24 dB) showed significant differences in DPOAE amplitudes compared to the 3<sup>rd</sup> experimental group. At the level of 65 dB SPL, the 3<sup>rd</sup> experimental group (51 dB) showed significant differences in DPOAE amplitudes compared to the control group (37 dB). At the level of 70 dB SPL, the 2<sup>nd</sup> experimental group (52 dB) and the 3<sup>rd</sup> experimental group (56 dB), respectively showed significant differences in DPOAE amplitudes compared to the control group (36 dB). The 4<sup>th</sup> experimental group (42 dB) showed significant differences in DPOAE amplitudes compared to the 3<sup>rd</sup> experimental group. At the level of 75 dB SPL, the 3<sup>rd</sup> experimental group (58 dB) showed significant differences in DPOAE amplitudes compared to the control group (43 dB). The 4<sup>th</sup> experimental group (46 dB) showed significant differences in DPOAE amplitudes compared to the 3<sup>rd</sup> experimental group. At the level of 60 dB SPL, there were no significant differences in the mean DPOAE amplitudes among different groups.

<Fig. 5> shows the cubic DPOAE amplitudes at f2=8 kHz at different groups. There were also significant differences in the mean DPOAE amplitudes among different groups. At the level of 50 dB SPL, the 3<sup>rd</sup> experimental group (29 dB) showed significant differences in DPOAE amplitudes compared to the control group (13 dB), the 1<sup>st</sup> experimental group (17 dB), and the 4<sup>th</sup> experimental group



**Fig. 5.** DPOAE amplitude at F2=8 kHz for control and different 4-OHPBN and NAC dosage groups.

(17 dB). At the level of 55 dB SPL, the 3<sup>rd</sup> experimental group (33 dB) showed significant differences in DPOAE amplitudes compared to the control group (19 dB), the 1<sup>st</sup> experimental group (23 dB), and the 4<sup>th</sup> experimental group (15 dB), respectively. The 4<sup>th</sup> experimental group showed significant differences in DPOAE amplitudes compared to the 2<sup>nd</sup> experimental group (28 dB). At the level of 60 dB SPL, the 3<sup>rd</sup> experimental group (36 dB) showed significant differences in DPOAE amplitudes compared to the control group (24 dB) while the 4<sup>th</sup> experimental group (22 dB) showed significant differences in DPOAE amplitudes compared to the 2<sup>nd</sup> experimental group (33 dB) and the 3<sup>rd</sup> experimental group. At the level of 65 dB SPL, the 2<sup>nd</sup> experimental group (41 dB) and the 3<sup>rd</sup> experimental group (42 dB), respectively showed significant differences in DPOAE amplitudes compared to the control group (31 dB). At the level of 70 and 75 dB SPL, there were no significant differences in the mean DPOAE amplitudes among different groups.

## Discussion and Conclusions

The objective of the present study was to investigate the therapeutic effect of a combined antioxidant drugs (4-OHPBN plus NAC) on AAT in terms of levels and frequency changes in DPOAE responses. The oral administration of 4-OHPBN plus NAC produced a significant increase in DPOAE amplitude. The improvement was correlated with

the dose of 4-OHPBN plus NAC. When the dose of 4-OHPBN and NAC was increased from 10 and 20 mg/kg to 50 and 100 mg/kg, respectively, the cubic DPOAE amplitude systematically increased. These results are consistent with previous studies reporting that IP injection of combined antioxidant drugs (4-OHPBN plus NAC) significantly reduced both permanent ABR hearing thresholds and OHC (Outer Hair Cell) loss (Choi et al., 2008). The increase of the cubic DPOAE amplitudes in our study is related with the OHC loss in the previous study because the DPOAE originates from OHC electromotility (Brownell, 1990; Choi & Oghalai, 2008; Choi, 2010).

It should be mentioned that the cubic DPOAE amplitude in the experimental group treated with 4-OHPBN (75 mg/kg) plus NAC (200 mg/kg) was decreased when it was compared to other experimental groups. It indicates that over dose of 4-OHPBN may be ototoxic in cochlea because it has not been reported that the dose (200 mg/kg) of NAC may damage the cochlea (Flyod et al., 2008). In addition, the increased amount in the cubic DPOAE amplitude for the experimental group treated with 4-OHPBN (50 mg/kg) plus NAC (100 mg/kg) is relatively smaller than the decreased amount in the ABR threshold shift for the same group in the previous study. These different results may result from the administration method. This study used oral administration while the previous study used IP injection. Furthermore, measurement methods may be another factor affecting the difference. The present study measured the cubic DPOAE while the previous study measured ABR thresh-

old shift. The ABR threshold shift is more appropriate to use for comparison than DPOAE because DPOAE is not very sensitive to change of the cochlear amplification (Choi & Oghalai, 2008; Kujawa & Liberman, 2001).

Since the antioxidant drug used in this study, 4-OHPBN is a major metabolite of phenyl-*N-tert*-butylnitone (PBN), the fundamental mechanisms of 4-OHPBN are very similar to those of PBN. The effect of PBN involves the reduction of oxidative stress. It has been reported that PBN has the spin trapping ability, its antioxidant properties, its action on important membrane enzymes (ion transport proteins), its role as a neuroprotectant, and its action as anti-inflammatory agent (Flyod et al., 2008). When PBN is compared to its other derivatives such as 4-hydroxy PBN (4-OHPBN), 3-hydroxy PBN (3-OHPBN), 2-hydroxy PBN (2-OHPBN), and 2-sulfoxy PBN (2-SPBN), the strongest effect on inhibiting hepatocarcinogenesis was reported in 4-OHPBN (Nakae et al., 2003). When PBN were applied to AAT, it decreased the permanent noise-induced hearing loss potentiated by several chemical compounds such as carbon monoxide, hydrogen cyanide, and acrylonitrile but did not show strong therapeutic effects on treating noise-induced hearing loss alone (Fechter et al., 1997; 2004; Rao & Fechter, 2000; Rao et al., 2001). However, when 4-OHPBN was applied to treatment of AAT, it reduced permanent hearing loss in a dose-dependent manner as well as the 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). However, the current study using the combination of 4-OHPBN and NAC did not show the same dose dependent effect as 4-OHPBN alone. Recently, 2,4-disulfonyl PBN (HPN-07, a derivative of 4-OHPBN) has been used to investigate its effect on AAT because HPN-07 has its relative safety compared to the effects of 4-OHPBN (Choi, 2011).

On the other hand, NAC involves a variety of mechanisms. NAC has its action as a ROS scavenger, its ability to reduce mitochondrial injury, its function to inhibit glutamate excitotoxicity, inflammation and apoptosis, and its role as neuroprotectant by improving levels of glutathione in the process of oxidative stress (Coleman et al., 2007; Henderson et al., 2006; Kopke et al., 2000; 2007). When NAC was applied to AAT, it has a good effect to prevent or

treat cochlear damage induced by AAT. In addition, NAC has been used to demonstrate the synergistic effects of the combination of antioxidant drugs [salicylate (a hydroxyl radical scavenger), ALCAR, and 4-OHPBN] in preventing or treating permanent hearing loss induced by AAT because each drug may have different mechanism and site of action (Choi et al., 2008; Kopke et al., 2000). A variety of pre-clinical tests related with oral administration of the combined antioxidant drugs (4-OHPBN plus NAC) should be performed because the clinical verification are further needed. Finally, although chinchilla used in this study has similar audiogram to human, it should be noticed that our results may not generalize to human.

Recently, the use of each antioxidant drug in combination of other antioxidant drugs is very popular because it brings the synergistic effects to maximize the prevention or treatment of AAT or NIHL. NAC and salicylate (a hydroxyl radical scavenger), salicylate and Trolox (a water soluble analog of  $\alpha$ -tocopherol), folate plus vitamin E plus acetyl-L-carnitine (ALCAR), free radical scavengers vitamins A, C, and E plus magnesium, 4-OHPBN plus NAC, 4-OHPBN plus NAC plus ALCAR have been used for these synergistic effects (Le Prell et al., 2007; Choi et al., 2008; Kopke et al., 2000; Yamashita et al., 2005; Dhitavat et al., 2005). More various combinations of antioxidant drugs will be continually sought to maximize the effects of antioxidant drug. The use of 4-OHPBN in combination with other antioxidant drug showed stronger effect to treat AAT. However, the maximal dosage of 4-OHPBN should be very careful because it may be ototoxic.

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