Reduced Phosphorylation of Histone Variant H2Ax in the Organ of Corti is Associated with Otoprotection from Noise Injury

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Abstract

Research on the molecular bases of noise induced hearing loss has revealed that noise exposure produces multiple independent and complementary biochemical cascades that could damage DNA. The phosphorylation of Ser139 of histone variant H2Ax (γ-H2Ax) occurs within one minute following DNA damage and spans two million DNA bases on either side of the damage. In the current study we investigated whether noise exposure could induce γ-H2Ax within the organ of Corti. Cumulative signal strength was employed to quantify the absolute level of γ-H2Ax in mathematical energy units. The results indicated that noise exposure could increase the level of γ-H2Ax in the organ of Corti. Furthermore, treatment with a DNA repair enhancing chemotype (carboxy alkyl ester) reduced the noise induced increase of γ-H2Ax which was associated with an accelerated rate of functional recovery from the noise exposure. The combined results implicate molecular mechanisms of DNA damage and repair in the pathophysiology of noise induced hearing loss.

Keywords: DNA damage and repair; Noise induced hearing loss; Carboxy alkyl esters

Introduction

Research on the molecular bases of Noise Induced Hearing Loss (NIHL) has revealed that noise exposure produces multiple independent and complementary biochemical cascades that may damage DNA [1-5]. For instance, chinchillas exposed to impulse noise revealed DNA strand breaks within five minutes following the exposure as detected by the TUNEL assay [2]. Furthermore, electrochemical high-performance liquid-chromatography revealed the presence of the 8-hydroxy-2’-deoxyguanosine DNA base adduct in the rat cochlea within eight hours post-noise exposure [6]. These DNA damage products are particularly toxic since they inhibit gene expression by stalling the procession of the RNA polymerase-II holocomplex during transcription [7,8]. Furthermore, the inhibition of gene expression or the manufacturing of mutated gene products would affect normal cell and tissue functions. One line of research has demonstrated that [Ca]1+ rapidly accumulates in the hair cells and supports cell following over-stimulation through an ATP regulated pathway that involves the release of [Ca]2+ reservoirs from mitochondria into the cytosol [9,10]. This is important because high cytosolic levels of [Ca]2+ may stimulate mitochondria to release enzymes that induce DNA strand breaks [11]. Indeed, previous demonstrations have shown that noise exposure stimulate mitochondria to release endonuclease-G and the apoptosis inducing factor [12,5]. Each of these enzymes can translocate to the nucleus and independently induce DNA strand breaks. Furthermore, noise stimulate both the intrinsic and extrinsic-caspase mediated cell death pathways and each pathway deploys the DNA fragmentation factor enzyme to precipitate DNA strand breaks [13,4].

Among the various types of DNA damage products, DNA double-strand breaks (DSB) are considered the most lethal because they threaten the stability of whole chromosomes [14]. Therefore, their appearance in a cell triggers a global response that involves the activation of at least three kinases, Ataxia Telangiectasia Mutated (ATM), DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia/RAD3 related (ATR), that phosphorylate Ser-139 of histone variant H2Ax (γ-H2Ax) at the site of the DSB [15]. Hundreds of γ-H2Ax may precipitate around the DSB within one minute [16]. For instance, in human cells γ-H2Ax can be detected as far as ~2 megabases on either side of the break [17]. Such amplification of a single DSB makes it possible to detect individual cells suffering episodes of DSBs. The specificity of γ-H2Ax for DSBs is an established phenomenon in DNA damage research and immunolabeling of γ-H2Ax in tissue sections is a standard approach for indentifying cells or tissues that are experiencing DSB [18,19]. Immunolabeling for γ-H2Ax is also used in the clinic as a biodosimeter of DSBs following exposure to radiotherapy, X-ray examinations or computed tomography (CT) scans [20]. Furthermore, it has been demonstrated that γ-H2Ax immunolabeling is the most sensitive method for detecting a DSB [21]. Since DSBs and other types of DNA damage products threaten the stability of the genome, a considerable amount of research has focused on identifying biomedical approaches to increase the repair of such lesions.

Small molecular weight compounds called Carboxy Alkyl Esters (CAEs) have been shown to maintain genomic stability under toxic conditions and have being standardized to increase DNA repair capacity when administered to humans and rodents [22-31]. In a randomized age and gender matched human study, two groups of subjects were treated with CAE (250 and 350 mg) for 8 consecutive weeks [30]. A third group of subjects served as control by not receiving CAEs. Blood cells were removed from the subjects in each group and exposed to hydrogen peroxide to induce free radical DNA damage. There was a statistically significant increase in enzymatic DNA repair activity as evidenced by higher levels of repaired DNA single strand breaks in the groups that received CAE treatment compared to the control group.

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Additionally, CAE had no detectable toxicity in humans. Further research on humans have confirmed these results, by demonstrating that CAE augments DNA repair capacity which then improves health outcomes such as immune function, anti-inflammation, osteoarthritis relief and positive psychotropic effects [32,26,29]. Animal experiments have also confirmed that CAE treatment augments DNA repair capacity. For instance, female W/Fu rats treated with CAE (up to 160 mg/kg) for 28 days by gastric gavage then exposed to 12 Gy whole body irradiation to induce wide-spread genomic damage, showed significantly (p < 0.05) improved DNA repair capacity relative to control rats who were not treated with CAE [29]. Several other studies in rats and mice have confirmed these results by showing that CAE augments DNA repair capacity which then increases whole-body defenses such as leukocyte recovery from DNA damage, improved mitogenic responses, improved proliferation of lymphocytes, improved life-span of cells and enhanced immune function [29,22]. A recent study has revealed that CAE treatment augments the recovery of auditory sensorineural functions following noise injury which suggest the possibility of reduced DNA damage in the cochlea with CAE treatment [33].

In the current study, we investigated whether or not noise exposure could increase immunolabeling of γ-H2Ax in the organ of Corti and whether CAE treatment would counteract the effect of the noise. We were particularly interested in an early time point (1 day) after the noise exposure because permanent cochlear impairment might be predicated on early genomic stress [2,6]. Therefore, we monitored cochlear function out to 4 weeks post-noise exposure to determine whether there was an association between events observed at 1 day post-exposure and the severity of permanent cochlear impairment.

Materials and Methods

Animals and experimental design

Experiments were conducted on male Long-Evans rats (250-300g) that were acquired from Harlan Laboratories, Inc. (Livermore, CA. USA). All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Loma Linda Veteran’s Affairs Hospital. A total of 48 animals were used in the experiments. After arriving at the vivarium, the animals were allowed to acclimate for one week. Baseline Distortion Product Otoacoustic Emissions (DPOAE) was then collected on each animal to verify cochlear function. The animals in the CAE and CAE+noise groups were administered 160 mg/kg of CAE by gastric gavage for 28 consecutive days [28,33]. The control group was also treated via gastric gavage with distilled water (dissolving agent for gavage) for 28 consecutive days [28,33]. The animals in the CAE and CAE+noise groups were administered 160 mg/kg of CAE by gastric gavage then exposed to 12 Gy whole body irradiation then 4 weeks of post-noise exposure. For instance, female W/Fu rats treated with CAE (up to 160 mg/kg) for 28 days by gastric gavage then exposed to 12 Gy whole body irradiation then 4 weeks of post-noise exposure showed significant improvement in DNA repair activity was prepared by Optigenex Inc (Hoboken, NJ, USA) as reported previously [31,33]. The animals in the CAE and CAE+noise groups were administered 160 mg/kg of CAE by gastric gavage for 28 consecutive days [28,33]. The control group was also treated via gastric gavage with distilled water (dissolving agent for CAE) for 28 consecutive days. The noise group did not receive any treatment beyond being exposed to the noise dose (see below). Noise exposure of the noise and CAE+noise groups occurred on the 29th day (one day after the 28 days of water or CAE treatment). Then post-exposure DPOAE measurements and tissue harvesting were conducted from each group on the 30th day (1-day after the noise exposure). Tissues were harvested to evaluate whether or not γ-H2Ax expression increased in the organ of Corti after noise exposure and whether or not CAE treatment could reduce the expression of γ-H2Ax in the organ of Corti at an early time point (1-day post trauma) after the exposure. To evaluate whether a putative effect on γ-H2Ax expression is associated with long-term recovery of cochlear function, some of the animals were allowed to survive for 4 weeks post-noise exposure and cochlear function was evaluated again with DPOAE. Table 1 describes the different animal groups, their treatment regimen and the experimental design.

Distortion Product Otoacoustic Emissions (DPOAE)

The cubic 2f1 - f2, DPOAE is particularly sensitive to cochlear noise damage. Therefore DPOAEs (2f1 - f2 level as a function of increasing stimulus frequency, commonly referred to as a DP-gram) were recorded as described previously [34]. Briefly, each animal was anesthetized with ketamine/xylazine (44/7 mg/kg, im.) while normal body temperature was maintained using a direct current (dc) heating unit built into the surgical table.

The cubic 2f1 - f2, DPOAE was recorded with two primaries, f1 and f2, : where f2 is higher than f1 at an f/f2 ratio of 1.25. The primaries (L) were presented in 0.1-octave increments from 3.2 to 63 kHz. The levels of the primaries were set to L - 10 = L1 or as indicated in the figures. The frequency and level ratios of the primaries were selected to maximize the 2f1 - f2, SPL recorded from the ear canal [35-37]. A customized signal presentation, acquisition and analysis program written in LabVIEW version 7.1 (National Instruments, Austin, TX, USA) was used to drive a PCI-4461 computer-based DSP board (National Instruments, Austin, TX, USA) for generation of the primaries and Fourier analysis of the response.

Noise exposure

Both the noise-only group and the CAE+noise group were exposed in the same noise exposure chamber at the same time. The noise exposure paradigm has been described previously [33]. Briefly, the animals were exposed to an octave band of noise (OBN) centered at 8 kHz. The intensity of the noise was 105 dB SPL and the duration was 4 hours. The animals were awake during the exposure and were free to move around in a wire-cloth enclosure within a 40 L noise chamber. The noise was generated by a Function Generator (Sanford Research System, Menlo Park, CA, USA) coupled to a Frequency Device (Frequency Device Inc., Haverhill, MA, USA). Vifa D25AG-05 speakers (Vifa International A/S, Videbaek, Denmark) located approximately 5 cm above the animals’ wire-cloth enclosure was used to present the noise. The frequency spectrum of the noise was verified in

<table>
<thead>
<tr>
<th>Groups</th>
<th>Baseline DPOAE</th>
<th>CAE treatment</th>
<th>Noise</th>
<th>DPOAE+ Sacrifice</th>
<th>DPOAE recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(N = 14)</td>
<td>water</td>
<td></td>
<td></td>
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<td>(N = 13)</td>
<td>water</td>
<td>noise</td>
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<td>N = 9</td>
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<tr>
<td>CAE</td>
<td>(N = 10)</td>
<td>CAE</td>
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<td>N = 8</td>
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<tr>
<td>Noise+CAE</td>
<td>(N = 11)</td>
<td>CAE</td>
<td>noise</td>
<td></td>
<td>N = 6</td>
</tr>
</tbody>
</table>

Abbreviations: CAE, Carboxy alkyl esters; DPOAE, distortion product otoacoustic emissions

Table 1: Experimental design.
the noise chamber containing the rats with a sound level meter (Quest Electronics, Oconomowoc, WI, USA) close to the animals’ pinnae.

**Immunolabeling**

**Animal and tissue preparation:** Immunolabeling of γ-H2Ax within cells in tissue sections is a standard method of detecting DSB [18-20]. Therefore, we employed γ-H2Ax immunolabeling and quantification to determine differences between the groups. Twenty anesthetized animals (control group = 5, noise group = 5, CAE group = 5 and CAE+noise group = 5) were sacrificed by transcardial perfusion with phosphate-buffered saline (PBS; 10 mM, pH 7.4) followed by periodate-lysine-paraformaldehyde fixative [38]. The heads were then removed, skinned and post-fixed in 4% paraformaldehyde overnight at 22°C. Formic acid (10%) was used for chemical decalcification of the heads as described previously [34]. Then the heads were paraffin embedded and sectioned at 5µm in the midmodiolar plane. The sections were incubated in a heated water bath and mounted on subbed slides for immunolabeling procedure. Kidney tissues were simultaneously harvested, postfixed, paraffin embedded, sectioned and mounted on subbed slides.

**Immunoperoxidase procedure**

Tissue sections were de-paraffinized in xylene, hydrated in graded ethanol alcohol and water then incubated in 0.9 % H₂O₂ for 10 minutes. They were then heated for 20 minutes at 90-98°C in a low pH (0.80-3.06) sodium citrate-citric acid buffer (antigen retrieval) and then rinsed thoroughly with PBS. Afterwards, the sections were pre-treated with a blocking solution of normal goat serum, 10% Triton X-100 and 2% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in PBS for 1 hour. The primary antibody was diluted in the blocking solution at a 1:100 concentration. The primary antibody (anti-γ-H2Ax, Ser139) for 1 hour. The primary antibody was diluted in the blocking solution with a blocking solution of normal goat serum, 10% Triton X-100 and 2% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in PBS for 1 hour. The primary antibody was diluted in the blocking solution at a 1:100 concentration. The primary antibody (anti-γ-H2Ax, Ser139) is commercially available (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The specificity of the antibody has been confirmed previously [39-41]. Nevertheless, negative control experiments were conducted such that sections were incubated with blocking serum without the primary antibody [42]. Such negative control sections were processed at the same time as the experimental sections and received simultaneous and identical treatments. Furthermore, positive control experiments were conducted by immunolabeling tissue that is known to exhibit constitutive expression of γ-H2Ax [43] (Figure 1). After incubating the sections with the primary antibody (or blocking serum for negative control sections) for 48 hours at 4°C, the sections were rinsed with PBS. They were then treated with a biotinylated anti-rabbit secondary antibody (Vector Laboratories, Temecula, CA, USA) diluted 1:100 in PBS + 2% BSA for 24 hours at 4°C. The sections were then rinsed in PBS, incubated with preformed avidin-biotin-peroxidase enzyme complexes (Vectastain ABC reagent; Vector Laboratories, Inc., Burlingame, CA, USA) for one hour, rinsed again with PBS and then treated with a solution of Trizma pre-set crystals (1.58 g; Sigma-Aldrich, St. Louis, MO, USA) to stabilize peroxidase enzyme reactions. The peroxidase enzyme complexes were then used to oxidize 3,3’-diaminobenzidine tetrahydrochloride to produce a brown chromogen.

**Quantitative morphometry**

**Equipment:** A Leica DM2500 upright microscope (Leica Microsystems Inc., Bannockburn, IL, USA) was used for brightfield microscopy. A Progres® CF® digital camera (JENOPTIK Laser, Jena, Germany) mounted on the Leica DM2500 was used for digital image capturing. Image-Pro® plus version 6.3 (Media Cybernetics Inc., Bethesda, MD, USA) for Windows™ was used to control image capturing, pixel thresholding and bitmap analyses. A Dell Optiplex GX620 with an Intel Core2 processor was used for software operations.

**Photomicroscopy:** The microscope current drain, light source and temperature were standardized to ensure accurate and consistent reading of each tissue section. The intensity of the light to a 1.4 megapixel charged-coupled device color sensor was monitored over an 8 hour period by capturing a blank field (cover slip, mounting media and glass slide). The light intensity fluctuated by only 0.1% (a modest difference in mean gray value of 0.2748) within the first hour of being turned on then stabilized when the lamp housing reached a temperature of 62 ± 1°C. All photomicrographs were taken when the microscope light intensity stabilized. To further ensure consistent spatial and temporal illumination for each tissue section during microscopy, the mean gray value (g) for blank fields adjacent to each tissue section was maintained at ≥ 254. In addition to providing consistent illumination, this high level illumination had the added advantage of masking background staining which was significantly lighter (light brown stain) than the antibody staining which exhibited a heavy brown stain and thus unmasked by the illumination. Photomicrographs of the organ of Corti were taken with an N-PLAN 40x/0.65 objective lens. These photomicrographs (680 x 512 pixels) were saved in uncompressed tagged-image file format for later retrieval. They were then converted from 48 bits/pixel to 24 bits for subsequent analyses. To further remove background staining from each photomicrograph a predetermined threshold criterion was applied via Image-Pro’s threshold algorithm. This threshold criterion was empirically determined from pilot experiments. For instance, the acellular tectorial membrane of Corti’s organ is sometimes stained after the immunolabeling procedure. This is due to the trapping of the secondary antibody within the microfibrillar matrix of the tectorial membrane and the failure of wash steps to completely dislodge the secondary antibody. The thresholding criteria employed in this study was trained on masking background levels of staining such as the level of background found in the tectorial membrane. Therefore, applying the threshold criteria increased the signal-to-noise ratio. After thresholding, an area of interest (AOI) field was selected within each organ of Corti. Bitmap readings were then taken within the AOI. The bitmaps record the positional matrix (n, x n) and brightness (b) of each primary colored (red, green and blue or RGB) pixel (n, x n, x 3). These bitmap readings were then used in the determination of absolute chromogen levels by the cumulative signal strength technique.

**Cumulative signal strength:** Cumulative signal strength is an objective technique that quantifies the absolute amount of immunolabeling (staining intensity) in photomicrographs [43,44]. This is achieved by measuring the mathematical energy (E) within each n, x n, x 3 pixel of an N, x N, x 3 photomicrograph. The computation is as follows [46].
\[
E = \frac{1}{N_1N_2} \sum_{n_1=1}^{N_1} \sum_{n_2=1}^{N_2} \left[I_g(n_1, n_2)\right]^2
\]
\[
+ \frac{1}{N_1N_2} \sum_{n_1=1}^{N_1} \sum_{n_2=1}^{N_2} \left[I_f(n_1, n_2)\right]^2
\]
\[
+ \frac{1}{N_1N_2} \sum_{n_1=1}^{N_1} \sum_{n_2=1}^{N_2} \left[I_f(n_1, n_2)\right]^2
\]

where \(I(n_1, n_2)\) is the energy from a particular hue (i = R, G or B) at position \(n_1\) x \(n_2\) in the AOI frame. In photomicrographs from bright field microscopy \(I(n_1, n_2) = 255 - b(n_2, n_2)\), where \(b\) is the brightness value or gray value. The \(E\) that reflects specific staining of the γ-H2Ax antibody \(E_{\text{H2Ax}}\) is the absolute value of the difference between antibody induced immunolabeling \(E_{\text{antibody}[\gamma-H2Ax]}\) and that induced by the absence of the antibody \(E_{\text{antibody}[\gamma-H2Ax]}\) also known as the negative control; \(E_{\gamma-H2Ax} = E_{\text{antibody}[\gamma-H2Ax]} - E_{\text{antibody}[\gamma-H2Ax]}\)

\[\text{(2)}\]

Statistical analyses

Statistical analyses were conducted with Prism 5, version 5.03 (GraphPad Software, Inc., La Jolla, CA, USA). The DPOAE data was analyzed for group effects such that analysis of variance (ANOVA) testing was conducted on 2f1 - f2 DPOAE levels to determine significant differences between the groups. Post-hoc testing employed Dunnett’s paired comparison analyses. The intensity of γ-H2Ax immunolabeling was recorded from midmodiolar cochlear sections. For each animal, duplicate readings were recorded and 10 cochlear sections were used for each group (a total of 40 sections). The apical, middle and basal cochlear coils were evident in the sections therefore, 120 (40 x 3) cochlear coils were studied. Statistical differences between groups were determined with ANOVA and Bonferroni’s multiple comparison testing.

Results

γ-H2Ax labeling

It is known that the kidney exhibit consistent immunolabeling of γ-H2Ax which is believed to represent DSB that form in noncoding regions of the genome called gene deserts [43,47]. Therefore, kidney sections (Figure 1) served as positive and negative controls for the immunolabeling experiments. Figure 1A reveals γ-H2Ax labeling in podocytes of the renal corpuscle (positive control). This labeling was abolished when the antibody is omitted from the immunolabeling procedure (Figure 1B, negative control). Immunolabeling for γ-H2Ax was also detected within the organ of Corti. The labeling was present under normal (control) conditions and after noise or CAE treatment. Figure 2A is a representative example of the staining. Several cochlear structures are labeled but labeling in the organ of Corti is particularly prominent. Panels B and C from Figure 2 demonstrate that γ-H2Ax labeling could be detected within hair cells and supporting cells with and without noise exposure. This persistent expression of γ-H2Ax is consistent with previous research that has shown persistent expression of DNA repair proteins in the organ of Corti [42,48,49].

γ-H2Ax levels

The prominence of γ-H2Ax labeling in the organ of Corti made it difficult to make subjective judgments on the level of expression between the groups. Therefore, to determine differences in staining intensity, the level of γ-H2Ax in the organ of Corti was quantified by determining the absolute amount of chromogen per pixel by employing the cumulative signal strength technique [44-46]. This technique measures the signal energy as a function of pixel, where energy is defined in the mathematical sense. Therefore, the values are unit less and thus reported as mathematical energy units per pixel \((E_{\gamma-H2Ax}/\text{pix})\). Figure 3A reveals that γ-H2Ax level in the control group was similar to that in the CAE group. However, noise exposure induced an increase in γ-H2Ax. This increase was also observed after co-treatment with CAE and noise. Note that γ-H2Ax level was lower in the CAE+noise treated group relative to that in the noise-only group. Figure 3B reveals γ-H2Ax levels for each cochlear turn (apical, middle and basal coils) as a function of the treatment groups. In all cochlear turns, γ-H2Ax levels were similar between the control and CAE groups. However, γ-H2Ax levels were highest among the noise and CAE+noise groups. In the middle and basal turn γ-H2Ax levels were higher in the noise group compared to the CAE+noise group. Therefore, inspection of individual cochlear turns further supported the notion that CAE treatment reduced noise induced levels of γ-H2Ax.

Statistical analyses were conducted on γ-H2Ax level measurements.
A one-way ANOVA where treatment condition served as a between subjects factor revealed that there was a statistically significant difference in γ-H2Ax levels between the four groups ($F_{(3,12)} = 30.33, p < 0.01$). Bonferroni pair wise contrast revealed no statistically significant differences between the control and CAE groups ($p > 0.05$). But these groups exhibited significantly lower γ-H2Ax levels compared to the noise exposed group ($p < 0.01$). Furthermore, the noise exposed group exhibited significantly higher γ-H2Ax levels compared to the CAE+noise group ($p < 0.05$). These statistical calculations suggest that CAE treatment reduced noise induced γ-H2Ax within the organ of Corti. This conclusion was further supported by statistical calculations on γ-H2Ax level measurements from individual cochlear turns. For instance, the control and CAE group showed no significant difference ($p > 0.05$) in γ-H2Ax levels regardless of cochlear turn (apical, middle or basal). However, the noise group and the CAE+noise group were significantly ($p < 0.05$) different at the basal and middle turns. In these cochlear turns, γ-H2Ax was significantly ($p < 0.05$) lower within the CAE+noise group. The pooled results indicate that CAE treatment reduced noise induced γ-H2Ax levels in the basal and middle cochlear turns. This is significant because basalward turns are known to be more vulnerable to cell death and the loss of cochlear function after exposure to the same noise dose used in the current study [33].

**Protection from noise injury**

To evaluate protection from noise injury, DP-grams of $2f_1 - f_2$ were recorded. These recordings were obtained at baseline and 1 day following noise exposure. Figure 4 shows the response of $2f_1 - f_2$, DPOAE as a function of $f_1$, frequency driven with primary levels ($L_p$) at 65/55 dB SPL. Baseline recordings revealed that all groups had large $2f_1 - f_2$ levels that exceeded the noise floor by at least 6 dB. At 1 day post-noise exposure, the control groups (vehicle-control and CAE treated groups) showed equivalent $2f_1 - f_2$ levels (two-way ANOVA: $F_{(1,42)} = 2.09, p > 0.05$). However, there was a significant difference (two-way ANOVA: $F_{(1,42)} = 56.37, p < 0.01$) between the noise treated groups where the CAE+noise group exhibited higher (better) levels than the noise-only group. Furthermore, the highest frequency components (≥ 32 kHz) of the noise-only group was suppressed into the noise floor (average = 1.27 dB above noise floor) while that of the CAE+noise remained above the noise floor (average = 7.31 dB above noise floor). These findings suggest that $2f_1 - f_2$, DPOAE levels from the CAE+noise group was better preserved than that of the noise group as early as 1 day post-noise exposure.

**Recovery from noise injury**

Recordings of the $2f_1 - f_2$, DPOAE were conducted out to 4 weeks post-noise exposure to evaluate functional recovery from the noise exposure. The stimulus primary levels were set at 55/35 ($L_p$) which increases the sensitivity of the $2f_1 - f_2$, recordings [50]. Figure 5 illustrates the change in $2f_1 - f_2$, levels for three time points (1-day, 1-week and 4-weeks post noise exposure). In the control group, $2f_1 - f_2$, levels were robust and exhibited modest variations between time points. However, in the noise exposed group there was wide-spread loss of $2f_1 - f_2$, levels at 1 day post exposure. For instance, the $2f_1 - f_2$, DPOAE levels were reduced to approximate the noise floor over the entire $f_2$ frequency range. At 1 week post-noise exposure there was some recovery in $2f_1 - f_2$, levels but the frequency range between ~8-24 kHz showed prominent loss. At 4 weeks post-noise exposure there was still a prominent loss in the ~8-24 kHz range. These results indicate that the noise exposure resulted in permanent loss 1½ -octave above the center frequency (8 kHz) of the OBN. Indeed, permanent cochlear loss in Long-Evans rats exposed to an 8 kHz OBN at 105 dB SPL for 4 hours typically occurs at 4 weeks post-exposure [51, 52].

The capacity of the CAE+noise group to recover from noise injury is shown in Figure 5. At 1 day post-noise exposure the CAE+noise group showed loss of $2f_1 - f_2$, levels. For instance, frequencies below ~16 kHz were suppressed in to the noise floor. However, high frequencies between ~16-32 kHz were not reduced into the noise floor which is in contrast with the noise-only treatment where the entire frequency spectrum was suppressed into the noise floor. These data support that of Figure 4 in suggesting that CAE may provide early protection against noise injury. Indeed, at only 1 week post-noise exposure the CAE+noise group showed recovery while the noise-only group still exhibited a prominent loss. One week following noise exposure
is considered an early time point because functional recovery is still occurring. Interestingly, the CAE+noise group showed almost complete recovery of 2f<sub>1</sub>-f<sub>2</sub> levels at 4 weeks post-noise exposure. This indicates that the CAE treated group was able to recover from the noise exposure. Statistical analyses were conducted on the 2f<sub>1</sub>-f<sub>2</sub> levels obtained at 4 weeks post noise exposure. A one-way ANOVA revealed significant (F<sub>4,120</sub> = 11.17, p < 0.01) main effects for the groups. Dunnett’s multiple comparison testing demonstrated that there was a statistically significant (p < 0.01) difference between the control and noise groups but there was no significant (p > 0.05) difference between the control and CAE+noise groups.

**Discussion**

In the current study, we found that noise exposure could increase immunolabeling of γ-H2AX in the organ of Corti. This is consistent with previous studies that have shown that noise exposure increase the level of DNA strand breaks and the 8-hydroxy-2'-deoxyguanosine DNA base adduct in the cochlea [2,6]. Furthermore, a previous study used immunoblots to demonstrate an increase in γ-H2AX expression in lysates from the ear following whole-body X-irradiation (20 Gy) of C57BL mice [53]. The current data also showed that, CAE treatment may reduce noise induced elevation of γ-H2AX. This observation is consistent with the DNA repair literature where CAE treatment has been shown to increase the repair of DNA damage products [22-31]. An interesting observation from the current study is that immunolabeling for γ-H2AX could be detected under normal conditions as well as after noise exposure. This indicates that the organ of Corti may be experiencing basal levels of DSBs under normal conditions. Indeed, previous research has shown a persistent DNA damage response among hair cells and supporting cell under normal conditions [38,49].

It is known that the normal metabolism of specific cell-types may perpetuate a persistent DNA damage response due to the precipitation of DSB as detected with γ-H2AX immunolabeling [43,54,55]. This basal (normal) level of DSB is believed to localize in gene deserts (non-protein coding genes) and do not affect the normal functions of the cell [47]. However, a consequence of this particular cellular phenotype is hyper-sensitivity to cell death because such cells are challenged with meeting the demands of endogenous genomic stress in addition to increased stress from exogenous stressors [55]. “This phenomenon has been described in a process called basal demand intereference and might help to explain the selective vulnerability of hair cells to a large variety of stressors [42,49,56]. Several endogenous mechanisms including, NADPH oxidase (NOX)-3 activity, oxidative redox activity and the functions of nitric oxide synthase-I, II and II may account for a large variety of stressors [42-56].” Ultimately, a persistent DNA damage response under normal conditions is known to preclude DNA repair during episodes of increased stress [42]. “This is particularly important because failure to repair damage DNA can result in mutated gene fragments (micosing) and/or altered DNA-protein interactions (epigenetic dys-regulation) that alter cellular functions and/or induce various forms of cell death [58,59].

It is known that noise exposure alters the expression of a large variety of genes and induce multiple forms of cell death [60-62]. Furthermore, noise exposure is associated with human neoplastic neuremas (e.g., acoustic neuromas and sonocarcinogenesis) which support the notion that noise stress can be mutagenic [63-65]. Therefore, efforts to increase the capacity of cells to repair damage DNA might be otoprotective against noise injury. For instance, it is known that cells with efficient DNA repair capacity can better resist mutagenesis, cell death and loss of cellular functions compared with cell that are less efficient [66,67]. Given that noise exposure damages DNA as early as 5 minutes post-exposure, it is tempting to speculate that this early genomic damage may underlie subsequent cascades that lead to miss-regulation of gene expression, cochlear dysfunction and ultimately cellular patterns of death and survival. If this is the case, then efforts to optimize DNA repair capacity in the initial stages of injury might provide a novel means of preserving cochlear function. For instance, the reduction in γ-H2AX that we observed at 1 day post noise exposure was associated with preserved cochlear function at 4 weeks after the noise exposure. Therefore, the current study provides a basis for further research focused on biomedical strategies of augmenting DNA repair capacity in the cochlea.

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