Effects of N-Acetylcysteine on Auditory Brainstem Response Threshold Shift in Rabbits Exposed to Noise and Carbon Monoxide

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Abstract: Problem statement: Noise Induced Hearing Loss (NIHL) is one of the most important occupational disease in worldwide. NIHL has been found to be potentiated by simultaneous Carbon monoxide (CO) exposure. Exposure to noise plus CO is common in occupational and environmental settings. Free radicals have been implicated in cochlear damage resulting from exposure to noise and due to CO hypoxia This study examined whether N-Acetylcysteine (NAC) administration cause attenuation of Auditory Brainstem Response (ABR) threshold shifts resulting from noise exposure and noise plus CO exposure. **Approach:** Forty-two rabbits were divided into seven groups including control, noise+ saline, noise+ CO +saline, noise+ NAC, noise+ CO+ NAC, CO+ NAC and NAC alone. ABR was assessed before exposure, 1 hour and 14 days post exposure. **Results:** The administration of 325 mg kg^{-1} of NAC prior, following and post exposure to noise or noise plus CO recovered permanent ABR threshold shift at 1 and 2 kHz almost to the baseline and provided significant attenuation in permanent ABR threshold shift at 4 and 8 kHz in subjects which were exposed to noise but it did not block the potentiating of threshold elevation by CO exposure (extra threshold loss by combined exposure) at 4 and 8 kHz. **Conclusion:** NAC provides protective effect against hearing loss resulting from noise exposure and simultaneous exposure to noise plus CO.

Key words: Noise induced hearing loss, antioxidant agent, NIHL potentiation, auditory brainstem response, NAC

INTRODUCTION

Excessive noise can cause many health problems in the workplace but Noise-Induced Hearing Loss (NIHL) is the most important effect of noise on the auditory system in occupational settings. NIHL is a widespread disease in the world with 16% of the disabling hearing loss in adults being attributed to occupational noise (Nelson *et al*., 2005). NIHL has been found to be potentiated by simultaneous exposure to noise and Carbon monoxide (CO), while CO exposure alone may not induce permanent hearing loss (Chen and Fechter, 1999; Rao and Fechter, 2000a). CO is the most abundant pollutant in air and exposure to noise plus CO

is common in many work environments. The mechanisms of NIHL can be classified into two major categories: direct mechanical trauma and metabolic damage to the organ of corti. The generation of Reactive Oxygen Species (ROS) is considered to be part of the second mechanism (Prell *et al*., 2007; Heinrich and Feltens, 2006; Fechter, 2005).

Early reports of noise-induced free radical formation in the cochlea led to the hypothesis that NIHL can be attenuated by exogenous antioxidants as well as endogenous ROS scavengers. Many compounds have been used for the prevention and treatment of NIHL (Kopke *et al*., 2005; Lynch and Kil, 2005). The mechanism underlying NIHL potentiation by CO is

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unclear but combined exposure to noise and CO is believed to increase free radical levels in the cochlea (Fechter *et al*., 2002; Rao and Fechter, 2000b). In many cases, engineering and administrative measures for reduction of noise and CO levels is very difficult; however, hearing protection devices is seldom used by workers because of inherent limitation. Hence, a pharmacological preventative measure for NIHL can be an important element of hearing conservation programs in the workplace. Many studies have demonstrated that N-Acetylcysteine (NAC) as an antioxidant and as a substrate for glutathione synthesis can effectively reduce NIHL in animal models (Kopke *et al*., 2000; 2004; 2005).

The present study has been designed to test the hypothesis that injury resulting from simultaneous exposure to noise plus CO is reduced by NAC in a rabbit model.

MATERIALS AND METHODS

Animals: Healthy male adult white New Zealand rabbits (2200-2500 g body weight) were acquired from the Pasture Institute of Iran. Animals were examined for possible otitis media prior to auditory assessments. Rabbits were maintained on a 12 h light/12 h dark cycle at 23-27°C and they had free access to food (Purina laboratory chow) and water. All procedures regarding the use and handling of animals were carried out in accordance with the principles of Helsinki and in accordance with animal use protocols approved by the appropriate institutional animal care and use committees.

Experimental protocol: Forty-two rabbits were divided into seven groups (n = 6, each group) according to type of experiment as follows: Group 1, control (no exposure to noise or CO and no injection); Group 2, exposed to noise alone and received saline intrapretoneal (i.p.) injection; Group 3, exposed to noise plus CO and received saline i.p. injection; Group 4, exposed to noise alone and received NAC 325 mg kg^{-1} body weight i.p. injection; Group 5, exposed to noise plus CO and received NAC 325 mg kg^{-1} body weight i.p. injection; Group 6, not exposed to noise or CO and received NAC 325 mg kg^{-1} body weight i.p. injection; Group 7, exposed to CO alone and received saline i.p. injection. The total duration of experimental procedures was 22 days. Noise and CO exposure was conducted for 5 consecutive days on day 4 through day 8. NAC or saline was injected once a day from day 1 through day 11 (3 days before the exposure, 5 days during the exposure and 3 days after exposure). Auditory Brain-

stem Response (ABR) threshold at 1, 2, 4 and 8 kHz were measured at three time points: day 1 (prior to experiment), day 8 (immediately at the end of exposure) and day 22 (14 days after exposure).

The exposure chamber was designed as a reverberant field which could accommodate six rabbits at a time. The chamber was equipped with stereo speakers for delivering noise. Air exchange rate in this chamber was adjusted to 12 air changes per hour using an exhaust fan. The ventilation system generated noise below 40 dB which was masked by the noise generated for exposure experiments. Experimental Group 2-5 were exposed to octave band noise centered at 4 kHz, 100 dB SPL, 8 h day^{-1} for 5 days consecutively. This octave band noise was generated and controlled with a custom software program (signal software) and then amplified and delivered with a computer system using Cool Edit software to two load speakers. Sound pressure level in the chamber was measured using a precision sound level meter (Cel-460, UK) at the level of animal head (15 cm above the chamber floor). Noise exposure levels in the chamber were monitored every 30 min. The variability of the noise within the animals' roaming area was less than 2 dB.

Animals exposed to noise plus CO or CO alone based on experimental design. The CO concentration in the exposure chamber was 700 ppm with an actual level achieved of 700±25 ppm (SD). Previous studies have shown that carboxyhemoglobin levels approach steady state within 30 min of exposure onset and have stabilized after 90 min of exposure (Chen and Fechter, 1999). Therefore, CO exposure began 90 min prior to the onset of noise to assure equilibration of carboxyhemoglobin. The total daily exposure time to noise was 8 h and the daily exposure time to CO was 9.5 h (8+1.5 h). Experimental Group 3, 5 and 7 were exposed to CO at 700 ppm concentration, 9.5 h day^{-1} for 5 days consecutively. CO gas was delivered into the chamber using a micro valve (Air Flow) and the CO level in the chamber was measured according to a standard procedure described in NIOSH: Method No 6604 by carbon monoxide monitor STICK (testo 317-3) (National Institute of Occupational safety and Health, 1999). The concentration of CO was monitored continuously. Experimental Group 4-6 received NAC325 mg kg^{-1} body weight once a day for 11 days by i.p. injection. During the exposure days, NAC was given 1 h prior to noise or noise plus CO exposure. Group 1-3 and 7 received saline with a similar volume and schedule. Protective effect of NAC against CO alone was not studied since CO alone does not cause any permanent auditory impairment (Chen and Fechter, 1999).

Auditory brainstem response testing: Auditory function was evaluated by Auditory Brainstem Response (ABR) threshold test. Before ABR test, animals were anesthetized with Xylazine (10 mg kg^{-1} body weight) and ketamin (40 mg kg^{-1} body weight) mixture by i.m. injection. The external ear canals and tympanic membranes were inspected to ensure the ear canal was free of wax. The body temperature of rabbits was maintained at 37±1°C using a heating blanket. An active needle electrode was inserted subcutaneously below the test ear, a reference electrode at the vertex and a ground electrode below the other ear. The test stimuli were 1, 2, 4 and 8 kHz tone bursts (1 ms Blackman rise/fall, 15 ms duration, alternating polarity) generated using Interacoustics EP25. Stimuli were routed through an insert phone fitted in the rabbit's ear canal. Near the threshold, the sound intensity was decreased in 5 dB steps and responses for 1024 tone presentations at 20/s were averaged at each intensity level. ABR threshold was defined as the lowest stimulus intensity that produces a reproducible ABR waveform peak 3 or 4. The average threshold of the right and left ear at a specific frequency was considered as the ABR threshold for a specific time point. All experimental groups were tested prior to experiment on day 1 to establish the baseline ABR threshold, day 11 (immediately at the end of exposure) and day 22 (14 days after exposure). Temporary Threshold Shift (TTS) was calculated at each frequency as the difference between baseline ABR threshold and ABR threshold on day 11 (0 day post exposure). The difference between baseline ABR threshold and ABR threshold on day 22 at each frequency was considered as the Permanent Threshold Shift (PTS).

Non-protein GSH measurement: Blood Glutathione (GSH) level was measured to evaluate the effects of NAC on glutathione synthesis in rabbit and compare blood GSH levels from control and treated rabbits. Total blood GSH was measure using Ellman's reagent (Mirzaee *et al.*, 2007). The whole blood (0.2 mL) was taken in a 10 mL test tube and was mixed by adding 1.8 mL of distilled water. Then 3.0 mL of the precipitation solution (1.67 g of glacial metaphosphoric acid, 0.2 g of disodium-EDTA and 30 g of NaCl in 100 mL water) was added. The mixture was allowed to stand for 5 min at room temperature and then filtered to remove proteins. Exactly 0.8 mL of the filtrate was mixed with 1.0 mL of DTNB (40 mg DTNB in 100 mL of sodium citrate solution). The intensity of the color was recorded at 412 nm using Shimadzu UV-3100 double beam spectrophotometer. The concentration of GSH in samples was calculated from a calibration curve which was prepared from freshly prepared standard solution of GSH.

This assay was performed at two time points: Day 4 (3 days after receiving NAC or saline and prior to noise or CO exposure) and day 8 (8 days after receiving NAC or saline and 5 days post exposure to noise or noise plus CO).

Statistical analysis: The Kolmogorov-Smirnov test was used to assess the normality of data in each group. The ABR threshold shift at each frequency was compared among groups using one-way Analysis of Variance (ANOVA) and the Tukey test was applied as a post hoc test for multiple comparisons between groups. Likewise, ANOVA was used for comparisons of ABR threshold shift between frequencies at each group and comparison of blood GSH level among groups. All statistical tests were performed using SPSS 15 for windows and $p<0.05$ was considered significant.

RESULTS

Blood Glutathione (GSH): The results indicate the blood GSH level in subjects which received NAC (Group 4-6) was significantly more than other groups on day 4 (prior to exposure) and day 8 (5 days after exposure). Blood GSH level on day 8 was found to be reduced significantly in Group 2, 3 and 7 as compared to Group 1 (control) (Fig. 1).

ABR measurement: Baseline ABR thresholds at each frequency were similar in all groups. The baseline ABR threshold at 8 and 4 kHz were significantly smaller than 2 and 1 kHz in all groups. ABR threshold shift on day 8 (1 h after exposure) and day 22 (14 days after exposure) at each frequency in experimental groups are represented in Fig. 2.

The ABR threshold shift of the control group was approximately 0 on day 8 and day 22. Statistical comparisons showed that the ABR threshold shifts of Group 6 (unexposed and received NAC) and Group 7 (CO exposure and received saline) at each frequency were not significantly different from Group 1 (control) at all time points. This indicates that CO exposure alone and receiving NAC alone could not alter ABR thresholds. The ABR threshold of Group 2 (noise + saline) had significant elevation compared to the control group at all frequencies on day 8 and day 22 but was significantly smaller than the ABR threshold of Group 3 (noise + CO + saline) ($p<0.01$).
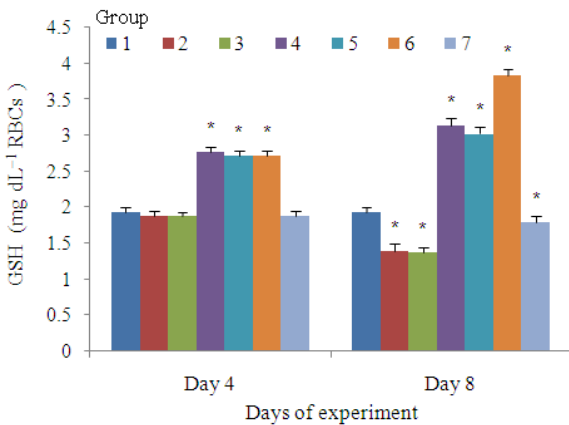
Fig. 1: Blood Glutathione (GSH) levels on day 4 (prior to exposure) and day 8 (5 days after exposure) in each group. Bars show means, error bars show ±1.0 SD. *: p<0.05, significantly different from Group 1. Groups: (1) control (no exposure to noise or CO, and no i.p. injection); (2) noise exposure + saline i.p. injection; (3) noise plus CO exposure + saline i.p. injection; (4) noise exposure + NAC 325 mg kg^{-1} i.p. injection; (5) noise plus CO exposure + NAC 325 mg kg^{-1} i.p. injection; (6) no exposure to noise or CO + NAC 325 mg kg^{-1} i.p. injection; (7) CO exposure + saline i.p. injection
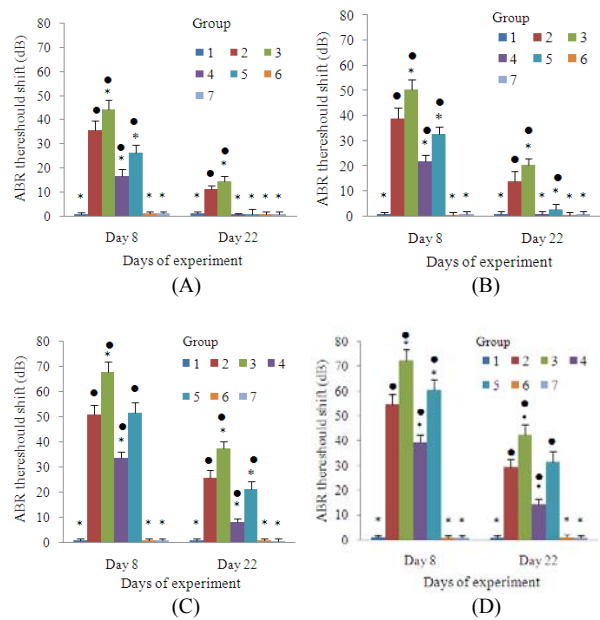

Fig. 2: ABR threshold shifts on day 8 (1 h post exposure to noise or noise plus CO) and day 22 (14 days post exposure to noise or noise plus CO) in each group at 1 KHz (A), 2 KHz (B), 4 kHz (C) and 8 KHz (D). Bars show means, error bars show ±1.0 SD. *: p<0.05, significantly different from group 2. •: p<0.05, significantly different from Group 1. Groups: (1) control (no exposure to noise or CO, and no i.p. injection); (2) noise exposure + saline i.p. injection; (3) noise plus CO exposure + saline i.p. injection; (4) noise exposure + NAC 325 mg kg^{-1} i.p injection; (5) noise plus CO exposure + NAC 325 mg kg^{-1} i.p. injection; (6) no exposure to noise or CO + NAC 325 mg kg^{-1} i.p. injection; (7) CO exposure + saline i.p. injection

The saline + noise group (Group 2) had a 44.8 dB threshold loss on day 8 and 20.1 dB threshold loss on day 22 averaged across frequencies of 1-8 kHz while animals receiving saline + noise + CO (Group 3) had a 58.5 and 28.6 dB average threshold loss on day 8 and day 22 respectively. The ABR threshold shifts of Group 3 at 4 and 8 kHz were significantly more prominent than at 1 and 2 kHz at all time points tested (p<0.01).The noise-induced ABR threshold shifts at all frequencies were found to be reduced significantly in animals which were exposed to noise and received NAC (Group 4) compared to subjects which were exposed to noise and received saline (Group 2) on day 8 and day 22 (p<0.01). This reduction at 1 and 2 kHz was significantly more than at 4 and 8 kHz (p<0.01).The average threshold elevation across frequencies 1-8 kHz of Group 4 was 27.7 dB on day 8 and 5.6 dB on day 22.

The ABR thresholds of animals in Group 4 at all frequencies were significantly greater than that of control group, 1 h after exposure while 14 days after exposure the thresholds at 1 and 2 kHz recovered almost to the baseline.

The ABR threshold of animals exposed to noise plus CO and treated with NAC (Group 5) at all frequencies was significantly smaller than that of animals which were exposed to noise plus CO and which received saline (Group 3) on day 8 and day 22. This indicates that treatment with NAC could reduce the temporary and permanent ABR threshold shift at all frequencies in subjects exposed to noise and CO simultaneously.

The ABR threshold loss of Group 5 was 42.5 dB on day 8 and 13.8 dB on day 22 averaged across frequencies 1-8 kHz. These values were significantly different from the control group (p<0.01). Comparison among experimental groups in Fig. 2 suggest that NAC

could attenuate ABR threshold shifts in animals exposed to noise plus CO, but it did not block the potentiating of threshold elevation by CO exposure (extra threshold loss by combined exposure) at 8 kHz on day 8.

DISCUSSION

The results showed that, when NAC was administered 3 days prior, following and 3 days post exposure to noise (or noise plus CO) by i.p. injection (325 mg kg^{-1} body weight), adequate protection was provided against noise induced hearing loss and appropriate protection was provided against combined exposure-induced hearing loss in rabbits.

Rabbits were used as the animal model in this investigation because the hearing frequency range is approximately 360-42000 Hz, which covers the frequency range of humans. Octave band noise centered at 4 kHz, 100 dB SPL, 8 h day^{-1} for 5 days consecutively was chosen to imitate continuous noise in the workplace. Likewise, maximum potentiation of NIHL by CO was reported at 100 dB SPL (Fechter *et al*., 2000).

However significant potentiation of NIHL by CO was reported with 500 ppm and higher concentrations of CO in a previous study (Fechter *et al*., 2000). Preliminary experiments demonstrate at least 700 ppm of CO exposure is required to provide more ABR threshold elevation than the noise alone. Rabbits exposed to simultaneous noise and CO had further elevation in temporary and permanent threshold shift at all frequencies as compared with those exposed to noise. This phenomenon was reported in several previous studies (Chen and Fechter, 1999; Fechter *et al*., 2002).

Administration of NAC was begun 3 days before noise (or noise plus CO) exposure for providing a stable and higher concentration of glutathione in the cochlea at beginning of the exposure. Biochemical assay showed that the blood GSH level at groups that received NAC were increased as compared with other groups at prior to exposure. Previous studies have indicated that GSH is an important factor in the modification of inner ear trauma following noise exposure (Ohinata *et al*., 2000; Yamasoba *et al*., 1998). However, the depletion of blood Glutathione (GSH) in rabbits exposed to noise has been previously reported (Mirzaee *et al*., 2007). NAC was given 3 days post exposure because there is evidence for delayed free radical formation, peaking 7-10 days following noise exposure and the finding that free radical scavengers administered as long as 3 days post-noise attenuate free radical formation and reduce NIHL (Prell *et al*., 2007).

NAC was administrated 325 mg kg^{-1} body weight by i.p. injection because many studies indicated that a dose of 325 mg kg^{-1} was most effective for attenuation of NIHL (Kopke *et al*., 2007; Duan *et al*., 2004).

Results from this study indicate that NAC attenuated temporary ABR threshold shift more significantly at 1 and 2 kHz than at 4 and 8 kHz in subjects exposed to noise (or noise plus CO) while noise (or noise + CO) could cause more temporary ABR threshold shift at 4 and 8 kHz than at 1 and 2 kHz. This finding is consistent with previous research showing that antioxidant agents were more effective at the frequencies away from the frequencies with greater threshold shift (Ohinata *et al*., 2000 ; Hou *et al*., 2003; Yamasoba *et al*., 1999) This may be because other factors, in addition to ROS formation.

The current work showed NAC recovered permanent ABR threshold shift at 1 and 2 kHz almost to the baseline and provided significant attenuation in permanent ABR threshold shift at 4 and 8 kHz in subjects exposed to noise. NAC as a GSH prodrug and antioxidant can ameliorate the cochlear damage through a variety of mechanisms, such as providing a substrate for cochlear GSH synthesis, free radical scavenging and inhibition of CDP pathway activation and necrosis.

The results showed that NAC can significantly attenuate the permanent ABR threshold shift in subjects exposed to simultaneous noise and CO.

We know that two major mechanisms are involved in NIHL including mechanical damage and metabolic alternations, but the mechanism of the potentiation of NIHL by CO is still unclear. CO exposure causes reduction of oxygen supply and makes recovery of outer hair cell function impossible, while subjects exposed to noise alone are capable of recovery from a temporary threshold shift (Chen and Fechter, 1999). Many studies indicated that an excitotoxic mechanism may be involved in CO ototoxicity such as CO inducing excessive glutamate release (Fechter *et al*., 1997). Excessive glutamate release has been identified as a basic mechanism of neurotoxicity through the process of excitotoxicity (Chen *et al*., 2001). Free radical generation seems also involved in CO ototoxicity. Production of hydroxyl radicals has been demonstrated directly in the hippocampus due to CO hypoxia (Piantadosi *et al*., 1997).

NAC-induced GSH synthesis may also be able to ameliorate the effects of glutamate excitotoxicity as well as protect cochlear mitochondria from injury. NAC has been shown to be an effective scavenger of hydroxyl radical (Kopke *et al*., 2007).

NAC, a thiol-containing amino acid derivative, is used in the United States as a nutritional supplement

and also a drug which has passed the stringent safety requirements for Food and Drug Administration (FDA) approval for prescription use only.

Thus, NAC may be an excellent candidate for the prevention of NIHL in humans. It must be mentioned that these findings are limited due to the one time point and one dosage level administration of NAC. Further studies of its action are needed.

CONCLUSION

NAC (325 mg kg^{-1}, i.p. injection) can attenuate temporary and permanent noise induced ABR threshold shifts and provides appropriate protection against ABR threshold shift resulting from exposure to noise plus CO in rabbits.

ACKNOWLEDGEMENT

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