ELSEVIER

Hearing Research 164 (2002) 29–38

HEARING RESEARCH

www.elsevier.com/locate/heares

Oxidative DNA damage is associated with intense noise exposure in the rat

Luann E. Van Campen [a,*], William J. Murphy [a], John R. Franks [a], Patricia I. Mathias [b], Mark A. Toraason [b]

[a] *Engineering and Physical Hazards Branch, Division of Applied Research and Technology, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, 4676 Columbia Parkway, Cincinnati, OH, 45226, USA*

[b] *Biomonitoring and Health Assessment Branch, Division of Applied Research and Technology, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, 4676 Columbia Parkway, Cincinnati, OH, 45226, USA*

Received 20 March 2001; accepted 12 September 2001

Abstract

Increasing evidence suggests that noise-induced hearing loss may be reduced or prevented with antioxidant therapy. Biochemical markers of reactive oxygen species (ROS)-induced damage can help elucidate possible treatment timing constraints. This study examined the time course of ROS damage following a 2-h, broad-band noise exposure resulting in permanent threshold shift in 35 Long–Evans rats. Cochlea, brain, liver, serum and urine were analyzed at 1, 3, 8, 72, and 672 h (28 days) after exposure. Oxidative DNA damage was assessed by measuring 8-hydroxy-2′-deoxyguanosine (8OHdG) by high performance liquid chromatography with electrochemical detection. Lipid peroxidation was measured via the thiobarbituric acid-reactive substances (TBARS) colorimetric assay for detection of aldehydes (e.g., malondialdehyde). Auditory brainstem response and distortion product otoacoustic emission thresholds showed progressive elevation for the 3- and 8-h groups, then notable recovery for the 72-h group, and some worsening for the 672-h group. 8OHdG was significantly elevated in cochlea in the 8-h group, and in brain and liver for the 72-h group. TBARS were significantly elevated in serum for the 72-h group. Based upon oxidative DNA damage present in cochlea following intense noise, we postulate that the first 8 h following exposure might be a critical period for antioxidant treatment. Published by Elsevier Science B.V.

Key words: Free radical; Reactive oxygen species; Oxidative stress; Cochlea; DNA; 8-Hydroxy-2′-deoxyguanosine; Thiobarbituric acid-reactive substance

1. Introduction

Oxidative stress is implicated as an important factor in the cascade of cochlear events resulting from noise- or medication-induced sensorineural hearing loss. Although reactive oxygen species (ROS), also known as free oxygen radicals, are normal byproducts of cellular aerobic metabolism, these unstable molecules can impair cellular lipids, proteins and nucleic acids in DNA if the balance of corresponding antioxidants is disrupted. The consequences of such disruptions can be detected biochemically and histologically, and can be demonstrated functionally.

There is both direct and indirect biochemical and histological evidence of cochlear oxidative stress. Direct evidence is derived from procedures that induce increased ROS activity by cellular infusion of ROS (Clerici et al., 1995; Clerici and Yang, 1996), exposure to noise (Yamane et al., 1995; Ohlemiller et al., 1999b) or ototoxic agents (Clerici et al., 1996; Lopez-Gonzalez et

* Corresponding author. Present address: Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA.
Tel.: +1 (317) 433-8859; Fax: +1 (317) 276-4730.
E-mail address: vancampen_l_e@lilly.com (L.E. Van Campen).

Abbreviations: ROS, reactive oxygen species; 8OHdG, 8-hydroxy-2′-deoxyguanosine; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; 2dG, guanosine base; PTS, permanent threshold shift; TTS, temporary threshold shift; HPLC-EC, high performance liquid chromatography with electrochemical detection; ABR, auditory brainstem response; DPOAE, distortion product otoacoustic emission; TDT, Tucker-Davis Technologies; Tris, Tris(hydroxymethyl)aminomethane

0378-5955/02/$ – see front matter Published by Elsevier Science B.V.
PII: S0378-5955(01)00391-4

al., 1998, 1999), or induced ischemia (Ohlemiller and Dugan, 1999). Indirect evidence is derived from procedures that detect changes in ROS levels by inducing increased antioxidant levels, blocking/reducing antioxidant production/activity (Yamasoba et al., 1998; Ohlemiller et al., 1999a), or inhibiting ROS production/activity (Seidman et al., 1993; Quirk et al., 1994; Clerici et al., 1995; Clerici and Yang, 1996; Fechter et al., 1997; Jacono et al., 1998; Kamimura et al., 1999; Yamasoba et al., 1999; Rao and Fechter, 2000).

While the direct evidence is necessary to determine ROS liability for subsequent tissue damage and functional impairment, much enthusiasm surrounds the indirect evidence because it suggests the possibility of prophylactic antioxidant treatment for cochlear dysfunction. The timing of such prophylaxis may be critical in preventing noise-induced hearing loss. To understand this critical timing, one first must understand the time course of oxidative damage to the cochlea. Biochemical markers of ROS-induced cellular damage can be utilized to delineate the time course of ROS-mediated damage as well as biologically effective dose(s). Biochemical markers of oxidative lipid and DNA damage have been well studied in many tissues, and several studies have employed biomarkers of lipid peroxidation for cochlear applications. However, the time course of oxidative damage to DNA has not been studied in cochlea.

The most common biomarker for lipid peroxidation is the thiobarbituric acid-reactive substances (TBARS) assay, which detects malondialdehyde (MDA) and other aldehydes (for review see Janero, 1990); however, more recently, the 8-isoprostane assay is being considered a sensitive and reliable biomarker (Morrow et al., 1990). Rybak and colleagues reported increased cochlear MDA following cisplatin administration in rats (Ford et al., 1997; Ravi et al., 1995; Rybak et al., 1995, 1997, 1999a,b), and She and Chen (1995) reported the same for kanamycin in guinea pigs. Only Liu (1992) has employed the TBARS assay following an intense noise exposure (i.e., blast), and reported increased MDA in guinea pigs at 1 h after explosion and again at 3 and 6 days. At 8 days MDA reduced to normal levels, then increased markedly at 12 days. These data were in accordance with auditory brainstem response (ABR) threshold changes.

Ohinata et al. (2000) employed an 8-isoprostane immunoassay in guinea pigs and demonstrated that a 4-kHz octave band noise exposure increased lipid peroxidation in a time-dependent manner related to the duration of the exposure. They additionally demonstrated that these levels dropped significantly within 30 min of noise cessation and returned to near baseline by 8 h post-exposure. Subsequent histology of various cochlear tissues implies a complex relationship between biochemical evidence of lipid peroxidation and tissue damage.

In addition to lipid peroxidation biomarkers, biomarkers of ROS-induced DNA damage are being increasingly utilized. For many applications, investigation of oxidative DNA damage is particularly compelling due to mutagenic consequences; however, for cochlear applications possible mechanisms of cell death are relevant. Low levels of damage as evidenced by base changes and deletions generally can be repaired efficiently, but high levels of oxidative stress can lead to cell death (Halliwell and Aruoma, 1992).

One of the most abundant oxidative DNA adducts is 8-hydroxy-2′-deoxyguanosine (8OHdG) (Breen and Murphy, 1995; Wang et al., 1995), which has been estimated to represent approximately 5% of all oxidative adducts (Beckman and Ames, 1997). 8OHdG is formed by a range of reactive species (e.g., hydroxyl radical and singlet oxygen) as they react with various forms of 2-deoxyguanosine (2dG) (Akiyama et al., 1989). When 8OHdG is incorporated into DNA, A:T to C:G transversions can result during the next DNA replication process (Cheng et al., 1992; Maki and Sekiguchi, 1992). Due to its prevalence and relative ease of measurement, the 8OHdG adduct is a widely used marker for oxidative damage. The extent of damage is expressed as a ratio of 8OHdG adducts to normal guanosine bases (8OHdG/2dG; ng/mg).

The purpose of this investigation was to determine whether an intense noise exposure (2-h broad-band noise) resulting in permanent threshold shift (PTS) in the rat produces measurable oxidative damage to cochlear DNA; and if so, to relate the time course of that damage to the time course of lipid peroxidation and functional impairment. To accomplish this, both DNA damage and lipid peroxidation were assessed within the same animal in bilateral cochlea and two control organs (brain and liver). A secondary purpose was to explore the process of repair following oxidative damage by assessing serum and urine for biochemical byproducts of damage and repair. 8OHdG was measured via high performance liquid chromatography with electrochemical detection (HPLC-EC), and lipid peroxidation was measured via the TBARS colorimetric assay. Auditory function was assessed with ABR to evaluate auditory sensitivity and with distortion product otoacoustic emissions (DPOAE) to evaluate outer hair cell function.

2. Methods

2.1. Animals

Thirty-five male Long–Evans pigmented rats (ap-

proximately 50–55 days of age; 226–250 g upon arrival) were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were divided randomly into five groups with one normal control animal per group. The groups were defined by time of sacrifice following noise exposure (or sham noise exposure for the control animal of the group): 1 h, 3 h, 8 h, 72 h (3 days), and 672 h (28 days). All animals received pre- and post-exposure ABR and DPOAE to assess auditory function. Pre-exposure (baseline) auditory assessments were obtained on a separate day from exposures and post-exposure auditory assessments were obtained within the hour before sacrifice (Table 1). Prior to testing, rats were anesthetized with ketamine (100 mg/kg i.m.) and xylazine (4 mg/kg i.m.).

Acquisition, maintenance and experimental use of rats were approved by the Animal Care and Use Committee at the National Institute for Occupational Safety and Health (Proposal #C88 VAN), which adheres to the guidelines of the Declaration of Helsinki. Rats were housed in standard polyurethane cages or metabolic cages in a temperature-regulated room with routine 12-h light/dark conditions. They had free access to water and food at all times. Average sound level in a typical vivarium room was 65 dB SPL.

2.2. Equipment

Tucker-Davis Technologies (TDT) BioSig and SigGen auditory software (200 MHz Pentium Dell computer; Windows 95) controlled a rack of TDT modular hardware. Right and left channel test signals were generated with a DA1 module then anti-alias filtered at 35 kHz, and cascaded through two PA4 attenuators. DPOAE signals then went directly to a Radio Shack 40-1377 tweeter and a Motorola KSN1165A piezoelectric tweeter. ABR signals were routed through a Crown 5515-750W power amplifier, then to the Radio Shack tweeter. Approximately 10 cm of Etymotic insert earphone tubing coupled the tweeters' inverted horn ports to the signal ports of an Etymotic Research ER-10B microphone, which served as the delivery system for both DPOAE and ABR stimuli.

The attenuator cascade design was used to compensate for the high-frequency spectral roll-off of the tweeters and to control signal output. Attenuator settings were established by measuring the output of the tweeter/ER-10B microphone system coupled to a tygon tube simulating a rat ear canal (3.175 mm inner diameter; 2.5 mm length). A Bruel and Kjaer 4138 1/8th inch microphone closed off the open end of the tygon tube and measured the sound pressure level of the system. The first attenuator adjusted for the transfer function of the system. The second attenuator controlled signal presentation level during animal testing. For DPOAE, the first attenuator was calibrated to yield 90 dB SPL at all test frequencies when the second attenuator was set to 0 dB. Similarly for ABR, the first attenuator was set to yield 110 dB SPL at all test frequencies with the second attenuator at 0 dB.

2.3. Auditory assessments

For both ABR and DPOAE, acoustic stimuli were delivered monaurally by coupling the ER-10B microphone to the animal's right ear canal with a piece of 1/8th inch diameter tygon tubing. Prior to ABR or DPOAE testing, pure-tone in situ measurements were made for each frequency with the ER-10B microphone. Ear tip placement was adjusted to yield a maximal intensity level at 32 kHz. ABR and DPOAE protocols were automated with tester intervention possible for repetitions or termination when no response was evident.

For ABR, subdermal needle electrodes were placed at vertex (non-inverting), midline jaw region directly under vertex (inverting), and cervical neck muscles (ground). ABR tone bursts were gated with a Blackman window, had a 1-ms rise–fall time and 3-ms duration, and were alternating in polarity. Stimuli were presented at a rate of 21.1/s. Test frequencies included 2, 4, 8, 16, and 32 kHz, and stimulus intensity ranged from 110 to 0 dB SPL (10- and 5-dB step sizes). Electrophysiologic activity was filtered from 200 to 2000 Hz and amplified $\times 200\,000$. Averaging was done during a 12-ms window with a 20-kHz sample rate over 512 stimulus presentations. Alternate polarity responses were col-

Table 1
Study design

Sacrifice interval	ABR and DPOAE evaluation intervals					
	Pre-exposure (baseline)	1 h post exposure	3 h post exposure	8 h post exposure	72 h post exposure	672 h post exposure
1 h post exposure ($n=6+1$ control)	X	X				
3 h ($n=6+1$ control)	X		X			
8 h ($n=6+1$ control)	X			X		
72 h ($n=6+1$ control)	X				X	
672 h ($n=6+1$ control)	X					X
Number of animals	35	7	7	7	7	7

lected simultaneously via two-bin averaging. Threshold was defined as the lowest intensity with repeatable waveform morphology as confirmed separately by two judges.

For DPOAE, stimuli were two simultaneous, continuous pure tones (F_1 and F_2). F_1 stimulus frequencies were 2, 4, 8, 16, and 32 kHz, and intensity ranged from 70 to 20 dB SPL (5-dB step size). The ratio of F_1/F_2 was 1.2 Hz, and the intensity of $F_2 = F_1 - 15$ dB. The ear canal signal was sampled at 128 kHz for 64 ms. DPOAEs were averaged over 24 stimulus presentations and were measured at 1.333, 2.666, 5.333, 10.666, and 21.333 kHz (i.e., $2F_1 - F_2$). A positive DP response was defined as amplitude 5 dB greater than the surrounding noise floor.

2.4. Exposure

Thirty noise-exposed animals received 120 dB SPL bandpass noise (7.5–15 kHz) for 2 h in a foam-lined, semi-anechoic enclosure. The uniformly distributed noise stimulus was produced with a TDT waveform generator, then band-pass filtered by a TDT programmable filter and attenuated by a TDT PA4 attenuator. The signal was amplified by a Mackie 1202 preamplifier and a Stewart PA-1400 power amplifier, then delivered by an Altec Lansing 908-8B high-frequency driver and horn coupled to a chamber. Exposure chamber microphones were calibrated daily and noise exposure levels were monitored each hour.

Animals were noise-exposed individually in one of two chambers that ran simultaneously. Attenuation within a chamber sufficiently isolated the exposure chambers from each another. The five normal control animals (one per experimental group) received sham treatments by being placed in an exposure chamber in quiet for 2 h.

2.5. Sample collection

At the specified post-exposure intervals, six experimental and one control rat(s) per group were anesthetized to re-evaluate auditory function and then sacrificed with CO_2. Bilateral cochlea, liver, brain and serum were collected and frozen in liquid nitrogen at the time of necropsy. Cochlear preparation included separating the cochlear-vestibular apparatus from temporal bone, dissecting away neural, connective, and circulatory tissue, and removing the vestibular apparatus from cochlea. All samples were stored at −80°C.

Urine samples were collected from experimental and control animals in the 8-, 72-, and 672-h groups. The 8-h animals resided in metabolic cages directly following their noise exposure until just prior to re-evaluation and sacrifice. The 72- and 672-h animals were placed in metabolic cages 12 h prior to re-evaluation and sacrifice. Samples were centrifuged, then frozen at −80°C. Urine was not collected for the 1- and 3-h groups due to insufficient volume.

2.6. Enzyme and chemical sources

Inorganic salts used in DNA extraction were from J.T. Baker (Phillipsburg, NJ, USA). Nuclease P1 was obtained from USB Corporation (Cleveland, OH, USA). Chemicals used in the TBARS assay and Tris-(hydroxymethyl)aminomethane (Tris)-equilibrated phenol were obtained from Sigma (St. Louis, MO, USA).

2.7. DNA extraction and 8OHdG analysis

Genomic DNA was extracted from left cochlea, brain and liver using a modification of the method of Hofer and Moller (1998). Tissues were homogenized using DNase-free pellet pestles (Kontes, Vineland, NJ, USA). Cochleae (46.11 ± 6.09 mg) were placed in DNase-free microtubes and crushed with the vinyl-coated handle of a microspatula. To simplify protein removal after protein cleavage with protease, phase-lock gel tubes (Eppendorf-5-Prime, Boulder, CO, USA) were used to recover DNA in the aqueous phase after protein precipitation with phenol.

Briefly, after protease treatment, DNA containing hydrolysate was transferred to a phase-lock gel 1 light microtube, and 500 μl of Tris-equilibrated phenol was added. Contents were mixed vigorously to form a homogeneous suspension. Tubes were centrifuged at $1500 \times g$ for 5 min at room temperature to isolate the upper aqueous phase. The aqueous phase was decanted into a second phase-lock gel tube, and 500 μl of Sevag reagent was added. The tubes were mixed as before and centrifuged at $1500 \times g$ to separate the phases. The aqueous phase was decanted into a third phase-lock gel tube, and Sevag treatment was repeated. After centrifugation, the aqueous phase was decanted to a clean microtube for DNA precipitation with ethanol and sodium chloride. From this point DNA precipitation and hydrolysis to nucleotides was completed as described by Hofer and Moller (1998).

To prevent loss of sample during transit, the nucleotide hydrolysate was transferred to a screw-cap 1.5-μl microtube after ultrafiltration, and frozen at −80°C. These tubes, as well as serum and urine samples, were shipped frozen on dry ice to ESA Laboratories (Chelmsford, MA, USA) for analysis of 8OHdG by HPLC-EC analysis. Urine 8OHdG ratios were calculated against creatinine levels.

2.8. Lipid peroxidation assay

The TBARS colorimetric assay method of Ohkawa et al. (1979) was employed for detection of ROS-induced lipid peroxidation in right cochlea, brain, liver, serum, and urine. The procedure was modified to adjust for small cochlear sample weight (46.17 ± 9.39 mg). Brain and liver sample weights were 20 mg to allow for approximate comparisons across tissues. Serum and urine samples were 25 μl.

Cochleae, serum, and urine were thawed on ice, then placed in a glass tube with 200 μl of 50 mM phosphate buffer with 0.1 mM ethylenediaminetetraacetic acid (pH 7.0). Cochleae were crushed and ground with a Teflon pestle. Brain and liver samples were thawed on ice, placed in a 1.5-ml tube with 200 μl of buffer, homogenized with a pellet pestle, then transferred to a glass tube. For tissues only, 50 μl of 8.1% sodium dodecylsulfate was added. After vortexing and incubation at room temperature, 375 μl of 20% acetic acid (pH 3.5) and 375 μl of 0.6% aqueous thiobarbituric acid reagent were added. The mixture was heated and cooled before adding 1.25 ml 1-butanol/pyridine, according to the method of Ohkawa et al. (1979).

Concentration of TBARS was determined with a 1,1,3,3-tetraethoxypropane standard. Fluorescence was read at excitation and emission wavelengths of 536 and 550 nm, respectively (Yin, 1995), using a Perkin Elmer LS 50B luminescence spectrometer. Results were expressed as nmol/g tissue or μmol/l serum. Due to intra-subject variability with serum and urine, analyses were done in triplicate, and the median value used for statistical applications. Urine TBARS were expressed as μmol/g creatinine.

2.9. Statistical analyses

Auditory and chemical data were analyzed with descriptive and inferential statistics that included analysis of variance and mixed linear models. For all auditory tests, no response was defined as 2.5 dB higher than output limits of the equipment. A priori probability of significance was $P \leq 0.05$. For both biochemical assays and ABR and DPOAE tests, data from the control animal from each of the five groups were pooled together to form a 'control group'. Therefore, this group was comprised of five normal, non-exposed animals that had different life spans.

2.10. Pilot study

Prior to the stated study design, 12 rats (same age, sex, and strain as previously stated) were used in a pilot study with a noise level of 125 dB SPL (2-h exposure; 7.5–15 kHz bandpass noise). Six exposed animals were sacrificed at 24 h and compared to six control animals. The pilot study targeted the time period of interest for the full study and determined the feasibility of using the 8OHdG assay for cochlear applications.

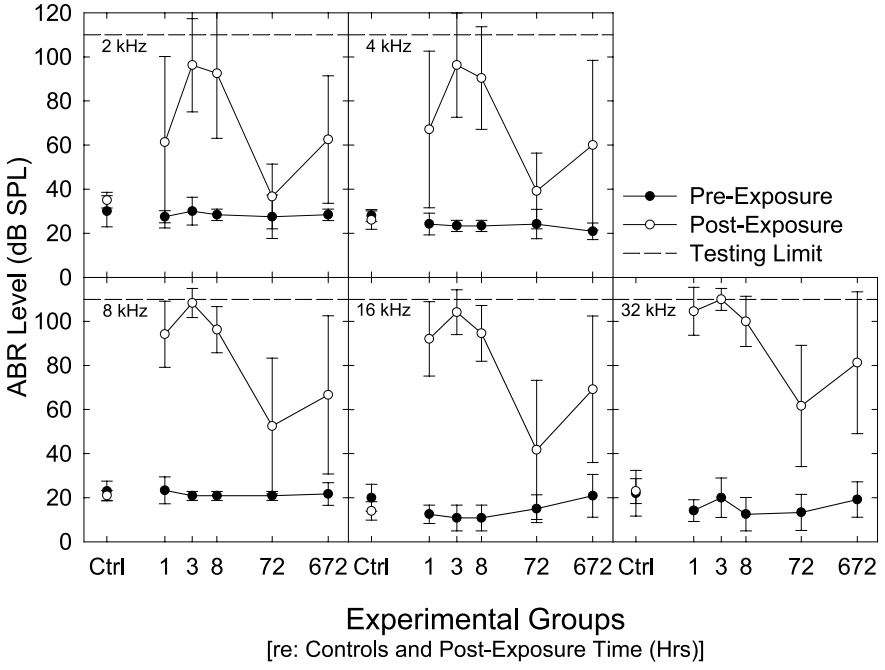

Fig. 1. Pre- and post-noise exposure ABR thresholds in dB SPL. The *x*-axis represents the group of control animals ($n = 5$) and experimental groups that were sacrificed at specified post-exposure time intervals ($n = 6$ per group). Post-exposure thresholds were measured within the hour prior to sacrifice. Time is displayed on a log scale. Values represent mean ± S.D. Maximum test signal output was 110 dB.

3. Results

3.1. Auditory function

The auditory thresholds measured by ABR and DPOAE both followed the same time course. Thresholds were elevated for the 1-h post-exposure group, remained high or progressed higher for the 3- and 8-h groups, decreased notably for the 72-h (3 days) group, and were elevated again for the 672-h (28 days) group (Figs. 1 and 2). The transient increase is consistent with a temporary threshold shift and the increase at 28 days is consistent with PTS. The elevated DPOAE thresholds confirmed outer hair cell dysfunction, as would be expected from noise-induced hearing loss.

In comparison to the pre-exposure (baseline) thresholds, all ABR and DPOAE post-exposure thresholds were significantly higher, except for the return to near-baseline thresholds at 2 and 4 kHz for the 72-h group. The 3-h group ABR thresholds were numerically the highest for all five frequencies tested, but were not statistically distinguishable from the 8-h group thresholds or from the threshold for the 1-h group, except for 2 and 4 kHz. DPOAE thresholds for the 1-, 3-, and 8-h groups were not statistically different from each other. The 72-h group had the lowest post-exposure thresholds for all frequencies with both methods. Thresholds for the 672-h group were higher than 72-h group thresholds for all frequencies, but only the ABR thresholds were significantly higher at 2, 4, 16, and 32 kHz.

The ABR pre-exposure (baseline) thresholds measured for each frequency in the control and exposed rats were not significantly different from each other. The control group post-sham-exposure ABR thresholds were not significantly different from their respective baseline thresholds for each frequency tested. The DPOAE pre-exposure (baseline) thresholds measured for each frequency in the control and exposed rats were not significantly different from each other except for the following: 3 h > 1 h at 2 kHz; 72 h > 672 h at 4 kHz; 3 h > 1, 8, 72, 672 h at 32 kHz; control > 1, 8, 72, 672 h at 32 kHz. The control group post-sham-exposure DPOAE thresholds were not significantly different from their respective baseline thresholds for each frequency tested.

3.2. 8OHdG

Oxidative DNA damage, as evidenced by the ratio of 8OHdG to 2dG (ng/mg), was present in cochlea, brain, and liver in animals exposed to noise. For cochlea, damage peaked with the 8-h group, then decreased for the 72- and 672-h groups (Fig. 3). The 8-h group mean ratio was significantly higher than both the 1-h and control groups. Cochlear ratio levels were approximately two to six times higher than brain and liver

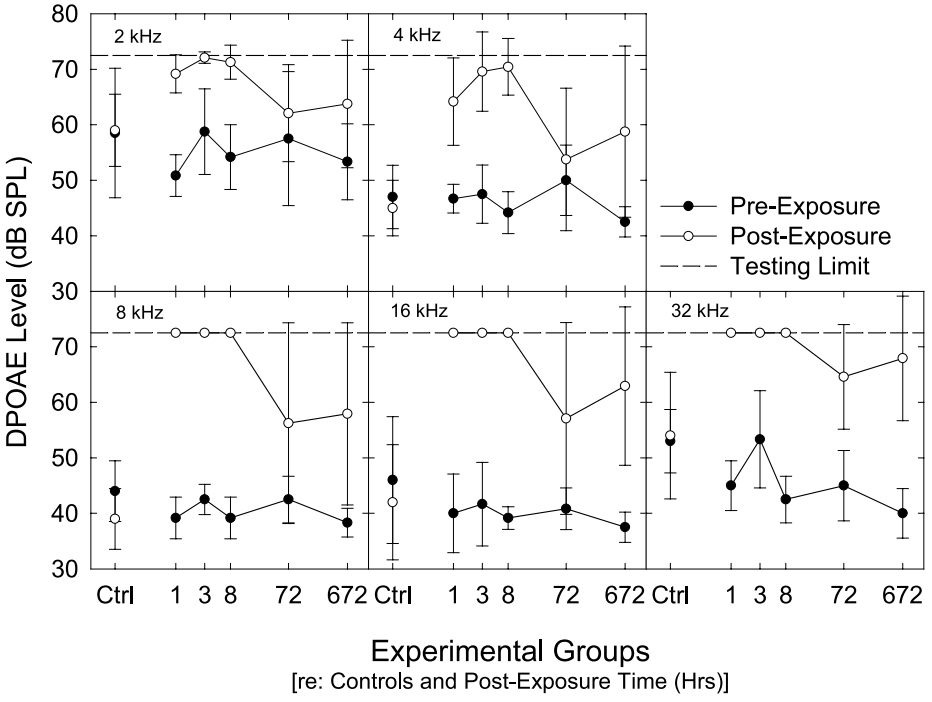

Fig. 2. Pre- and post-noise exposure DPOAE thresholds in dB SPL. The *x*-axis represents the group of control animals ($n = 5$) and experimental groups that were sacrificed at specified post-exposure time intervals ($n = 6$ per group). Post-exposure thresholds were measured within the hour prior to sacrifice. Time is displayed on log scale. Values represent mean ± S.D. Maximum test signal output was 70 dB.

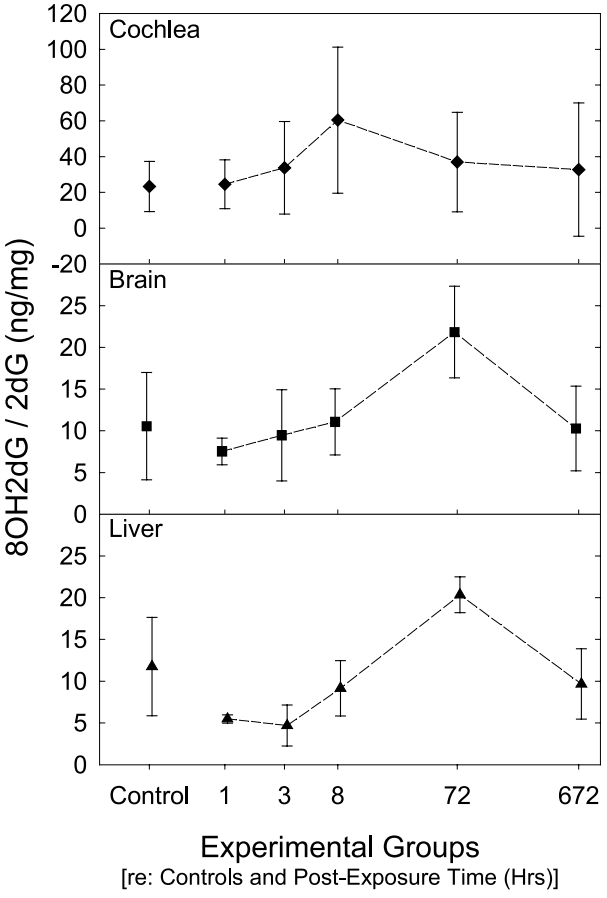

Fig. 3. Control and post-noise exposure 8OHdG ratio levels for cochlea, brain and liver. The *x*-axis represents the group of control animals ($n = 5$) and experimental groups that were sacrificed at specified post-exposure time intervals ($n = 6$ per group). Time is displayed on log scale.

levels and variability was notably higher for cochlea than for brain or liver. The variability for cochlea could reflect individual susceptibility to noise. Also possible are technical challenges of extracting DNA from the small amount of cochlear tissue, and extracting the DNA from a background of bone. The background of bone would be reflected in slight differences of cochlear weight (46.11 ± 6.09 mg). The pilot study revealed a significant increase in mean cochlear 8OHdG ratio levels for the six experimental rats (120.71 ± 54.25 ng/mg) compared with the six control rats (40.81 ± 14.09 ng/mg) 24 h after noise exposure.

For brain and liver, oxidative damage peaked with the 72-h group (Fig. 3). In both organs, the 72-h group mean ratio was significantly higher than all other groups. In liver, there were additional significant differences between groups. The 8-h group mean ratio was significantly higher than the 3-h group, and both the 672-h group and control group were significantly higher than the 1- and 3-h groups. Urine levels of 8OHdG to creatinine (μg/g) showed no significant differences between the 8-, 72-, 672-h, or control groups. The control group mean was 22.2 ± 6.8. In serum, 2dG and 8OHdG levels were below the level of detection.

3.3. TBARS

Oxidative lipid damage, as evidenced by TBARS, was present only in liver and serum. Cochlea, brain and urine showed no significant differences in TBARS levels for any groups. For liver, the only significant difference was that the control group mean (12.6 ± 1.1 nmol/g) was significantly higher than the 672-h group mean (11.0 ± 1.7). With serum, the 72-h group mean (4.6 ± 1.1) was significantly higher than 1-h (3.5 ± 0.5) and control group (3.4 ± 0.6) means. Although urine levels were not statistically significant, it is notable that they showed the same general trend as serum, with a peak for the 72-h group.

4. Discussion

These data indicate that oxidative DNA damage is present in cochlea following intense noise exposure, and that the time course of damage corresponds to the time course of functional impairment. In rats exposed to a 120-dB SPL broad-band noise for 2 h, ABR and DPOAE thresholds showed progressive deterioration in groups sacrificed at 3 and 8 h post-exposure, and 8OHdG ratios peaked with the 8-h group. Therefore, oxidative DNA damage peaked during or just after the peak auditory threshold shift implying that oxidative pathways are involved in mechanisms of noise-induced auditory dysfunction. It is not possible to adequately discern ROS contributions to temporary threshold shift (TTS) and PTS from this study, however, the peak and subsequent decline in 8OHdG ratios seem to correlate with the TTS component of the ABR and DPOAE results. Although the time course is different, Liu's (1992) blast exposure data also showed a correlation between oxidative stress (lipid peroxidation) and auditory dysfunction (ABR).

Additional evidence for noise-induced ROS DNA damage was obtained from the pilot study, which showed noise-exposed rats had a significant increase in 8OHdG ratios compared to control rats, 24 h following broad-band noise exposure. Since the pilot group had a different exposure level than the main study, and the chemistry was done separately from the large group, direct comparisons cannot be made. However, these data help confirm that cochlear oxidative DNA damage is detectable within a day of intense noise exposure.

Clerici et al. (1996) counseled against placing all liability for induced hearing loss on ROS activity since most antioxidant studies show only partial protection.

Very likely, there are multiple mechanical and biochemical cochlear activities following an ototoxic insult; however, others have demonstrated complete protection from aminoglycoside ototoxicity in the presence of antioxidants or iron chelators (see Schacht, 1999 for review). Clerici et al. (1996) did demonstrate that direct ROS-induced hearing impairment is predominantly high-frequency in nature and Ohinata et al. (2000) stated that a causal relationship between ROS activity and auditory dysfunction is implied by a tonotopic relationship between ROS lipid peroxidation and hair cell loss in the organ of Corti. In the present study, the elevated DPOAE thresholds, the time course and high-frequency nature of the auditory dysfunction, and the time course of DNA damage provide additional evidence of a relationship between outer hair cell damage and ROS activity. This evidence should be weighed with the knowledge that oxidative DNA damage can also be caused by cell damage that is non-oxidative in origin.

Over 20 specific oxidized DNA adducts have been identified to date, and of these, 8OHdG is the most prominent modification (Dizdaroglu, 1992). It has become, therefore, a widely used biomarker of DNA oxidative stress. It is also considered one of the most mutagenic oxidized adducts, associated with formation and progression of neoplasms (for review see Halliwell and Aruoma, 1991). Since neoplasms are not an issue within cochlea, there must be other implications for increased 8OHdG levels in this organ.

One possibility is that DNA oxidative stress may contribute to, or result from, biochemical dysfunction occurring in necrotic and apoptotic cell death. DNA can be an early target of damage in mammals, such that strand breakage occurs before detectable lipid peroxidation or protein damage (Schraufstatter et al., 1986). The damage may result from direct ROS activity or may be an indirect result of ROS lipid peroxidation. Excessive strand breakage can lead to a depletion of compounds necessary for strand repair and ATP synthesis, and subsequently to cell death (Szabo et al., 1996). Additionally, the noise-induced DNA damage reported here is not unprecedented. Quirk et al. (1999) presented unpublished data to support this concept. They found a 4834-bp deletion in mitochondrial DNA in rat following 40 days of daily noise exposure. Although our data represent nuclear DNA, and Quirk et al.'s mitochondrial DNA, taken together these studies provide intriguing evidence for a noise–ROS–DNA damage linkage.

The methodology for detection of 8OHdG deserves discussion. While cochlear results were statistically significant, and the trend of increasing levels at 8 h post-exposure (and 24 h for the pilot group) is notable, within groups variability was rather high. This may reflect individual susceptibility to noise, especially since the 8-h group had the highest standard deviation and the 1-h and control groups had the least. Standard deviation values for ABR and DPOAE thresholds in the noise-exposed groups also had high variability, which further supports the concept of individual noise susceptibility. Another source of 8OHdG variability could be the DNA extraction and hydrolysis from cochlea. Overall, there was not a high level of 2dG and at times the level of 8OHdG was just at or above the level of detection, possibly due to the amount of tissue available or the background of bone. It is also possible that auto-oxidation during cochlear DNA extraction and hydrolysis contributed to the higher level of 8OHdG in cochlea versus liver or brain. High ratios can also reflect the redox state of the cell, or the presence of transition metals under oxic conditions (Halliwell, 1998).

Besides DNA, cellular lipids and proteins are vulnerable to ROS activity. The byproducts of lipid peroxidation (e.g., epoxides, saturated aldehydes, unsaturated aldehydes, ketones, and hydrocarbons) can cause severe damage to cell membrane lipids and proteins (for review see Halliwell and Gutteridge, 1999). The present study did not show significant differences in lipid peroxidation aldehydes, as measured by the TBARS assay, between control and experimental animals. This was unexpected since Liu (1992) demonstrated increased TBARS following blast exposure, and Rybak and colleagues (Ford et al., 1997; Ravi et al., 1995; Rybak et al., 1995, 1997, 1999a,b), and She and Chen (1995) reported similar results for cisplatin and kanamycin, respectively. It is possible that the TBARS assay may not be optimal for all cochlear applications or that the time course utilized in the present study was not optimal for lipid peroxidation detection. Ohinata et al. (2000) employed the 8-isoprostane immunoassay in guinea pig and reported increased noise-induced lipid peroxidation that was time-dependent during exposures. They also reported that levels dropped rapidly within the first 30 min following noise cessation and returned to near baseline by 8 h post-exposure, the same time that DNA damage peaked in the present study. Taken together, these results support Halliwell and Gutteridge's (1999) comment that there is no universal 'marker' of oxidative stress. Failure to detect one variation does not preclude damage to that or other parts of the cell.

In addition to cochlea, oxidative DNA and lipid damage was investigated in brain, liver, serum and urine. Brain was selected as a control organ because of neural connections to the ear, while liver was selected due to its high metabolic function. Both organs showed a statistically significant peak in oxidative DNA damage at 72 h post-exposure. It is notable that even with this very small sample weight (20 mg), standard devia-

tions and 8OHdG ratios were much smaller for both organs than for cochlea in all groups. These differences might reflect a generally lower level of oxidative activity, but likely represent better DNA extraction/hydrolysis as the cochlear mass included bone.

The positive DNA findings for brain and liver were somewhat unexpected if interpreted as direct consequences of cochlear insult. However, it is well documented that noise is a psychophysiological stressor. Ising and Braun (2000) propose a compelling theory of noise exposure reactions. According to them, habitual noise produces sympathetic activation and a chronic increase in noradrenaline. Non-habitual noise produces a fight/flight reaction with an acute increase in adrenaline and noradrenaline. Extremely intense noise at or above the level of pain produces a defeat reaction with an increase in cortisol, an adrenal stress hormone. In the present study, animals likely had a defeat reaction due to the intensity and duration of the noise presentation. If so, the delayed (72-h) peak in oxidative stress may be time-dependent upon the rising stress hormone level. The present study's liver results agree with a previous study by Adachi et al. (1993) that showed increased levels of liver 8OHdG in rats exposed to psychological stress.

Serum and urine were analyzed for detection of DNA repair products and excretion of lipid peroxidation products. Although these circulating products represent the oxidative status of the whole body, they can serve as biomarkers of oxidative stress if elevated above normal levels, and therefore can provide additional details regarding the time course of ROS damage (Collins et al., 1996). Unfortunately, DNA repair products could not be adequately extracted from serum in our samples, and urine showed no significant differences among groups. However, lipid peroxidation products (MDA) were detected in serum and urine. Although these elevations cannot be directly correlated to cochlear damage, they did corresponded in time period with ABR and DPOAE threshold recovery, which returned to near baseline at some frequencies. It is also the time period when brain and liver 8OHdG ratios were at their highest. Neither the serum and urine data, nor the cochlear data provided a clear explanation for the rebound of ABR and DPOAE thresholds to higher levels at 4 weeks post-exposure.

In summary, the present study contributes further understanding of the time course of oxidative cochlear damage following intense noise exposure. It provides evidence that ROS DNA damage corresponds to auditory dysfunction following an acoustic insult, and that the first 8 h post-exposure may be a critical period for antioxidant treatment. The potential for prevention of noise-induced hearing loss, possibly through antioxidant treatment, is an exciting clinical concept with widespread implications. Fuller comprehension of its feasibility will depend on future biomarker studies that will assess noise exposure with finer time increments, varying doses and types of noise, and include the use of antioxidants. Additionally, more complete assessments of the cascade of cellular events, including gene activation in affected structures, will be necessary.

Acknowledgements

We thank the following people for advice, technical assistance, and professional judgement: John Clark, Rickie Davis, Richard Edwards, Larry Erway, Chuck Kardous, James Kesner, Edward Knecht, Richard Salomon, Diana Snyder, Sue Stanton, Cynthia Walker, and James Yiin. We also thank Christa Themann for serving as a judge of ABR data, Edward Krieg for statistical advice and applications, and Larry Fechter, Sandra McFadden, Leonard Rybak, and Howard Wey for reviewing an earlier version of the manuscript.

References

Adachi, S., Kawamura, K., Takemoto, K., 1993. Oxidative damage of nuclear DNA in liver of rats exposed to psychological stress. Cancer Res. 53, 4153–4155.

Akiyama, M., Maki, H., Sekiguchi, M., Horiuchi, T., 1989. A specific role of MutT protein: to prevent dG:dA mispairing in DNA replication. Proc. Natl. Acad. Sci. USA 86, 3949–3952.

Beckman, K.B., Ames, B.N., 1997. Oxidative decay of DNA. J. Biol. Chem. 272, 19633–19636.

Breen, A.P., Murphy, J.A., 1995. Reactions of oxyl radicals with DNA. Free Radical Biol. Med. 18, 1033–1077.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., Loeb, L.A., 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G6T and A6C substitutions. J. Biol. Chem. 267, 166–172.

Clerici, W.J., Yang, L., 1996. Direct effects of intraperilymphatic reactive oxygen species generation on cochlear function. Hear. Res. 101, 14–22.

Clerici, W.J., DiMartino, D.L., Prasad, M.R., 1995. Direct evidence of reactive oxygen species on cochlear outer hair cell shape in vitro. Hear. Res. 84, 30–40.

Clerici, W.J., Hensley, K., DiMartino, D.L., Butterfield, D.A., 1996. Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. Hear. Res. 98, 116–124.

Collins, A.R., Dusinska, M., Gedik, C.M., Stetina, R., 1996. Oxidative damage to DNA: Do we have a reliable biomarker? Environ. Health Perspect. 104, 465–469.

Dizdaroglu, M., 1992. Oxidative damage to DNA in mammalian chromatin. Mutat. Res. 275, 331–342.

Fechter, L.D., Liu, Y., Pearce, T.A., 1997. Cochlear protection from carbon monoxide exposure by free radical blockers in guinea pig. Toxicol. Appl. Pharmacol. 142, 47–55.

Ford, M.S., Nie, Z., Whitworth, C., Rybak, L.P., Ramkumar, V., 1997. Up-regulation of adenosine receptors in the cochlea by cisplatin. Hear. Res. 111, 143–152.

Halliwell, B., 1998. Can oxidative DNA damage be used as a bio-

marker of cancer risk in humans? Problems, resolutions and preliminary results from nutritional supplementation studies. Free Radical Res. 29, 469–486.
Halliwell, B., Aruoma, O.I., 1991. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. 281, 9–19.
Halliwell, B., Aruoma, O.I., 1992. DNA damage by oxygen-derived species: Its mechanism, and measurement using chromatographic methods. In: Scandalios, J.G. (Ed.), Molecular Biology of Free Radical Scavenging Systems. Cold Spring Harbor Laboratory Press, Plainview, NY.
Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 246–350.
Hofer, T., Moller, L., 1998. Reduction of oxidation during the preparation of DNA and analysis of 8-hydroxy-2′-deoxyguanosine. Chem. Res. Toxicol. 11, 882–887.
Ising, H., Braun, C., 2000. Acute and chronic endocrine effects of noise: Review of the research conducted at the Institute for Water, Soil and Air Hygiene. Noise Health 7, 7–24.
Jacono, A.A., Bohua, H., Kopke, R.D., Henderson, D., Van De Water, T.R., Steinman, H.M., 1998. Changes in cochlear antioxidant enzyme activity after sound conditioning and noise exposure in the chinchilla. Hear. Res. 117, 31–38.
Janero, D.R., 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radical Biol. Med. 9, 515–540.
Kamimura, T., Whitworth, C.A., Rybak, L.P., 1999. Effect of 4-methylthiobenzoic acid on cisplatin-induced ototoxicity in the rat. Hear. Res. 131, 117–127.
Liu, Z., 1992. Experimental study on the mechanism of free radical in blast trauma induced hearing loss. Chung Hua Erh Pi Yen Hou Ko Tsa Chih (Chin. J. Otolaryngol.) 27, 24–26.
Lopez-Gonzalez, M.A., Lucas, M., Delgado, F., Diaz, P., 1998. The production of free oxygen radicals and nitric oxide in the rat cochlea. Neurochem. Int. 33, 55–59.
Lopez-Gonzalez, M.A., Delgado, F., Lucas, M., 1999. Aminoglycosides activate oxygen metabolites production in the cochlea of mature and developing rats. Hear. Res. 136, 165–168.
Maki, H., Sekiguchi, M., 1992. MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature 355, 273–275.
Morrow, J.D., Hill, K.E., Burk, R.F., Nammour, T.M., Badr, K.F., Roberts, L.J., II, 1990. A series of prostaglandin F_2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc. Natl. Acad. Sci. USA 87, 9383–9387.
Ohinata, Y., Miller, J., Altschuler, R., Schacht, J., 2000. Intense noise induces formation of vasoactive lipid peroxidation products in the cochlea. Brain Res. 878, 163–173.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Ohlemiller, K.K., Dugan, L.L., 1999. Elevation of reactive oxygen species following ischemia-repurfusion in mouse cochlea observed in vivo. Audiol. Neurootol. 4, 219–228.
Ohlemiller, K.K., McFadden, S.L., Ding, D.L., Flood, D.G., Reaume, A.G., Hoffman, E.K., Scott, R.W., Wright, J.S., Putcha, G.V., Salvi, R.J., 1999a. Targeted deletion of the cystolic Cu/Zn-superoxide dismutase gene (Sod1) increases susceptibility to noise-induced hearing loss. Audiol. Neurootol. 4, 237–246.
Ohlemiller, K.K., Wright, J.S., Dugan, L.L., 1999b. Early elevation of cochlear reactive oxygen species following noise exposure. Audiol. Neurootol. 4, 229–236.
Quirk, W.S., Shivapuja, B.G., Schwimmer, C.L., Seidman, M.D., 1994. Lipid peroxidation inhibitor attenuates noise-induced temporary threshold shifts. Hear. Res. 74, 217–220.
Quirk, W.S., Komjathy, D.A., Malamud, R.Y., Bai, U., Seidman, M.D., 1999. Noise-induced changes in auditory thresholds and mitochondrial DNA. Poster presented at the Association for Research in Otolaryngology Midwinter Meeting, St. Pete Beach, FL.
Rao, D., Fechter, L.D., 2000. Protective effects of phenyl-*N*-*tert*-butylnitrone on the potentiation of noise-induced hearing loss by carbon monoxide. Toxicol. Appl. Pharmacol. 167, 125–131.
Ravi, R., Somani, S.M., Rybak, L.P., 1995. Mechanism of cisplatin ototoxicity: antioxidant system. Pharmacol. Toxicol. 76, 386–394.
Rybak, L.P., Ravi, R., Somani, S.M., 1995. Mechanism of protection by diethyldithiocarbamate against cisplatin ototoxicity: antioxidant system. Fund. Appl. Toxicol. 26, 293–300.
Rybak, L.P., Husain, K., Evenson, L., Morris, C., Whitworth, C., Somani, S., 1997. Protection by 4-methylthiobenzoic acid against cisplatin-induced ototoxicity: Antioxidant system. Pharmacol. Toxicol. 81, 173–179.
Rybak, L.P., Husain, K., Whitworth, C., Somani, S., 1999a. Dose dependent protection by lipoic acid against cisplatin-induced ototoxicity in rats: Antioxidant defense system. Toxicol. Sci. 47, 195–202.
Rybak, L.P., Whitworth, C., Somani, S., 1999b. Application of antioxidants and other agents to prevent cisplatin ototoxicity. Laryngoscope 109, 1740–1744.
Schacht, J., 1999. Antioxidant therapy attenuates aminoglycoside-induced hearing loss. Ann. NY Acad. Sci. 884, 125–130.
Schraufstatter, I.U., Hinshaw, D.B., Hyslop, P.A., Spragg, R.G., Cochrane, C.G., 1986. Oxidant injury of cells. DNA strand-breaks activate polyadenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. J. Clin. Invest. 77, 1312–1320.
Seidman, M.D., Shivapuja, B.G., Quirk, W.S., 1993. The protective effects of allopurinol and superoxide dismutase on noise-induced cochlear damage. Otolaryngol. Head Neck Surg. 109, 1052–1056.
She, W.D., Chen, Z.H., 1995. Effect of ligustrazine on kanamycin ototoxicity. Chung Kuo Chung Hsi I Chieh Ho Tsa Chih 15, 609–611.
Szabo, C., Zingarelli, B., O'Connor, M., Salzman, A.L., 1996. DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. Proc. Natl. Acad. Sci. USA 93, 1753–1758.
Wang, Y.J., Ho, Y.S., Lo, M.J., Lin, J.K., 1995. Oxidation modification of DNA bases in rat liver and lung during chemical carcinogenesis and aging. Chem. Biol. Interact. 94, 135–145.
Yamane, H., Nakai, Y., Takayama, M., Iguchi, H., Nakagawa, T., Kojima, A., 1995. Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. Eur. Arch. Otorhinolaryngol. 252, 504–508.
Yamasoba, T., Nuttall, A.L., Harris, C., Raphael, Y., Miller, J.M., 1998. Role of glutathione in protection against noise-induced hearing loss. Brain Res. 16, 82–90.
Yamasoba, T., Schacht, J., Shoji, F., Miller, J.M., 1999. Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. Brain Res. 815, 317–325.
Yin, D., 1995. Appropriate excitation/emission wavelengths for fluorometric determination of thiobarbituric acid-reactive substances. Clin. Chem. 41, 329–330.