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Research Report

Carboxy alkyl esters of *Uncaria tomentosa* augment recovery of sensorineural functions following noise injury

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ABSTRACT

This study tested the hypothesis that hydrophilic chemotypes of the medicinal vine *Uncaria tomentosa* (*UT*) would facilitate recovery of sensorineural functions following exposure to a damaging level of noise. The particular chemotypes investigated were carboxy alkyl esters (CAE) which are known to exhibit multifunctional cytoprotective properties that include: enhanced cellular DNA repair, antioxidation and anti-inflammation. Long–Evans rats were divided into four treatment groups: vehicle-control, noise-only, CAE-only and CAE+noise. The noise exposure was an 8 kHz octave band of noise at 105 dB SPL for 4 h. Outer hair cell (OHC) function was measured with the cubic $2f_1$–f_2 distortion product otoacoustic emissions (DPOAE) at the start of the study (baseline) and at time-points that corresponded to 1 day, 1 week and 4 weeks post-noise exposure to determine within-group effects. Compound action potentials to puretone stimuli were recorded from the VIIIth craniofacial nerve at 4 weeks post-noise exposure to determine between-group effects. Additionally, cytocochleograms were constructed for each row of OHCs from each group. Noise exposure produced significant sensorineural impairments. However, CAE treatment facilitated almost complete recovery of OHC function and limited the magnitude of cell loss. The loss of neural sensitivity to puretone stimuli was inhibited with CAE treatment. Therefore, it appears that the multifunctional cytoprotective capacity of CAE from *UT* may generalize to otoprotection from acoustic over-exposure.

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1. Introduction

Uncaria tomentosa (*UT*) also known as uña de gato or cat's claw is a multifunctional medicinal vine that has been used for over 2000 years by ancient civilizations including that of the Tahuantinsuyo (Inca) empire (Pilarski et al., 2005). The bioactive components of *UT* can be divided into hydrophobic and hydrophilic chemotypes (Desmarchelier et al., 1997; Pilarski et al., 2005). The hydrophobic chemotypes include uncarine F, speciophylline, mitraphylline, isomitraphylline, pteropodine and isopteropodine (Bacher et al., 2006; Laus, 2004; Pilarski et al., 2005; Wagner et al., 1985). These hydrophobic chemotypes are derived from tincture preparations and have received considerable attention for their role in immunomodulation, antimicrobial defense, anti-inflammation and antimutagenicity (Keplinger et al., 1999). However, these hydrophobic chemotypes are not representative of medicinal decoctions consumed by ancient and indigenous peoples. For instance, the Asháninka Indians of the Amazon basin typically boiled *UT* in water and consumed the resulting

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hydrophilic chemotypes (Keplinger et al., 1999; Mammone et al., 2006). Recent experiments have demonstrated that carboxy alkyl esters (CAEs; see Fig. 1) are the bioactive components of these hydrophilic chemotypes (Akesson et al., 2005; Sheng et al., 2005). The documented health benefits of CAEs include: antioxidant protection, augmentation of DNA repair, anti-inflammation and immunomodulation (Akesson et al., 2003a, 2003b; Sandoval et al., 2002). These and other health benefits are based on the potency of CAEs to potentiate several biochemical cascades in order to increase the overall capacity of cells to survive and maintain functional integrity (Pero, 2010; Pero et al., 2009; Pero and Lund, 2010). Furthermore current human, animal and in vitro research has supported a role for CAEs in augmenting cellular repair from various physical or chemical exposures (Akesson et al., 2003a, 2003b; Belkaid et al., 2006; Gurrola-Díaz et al., 2010; Lemaire et al., 1999; Mammone et al., 2006; Pero et al., 2002). However, a role for CAEs in preserving auditory function following noise injury has not been studied.

Exposure to high levels of sound may induce a multiplicative array of biochemical cascades that perpetuate cell death and/or loss of auditory function (Le Prell et al. 2007; Ohlemiller, 2008). These biochemical cascades may propagate within minutes following exposure and are driven by processes such as ionic dyshomeostasis, mitochondriopathy, energy catastrophe and the proliferation of free radicals. For instance, loud-sound exposure may alter cochlear homeostasis of Ca^{2+}, K^{+}, and Na^{+} particularly through glutamate excitotoxicity (Hakuba et al., 2000; Le Prell et al. 2007). Mitochondriopathy is evidenced by sound induced increase in mitochondrial permeability and the independent release of at least two mitochondrial nucleases, endonuclease-G and apoptosis-inducing-factor (Han et al., 2006; Yamashita et al., 2004b). Furthermore, it is known that loud-sound exposure activates the mitochondria-mediated caspase-dependent cell death pathway (Nicotera et al., 2003; Wang et al., 2007). Energy catastrophe relates to depleted stores of high energy phosphates (e.g., ATP) following loud-sound exposure (Minami et al., 2007). The proliferation of free radicals is exemplified by increased production of reactive lipid, oxygen and nitrogen species (Ohlemiller et al., 1999; Yamashita et al., 2004a). These combined processes (ionic dyshomeostasis, mitochondriopathy, energy catastrophe and free radical production) complement each other to elicit acute and chronic inflammation that ultimately results in cell death and/or loss of auditory function (Masuda et al., 2006; Ohlemiller, 2008). The clinical manifestation of this combinatorial process includes permanent sensorineural hearing loss, tinnitus, loudness recruitment, hyperacusis, dyploacusis and speech intelligibility deficits (Basta et al., 2005; Pienkowski and Eggermont, 2010). These auditory impairments reduce an individual's quality of life and work productivity such that the economic burden to society may average $297,000 over an individual's life span (Mohr et al., 2000).

A major goal in audiologic rehabilitation and neuro-otologic medicine is the development of biomedical strategies that preserve auditory sensory and/or neural function following loud-sound exposure. To this end an impressive mosaic of pharmaceuticals that target individual pathophysiologic cascades has been employed (Le Prell et al. 2007; Ohlemiller, 2008). For instance, Ca^{2+} blockers have been used to regulate ionic homeostasis, creatine supplementation has been employed to restore energy and several types of free radical scavengers have been tested (Minami et al., 2007; Shen et al., 2007). Unfortunately, none of these single-target approaches has gained wide-spread clinical acceptance due in part to inconsistent outcomes. Since loud-sound exposure induces multiple pathologic cascades, then, an alternative approach might be to employ a multifunctional agent that simultaneously targets several pathophysiologic mechanisms. Given that CAEs of *UT* have demonstrated efficacy as a multifunctional cytoprotective agent in both human and animal studies, we speculated that CAEs might be otoprotective. Therefore, in the current experiment we tested the hypothesis that CAEs of *UT* will augment recovery of sensory and neuronal functions following noise injury.

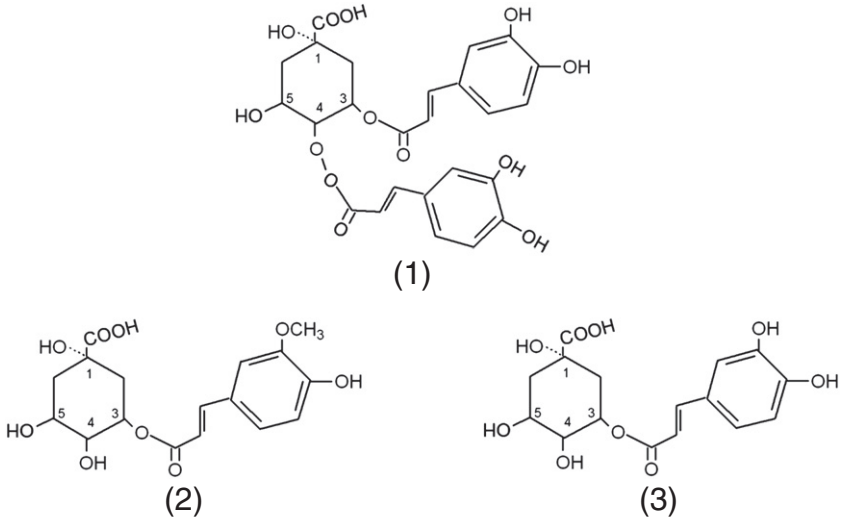

Fig. 1 – Chemical structure. The structure of three representative carboxy alkyl esters are shown: (1) 3,4-O-dicaffeoylquinic acid; (2) 3-O-feruloylquinic acid; and (3) 3-O-caffeoylquinic acid.

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2. Results

2.1. *Sensory function*

Sensory function as determined by distortion product otoacoustic emission (DPOAE) revealed that CAE treatment preserved outer hair cell (OHC) activity following noise exposure. All groups started and ended the study at the same time therefore DPOAE measurements from vehicle-only and CAE-only groups followed the same time schedule as the noise exposure groups. Fig. 2A shows DPOAE measurements for the vehicle-only group (control). These measurements reveal that DPOAE levels exhibited little variability across time-points. This small variability is representative of normal physiologically active OHCs (Wagner et al., 2008). Fig. 2B shows DPOAE levels for the noise treated group. Note that Fig. 2B shows a significant loss in DPOAE levels at 1 day following noise exposure. At 1 week following noise exposure DPOAE levels of apical (low frequency: 3–7 kHz) and basal (high frequency: 25–34 kHz) components showed marked recovery. However, the middle frequency components (8–24 kHz) and the highest frequency components (frequencies greater than 34 kHz) failed to recover to the same extent as the other frequency components. At 4 weeks post-noise exposure there was a slight improvement in DPOAE levels for the middle frequency components, however, these DPOAE components and the highest frequency components are still significantly depressed relative to that at baseline. These remaining depressions in DPOAE levels are consistent with a permanent sensory impairment. The combined results suggest that the noise exposure induced permanent loss of DPOAE levels 1–4 weeks following noise-treatment.

Fig. 2C shows that CAE treatment alone does not adversely affect OHC function and similar to the control group, DPOAE levels exhibited little variability between time-points. Fig. 2D shows DPOAE levels for the CAE + noise treated group. Note the significant loss in DPOAE levels at 1 day following noise exposure. At 1 week following noise exposure DPOAE levels across-frequencies showed marked recovery. Interestingly, this recovery was almost complete at only 1 week following noise exposure which indicates significant preservation of OHC function. Additionally, at 4 weeks post-noise exposure DPOAE levels across frequencies further improved to approximate DPOAE levels recorded at baseline. The combined results suggest that CAE treatment facilitated almost complete recovery of OHC function following noise injury.

Statistical analyses were conducted on DPOAE levels. Table 1 provides a summary of the *F*-ratios from several two-way repeated measures ANOVAs where time (baseline,

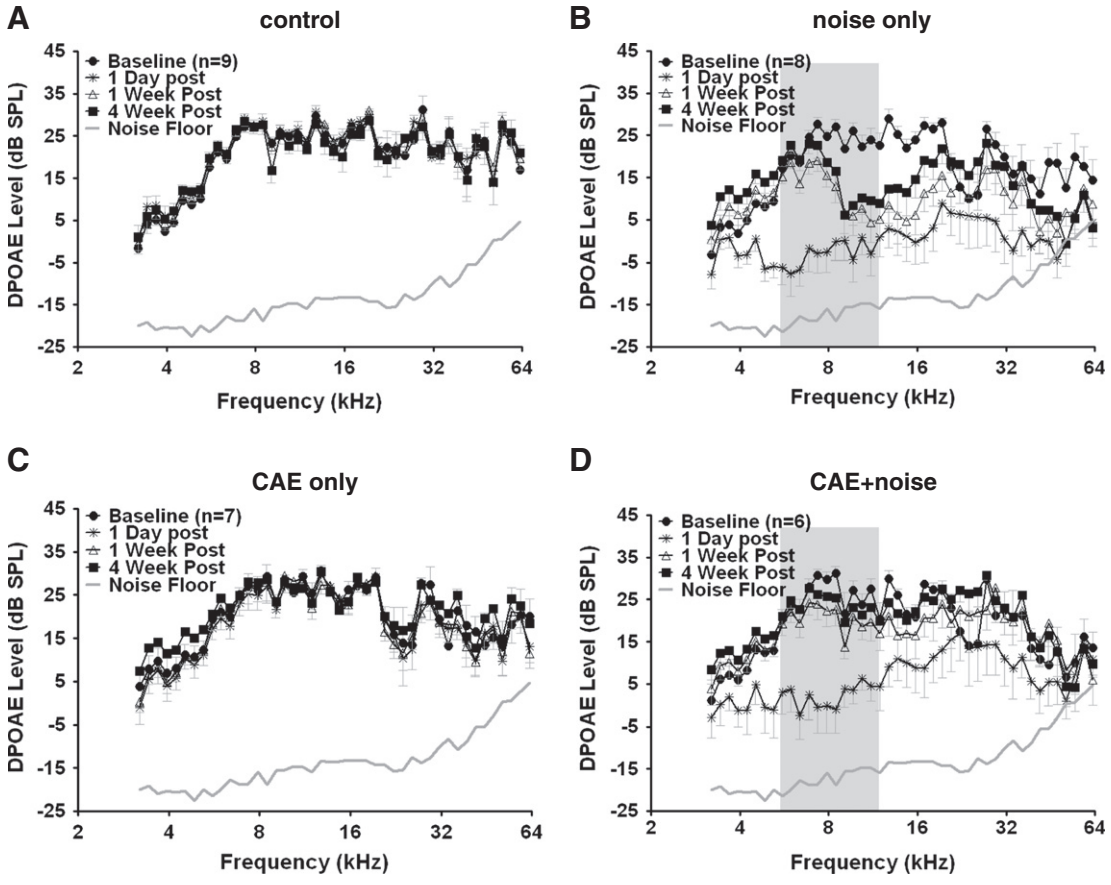

Fig. 2 – Sensory function. DPOAE levels as a function of f_2 frequency are shown for each treatment group. The gray bars in this and subsequent figures represent the frequency range of the damaging noise. Note that the noise-only group showed depressed DPOAE levels out to 4 weeks after noise exposure while the CAE + noise group showed almost complete recovery as early as 1 week following the noise exposure. Errors bars are standard errors of the means.

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Table 1 – Results of two-way repeated measures analysis of variance on DPOAE levels.

Source	df	F-values			
		Control	Noise	CAE	CAE+noise
Time (T)	3	12.33 [a]	128.3 [a]	11.82 [a]	114.9 [a]
Frequency (F)	15	2.386 [a]	0.621	2.863 [a]	0.3557
T×F	45	0.2471	1.514 [b]	3.072 [a]	1.576 [b]
MS_{ERROR}		(28.49)	(77.34)	(24.67)	(44.93)

The F-ratios are shown for separate analyses on DPOAE levels from the four treatment groups (control group, noise-only group, CAE-only group and CAE+noise group). Significant main effects of time (baseline, 1-day, 1-week and 4-weeks post-noise exposure) and frequency (8–24 kHz) and the significant time×frequency interactions are indicated.

[a] $p<0.01$.

[b] $p<0.05$.

1 day, 1 week and 4 weeks) and frequency (8–24 kHz) served as main effects for the four groups (control, noise, CAE, CAE +noise). For each group Dunnett's post-hoc testing was used to compare the mean DPOAE levels at baseline to the other three time points. Fig. 2 shows that the noise only group exhibited the largest DPOAE amplitude shifts (Dunnett $p<0.05$) between baseline and the other three time points.

2.2. *Neural function*

Compound action potential (CAP) recordings suggested that CAE treatment limited neural impairment following noise exposure. Fig. 3 reveals neural sensitivity within a frequency range of 2–40 kHz for the four treatment groups at 4 weeks post-noise exposure. Note that the noise exposure produced a significant loss in neural sensitivity while the CAE treatment provided significant protection (ANOVA main effect of treatment; $F_{3,264}=65.22$, $p<0.01$). In the noise-only group, neural sensitivity was significantly impaired between 8.0 and 40.0 kHz where the average sensitivity loss was 28 dB relative to untreated control subjects (Dunnett's post-hoc contrast; $p<0.05$). For the CAE treated group, neural sensitivity was slightly elevated between 8.0 and 40.0 kHz where the average sensitivity loss was only 7 dB relative to untreated control subjects (Dunnett's post-hoc contrast; $p>0.05$). This slight shift of 7 dB indicates that systemic CAE treatment affects inner ear neurons possibly by potentiating endogenous mechanisms (see Discussion section). In the CAE+noise group neural sensitivity was mildly impaired between 8.0 and 40.0 kHz where the average sensitivity loss was 13 dB relative to untreated control subjects (Dunnett's post-hoc contrast; $p<0.05$). This mild impairment suggests that the noise treatment had a damaging effect in this group, but the damage was less than that of the noise-only group.

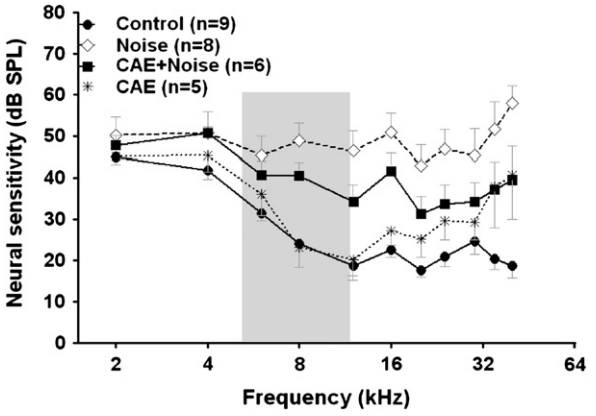

Fig. 3 – Neural function. CAP recordings of neural sensitivity in dB SPL are shown for each group. Note that the CAE+noise group exhibits better neural sensitivity than the noise-only group. Errors bars are standard errors of the means.

2.3. *Cytocochleogram*

Cytocochleograms of OHC counts from each group of animals revealed that the noise exposure induced cellular loss, but CAE treatment limited the magnitude of the loss. Fig. 4 reveals OHC loss (for rows 1–3) along the length of the cochlear neurosensory epithelium for the different treatment groups (control, noise-only, CAE-only and CAE+noise) at a 4 week post-noise exposure survival time. There were statistically significant differences between the four treatment groups (ANOVA main effect of treatment; $F_{3,232}=21.45$, $p<0.01$). Cell loss as a function of distance along the neurosensory epithelium of the control group provided a reference for determining the effect of individual treatment (noise-only, CAE-only or CAE+noise). In the noise-only group cell loss was significant toward the basal end of the neurosensory epithelia relative to that of the untreated control subjects (Dunnett's post-hoc contrast; $p<0.01$). This suggests that the noise exposure produced permanent cell loss which was more prominent at the basal coil (high-frequency responsive area). For the CAE treated group cell loss was similar to that of untreated control subjects (Dunnett's post-hoc contrast; $p>0.05$). This reveals that the CAE treatment does not adversely affect the OHCs. In the CAE +noise group there was a mild increase in cell loss relative to untreated control subjects (Dunnett's post-hoc contrast; $p<0.05$). This mild loss suggests that the noise treatment had a damaging effect in this group, but the damage was less than that of the noise-only group.

3. Discussion

This study provides the first demonstration that treatment with CAE of *UT* may augment recovery of sensory and neural functions following noise injury. Exposure to an 8 kHz octave-band of noise (OBN) at 105 dB SPL for four hours significantly reduced DPOAE levels in all animals at 1 day post-exposure. However, at 1 week post-exposure the CAE treated animals showed almost complete recovery of DPOAE levels while the animals which received only noise failed to show significant recovery. At 4 weeks post-noise exposure the CAE treated animals exhibited additional recovery in DPOAE levels while the animals which received only noise continued to experience reduced DPOAE levels. These findings suggest that CAEs may enhance the recovery of OHC function from both temporary (1 week) and permanent (4 weeks) noise-injury. Indeed,

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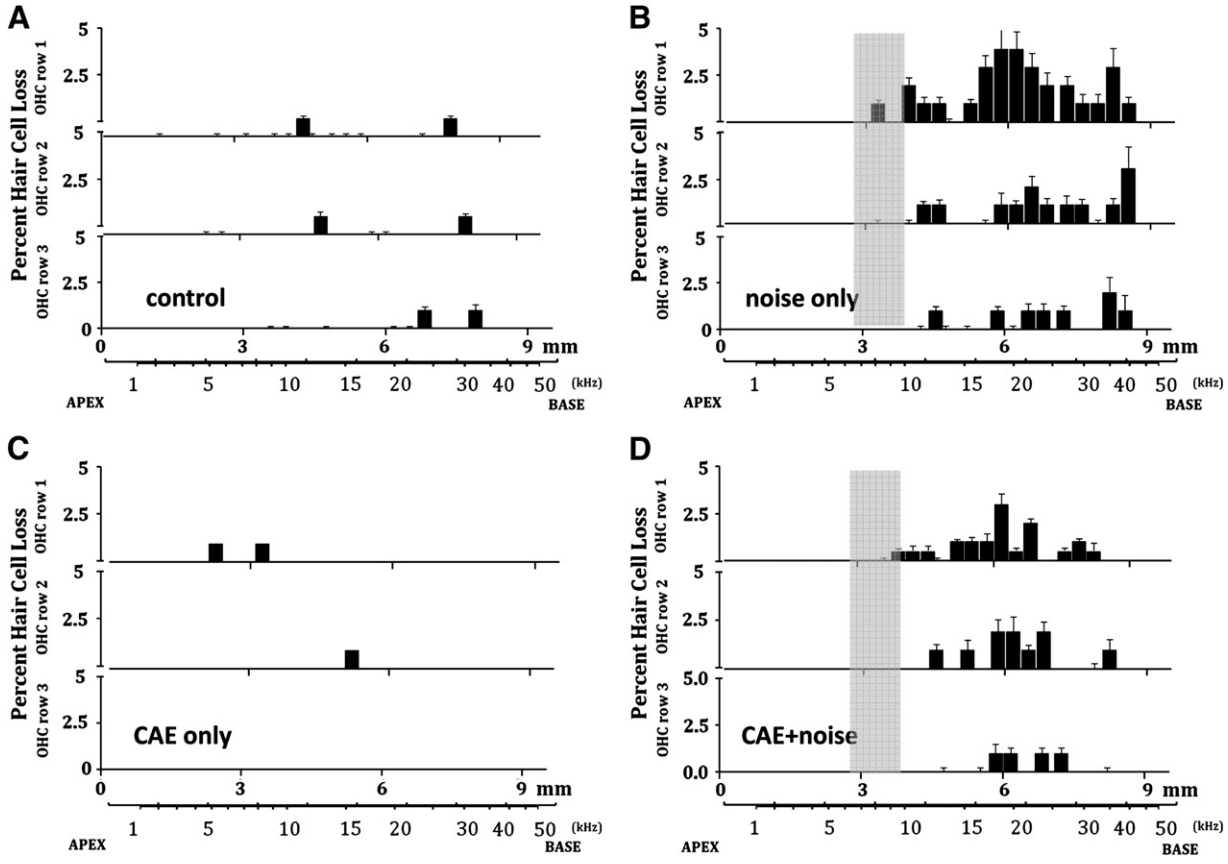

Fig. 4 – Cytocochleograms. These cytocochleograms reveal the percent of missing OHCs from rows 1–3 as a function of distance (0–9 mm) and frequency (1–50 kHz). Note that the noise exposure produced OHCs loss, however the level of missing OHCs is less in the CAE+noise group compared to the noise-only group. Errors bars are standard errors of the means.

cytocochleograms of the percent of missing OHCs revealed that CAE treated animals suffered from less OHC loss compared to animals which received only noise. The functional and structural preservation observed for OHCs was consistent with round-window recordings of action potentials. Here, CAE treatment prevented significant impairment in neural sensitivity following noise exposure. The combined results imply an otoprotective role for CAE in noise-injury. The basis for this otoprotection is unknown. However, loud-noise exposure simultaneously elicits several injury inducing mechanisms which indicates that otoprotection would require the targeting of several pathophysiologic mechanisms. CAEs of *UT* are multifunctional and known to suppress several pathophysiologic mechanisms (Belkaid et al., 2006; Gurrola-Díaz et al., 2010; Lemaire et al., 1999; Pero et al., 2002).

3.1. *Oxidation and antioxidant protection*

A ubiquitous pathophysiologic mechanism induced by loud-sound exposure is the proliferation of free radicals. Free radicals are perpetuated in the cochlea through processes such as Ca^{2+} dyshomeostasis, mitochondriopathy and energy catastrophe (Le Prell et al. 2007; Ohlemiller, 2008). Free radicals induced by noise exposure include potent reactive oxygen species (ROS) such as the hydroxyl radical (HO.) and the superoxide anion radical ($O_2^{\cdot-}$) (Ohlemiller et al., 1999; Yamane et al., 1995). Therefore, antioxidants are needed to protect endogenous biomolecules such as DNA, proteins and lipids. Several human experiments have supported a role for CAE in antioxidation (Pero et al., 2002, 2009; 2010). For instance, humans treated with CAE showed a significant increase in blood plasma antioxidation levels which lead to beneficial systemic effects such as increased energy and concentration coupled with reduced incidence of headaches, general pain, sore throat, diarrhea and fatigue (Pero et al., 2002, 2005, 2009, 2010). Additionally, CAE has been shown to protect rats treated with oxidative stress inducing drugs (Sheng et al., 2005). Therefore, it is tempting to suggest that CAE may induce otoprotection by increasing antioxidation. However, antioxidation would not be expected to provide maximal protection for at least three reasons. First, decades of research on antioxidants optimized for dose, route of administration and timing have not yielded acceptable and consistent levels of otoprotection (Ohlemiller, 2003). Second, transgenic mice with elevated levels of antioxidant genes fail to exhibit protection from noise-injury and are more vulnerable to noise-damage (Coling et al., 2003; Endo et al., 2005). Furthermore, mice with targeted deletions of antioxidant genes have been shown to be more resistant to noise-injury than wild-type strains (McFadden et al., 2001). These findings suggest that ROS are a necessary component of a cell's ability to maintain homeostasis following stress. Indeed ROS serve as signaling molecules for normal cell functions and strong antioxidants may actually facilitate cytotoxicity (Chung

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et al., 2009). CAE and other hydrophilic extracts of *UT* are weak antioxidants and their antioxidant capacity is ~10% less than the otoprotective antioxidant *N*-acetylcysteine (Desmarchelier et al., 1997; Gonçalves et al., 2005; Sandoval et al., 2002). Third, free radicals are short lived and their proliferation is buffered by endogenous reducing molecules (e.g., antioxidants, thiols, sulfhydryls). Although CAE could protect cochlear cells through antioxidation this mode of action by itself would not be sufficient. Furthermore, ROS production is only one of several injury mechanisms that result from loud-sound exposure (Le Prell et al. 2007; Ohlemiller, 2008).

3.2. *DNA damage and repair*

Genomic integrity is important for cellular functions and loud-sound exposure is known to induce genetic damage. For instance, chinchillas exposed to impulse noise demonstrate genomic DNA strand breaks within 5 min following the exposure as detected by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Hu et al., 2006). Furthermore, electrochemical high performance liquid chromatography (HPLC) has revealed the presence of the 8-hydroxy-2′-deoxyguanosine DNA base adduct in the rat cochlear genome within hours post-noise exposure (van Campen et al., 2002). These findings are important because both DNA strand breaks and the 8-hydroxy-2′-deoxyguanosine DNA base adduct are particularly toxic since they inhibit gene expression and produce mutated gene products (Satou et al., 2009; Schildkraut et al., 2005). Augmenting endogenous DNA repair capacity would be needed to counterbalance this noise-induced surge in genomic damage. A notable volume of human, animal and *in vitro* experiments has demonstrated that treatment with CAEs of *UT* specifically augment endogenous DNA repair capacity which then restores cellular function after exposure to a variety of physical and chemical stressors (Akesson et al., 2003a, 2003b; Emanuel and Scheinfeld, 2007; Mammone et al., 2006; Pero et al., 2009; Pero et al., 2002, 2005; Sheng et al., 2000a, 2000b, 2001, 2005). For instance, whole-body irradiation of female Wistar Furth rats with 12 Gy exhibited significant levels of DNA strand breaks in splenocytes (Sheng et al., 2000a). However, supplementation with CAEs resulted in almost complete strand break repair within 3 h post irradiation. This augmented DNA repair capacity exhibited a dose-dependent effect and was also confirmed in experimental studies on humans (Sheng et al., 2001). In addition to DNA strand breaks, treatment with CAE has been shown to augment the repair/-removal of the 8-hydroxy-2′-deoxyguanosine DNA base adduct (Pero et al., 2005; Pero et al., 2002). This is particularly relevant given the recent discovery of inducible DNA repair enzymes in the mammalian cochlea (Guthrie, 2009; Guthrie and Carrero-Martínez, 2010). It is possible that *UT* may augment the translational efficiency of these endogenous repair enzymes (Pero, 2010). However, augmenting endogenous DNA repair capacity by itself may not provide complete protection.

3.3. *Anti-inflammation*

Cochlear inflammation is a major consequence of loud-sound exposure. A resident population of phagocytic-macrophages that are specific to the cochlea have been identified in the spiral ligament, stria vascularis and optic capsule (Hirose et al., 2005). Loud-sound exposure induces an increase in the abundance of these cells as they spread throughout the cochlea. This is consistent with noise-induced activation of the transcription factor NF-kB that regulates the expression of proinflammatory cochlear genes (Masuda et al., 2006). Furthermore, noise increases the activation of inflammatory cytokines such as TNFα and interleukin-1β (Fujioka et al., 2006). Therefore, inhibition of cochlear inflammation would be needed in order to preserve auditory function. CAE of *UT* have been shown to inhibit inflammation by directly altering the activation of NF-kB and suppressing the production of TNFα and interleukins-1β (Akesson et al., 2003a, 2005; Pero et al., 2002). Therefore, it is reasonable to speculate that CAEs may provide otoprotection by inhibiting cochlear inflammation. But given the multiplicative nature of noise injury, we would not expect the inhibition of inflammation (by itself) to provide enough protection. A more likely explanation for the otoprotection we observed is that CAE treatment simultaneously potentiated multiple pathophysiologic mechanisms.

Beyond a role in antioxidation, DNA repair and anti-inflammation; CAEs are known to regulate endogenous molecules such as, tryptophan, nicotinamide, collagen III, thiols, amyloid, prostaglandin-E2 and glucose-6-phosphate translocase while potentiating several signaling cascades (Belkaid et al., 2006; Gurrola-Díaz et al., 2010; Lemaire et al., 1999; Pero et al., 2002). Furthermore, CAEs are known to function as protein synthesis enhancers, neuroprotectants, antibiotics, cytoprotectors and tumor inhibitors (Akesson et al., 2003a, 2003b). Therefore, CAE induced otoprotection may encompass multiple interacting mechanisms.

In summary, noise exposure induces multiple pathophysiologic mechanisms; therefore multifunctional strategies that target several mechanisms may achieve otoprotection. CAEs are known to target several pathophysiologic mechanisms to achieve cytoprotection. Therefore in the current study we test the hypothesis that CAE will augment recovery of sensorineural functions following noise injury. The results showed that animals treated with CAE exhibited better OHC and neural functions after noise injury compared to control animals. Therefore, it appears that CAEs may be important for future research efforts aimed at preventing noise induced hearing loss.

4. Experimental procedures

4.1. *Materials*

CAEs of *UT* were prepared by Optigenex Inc. (Hoboken, NJ. USA) as AC-11® (also known as C-MED-100®). The extraction and purification protocol followed a patented (US patents 6,039,649; 6,238,675 B1; 6,361,805 B2 and 6,964,784 B2) process that partly mimics the extraction procedure used by the Asháninka Indians. The procedure has been published previously (Sheng et al., 2000a, 2000b, 2005). Briefly, the bark (~150 g) of *UT* is heated in water for 12–24 h at 90–100 °C and the soluble extracts are decanted and ultra-filtered to remove components with a molecular weight that is greater than 10 kDa (e.g., tannins and flavanoids) while the remaining low molecular weight

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components are spray dried on maltodextrin. Bartos chemistry was used to demonstrate the presence of CAEs (Bartos, 1980). For instance, the extracts react with hydroxylamine (10% hydroxylamine hydrochloride in methanol, 10% sodium hydroxide in methanol, pH 10) to produce hydroxamic acid which was then reacted with ferric chloride (0.3% ferric chloride hexahydrate) to exhibit a chromophore with absorbance at 490 nm (Lamm et al., 2001; Sheng et al., 2005). To further confirm the presence of CAE, the extracts produced a 200 nm UV absorption maxima which was standardized against dioctyl phthalate, a typical benzoic acid-type CAE (Sheng et al., 2005). Lastly, a NaOH neutralization procedure served as a third method to verify the presence of CAE. Here NaOH is used to neutralize the extracts in order to determine the base equivalents needed to adjust the pH to 7. Up to 20% of the extracts are CAEs which are the only bioactive constituents (Mammone et al., 2006; Sheng et al., 2005). In the current experiment, the extracts (Optigenex, lot # 280809.1785) contained 10.25% CAE. All animals in the CAE treatment groups were treated with this particular lot of extracts.

4.2. *Animals*

Thirty pigmented male Long–Evans rats (250–300 g at 2 months old) were acquired from Harlan Laboratories, Inc. (Livermore, CA. USA) and served as subjects in these experiments. The animals were housed at the Veterinary Medical Unit (VMU) at the Loma Linda Veteran's Hospital (Loma Linda, CA. USA). The VMU is accredited by the Association for Assessments and Accreditation of Laboratory Animal Care (AAALAC) and is staffed with a medical veterinarian and veterinary technicians. The animals were maintained in a low-stress and physically-enriched environment where they had free access to food and water. The environmental temperature was maintained at 21 °C±1 °C and the lighting followed a 12 hour light/dark cycle (6:30 am to 6:30 pm). All experimental protocols were conducted during the light cycle and each protocol was approved by the Hospital's Institutional Animal Care and Use Committee. The experimental protocols were designed to minimize the number of animals used, pain and discomfort. Table 2 describes the different animal groups, their treatment regimen and the experimental design. After arriving from Harlan the animals were given 1 week to acclimatize to the VMU. Baseline DPOAEs were then collected on each animal to verify auditory function. The animals were then assigned to one of four groups based on their DPOAE measurements to counterbalance auditory function between groups. CAEs were dissolved in double-distilled water at a concentration of 160 mg/mL to produce a homogenous solution suitable for gastric gavage (Sheng et al., 2000a). A 20-gage animal feeding stainless steel needle was used to intubate alert animals in order to administer 160 mg of the CAE solution per kilogram of animal weight. Fresh solutions were prepared each day and administered via gastric intubation for 28 consecutive days. A control group of animals received water-vehicle (volume/body weight) via gastric intubation instead of the CAE solution. Two groups (CAE+noise and noise-only) were exposed to noise (see Noise exposure) on the 29th day. DPOAE was measured again at 1 day, 1 week and 4 weeks following the noise exposure. CAP recordings and tissue collection for hair cell counts (cytocochleograms) were obtained at 4 weeks (end of the study) post-noise exposure.

4.3. *Noise exposure*

In order to elicit noise induced auditory dysfunction, the animals in the CAE+noise and noise-only groups were exposed to an 8 kHz OBN at 105 dB SPL for 4 h. This noise exposure exceeds the permissible doses for work-place safety in the United States and is known to produce permanent sensorineural auditory dysfunction in rats (Chen and Fechter, 2003; Lorito et al., 2006). Awake and alert animals were placed in a small wire-cloth enclosure (15×13×11 cm) within a reverberant 40 L chamber. Broadband noise was driven by a DS335 Function Generator (Stanford Research System, Menlo Park, CA. USA) and bandpass filtered with a Frequency Device 9002-Dual-Channel Filter/Amplifier Instrument (Frequency Device Inc., Haverhill, MA. USA) with a roll-off of 48 dB/octave to produce an OBN with center frequency at 8 kHz. This OBN was then amplified by a HCA1000A Parasound Amplifier (Parasound Products, Inc., San Francisco, CA, USA) and delivered to Vifa D25AG-05 speakers (Vifa International A/S, Videbaek, Denmark) located approximately 5 cm above the animals' wire-cloth enclosure. Sound pressure levels measured at the rats' pinnae were 105 dBlin SPL in the octave band centered around 8 kHz. These sound pressure measurements were made using an OB-300 Quest Type-1 Sound Pressure Meter with 1/3 octave filter set (Quest Electronics, Oconomowoc, WI. USA).

4.4. *Assessment of sensory function*

DPOAE was used to assess the function of the OHCs in the right ear. Each animal was lightly anesthetized with ketamine (44 mg/kg) and xylazine (7 mg/kg) while normal body temperature was maintained using a direct current (dc) heating unit built into the surgical table. All measurements were obtained in

Table 2 – Experimental design.

	Baseline DPOAE testing			1-day, 1-week and 4 week post-noise exposure data collection (DPOAE+CAP+hair cell count)
Groups		28 days of gavage	Noise exposure	
CAE+noise	+	CAE	105 dB OBN	+
CAE-only	+	CAE		+
Noise only	+		105 dB OBN	+
Control	+	Water		+

Abbreviations: CAE, carboxy alkyl ester; DPOAE, distortion product otoacoustic emission; CAP, compound action potential; 105 dB OBN, 105 decibel (dB) octave band noise centered at 8 kHz for 4 h.

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a double-walled sound-isolation chamber (Industrial Acoustics Company Inc., Bronx, NY. USA). The cubic $2f_1$–f_2 DPOAE was recorded with two primaries, f_2 and f_1; where f_2 is basal to f_1 at an f_2/f_1 ratio of 1.25. The f_1 and f_2 frequencies were swept basalward in 0.1-octave increments of f_2 along the cochlear spiral from f_2=3.2 to 63 kHz. The sound pressure level (SPL) for the f_1 primary was 65 dB SPL (L_1) and that for the f_2 primary was 55 dB SPL (L_2) with a level ratio of 1.18 (L_1/L_2). These combined frequency and level ratios were selected to maximize the $2f_1$–f_2 SPL recorded from the external auditory meatus (Whitehead et al., 1995a, 1995b, 1995c.). Two separate realistic dual radial horn tweeters (Radio Shack, Tandy Corp., Ft Worth, TX, USA) were used to present the primaries, f_2 and f_1. The primaries were acoustically mixed in the external auditory meatus to avoid artifactual distortion. An ER-10B+emissions microphone assembly (Etymotic Research, Elk Grove Village, IL. USA) was used to capture SPLs in the external auditory meatus. 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). This allowed for the delivery of the primaries, synchronous averaging and Fourier analysis of $2f_1$–f_2 dB SPLs as a function of f_2 frequencies ranging from 3.2 to 63 kHz in 0.1-octave increments. The noise floor was computed by averaging SPLs from the external auditory meatus for frequency bins above and below the $2f_1$–f_2 bin (±3.75 Hz). The measuring microphone assembly and the stimulus delivery system were extended to a probe that was physically and acoustically coupled to each animal's external auditory meatus. A 0.2 cm^2 hard-walled cavity that approximates the rat's external auditory meatus was used to calibrate the DPOAE recordings. These calibrations were free of artifacts and did not produce DPOAE SPLs that exceeded the noise floor. A DPOAE is considered to be present when the SPL exceed the noise floor by at least 3 dB.

4.5. *Assessment of neural function*

The CAP was used to access the sensitivity of the auditory branch of the VIIIth craniofacial nerve in the right ear. This procedure is terminal (nonsurvival) and therefore deployed at the end of the study (4 weeks post-noise exposure). The animals were anesthetized with xylazine (13 mg/kg, im) and ketamine (87 mg/kg, im) while normal body temperature was maintained using a dc heating unit built into the surgical table. All recordings were obtained in a double-walled sound-isolation chamber (Industrial Acoustics Inc.). The auditory bulla was approached and opened via a ventrolateral surgical approach. The cochlea was warmed using a low-voltage high-intensity lamp. A fine Teflon-coated silver-wire-recording electrode (A-M Systems, Inc., Carlsborg, WA, USA) with an outer-diameter of 0.1 mm was placed on the round window membrane while a silver chloride electrode (ground) was inserted into neck musculature. A speaker-probe assembly was acoustically coupled to the surgically-resected external auditory meatus. A customized program written in LabVIEW 7.1 (National Instruments) was used to drive a SoundMax Integrated Digital Audio board in order to generate and shape stimulus frequency, timing and intensity. Stimulus frequencies were shaped as a 10 ms burst with ramps of 1 ms on/off-sets. Frequencies between 2 and 40 kHz in approximately 1/2 octave steps were presented through the probe assembly at a rate of 9.7/s per frequency. The intensity of the stimulus was adjusted in 1 dB steps until an action potential was discernable on a TDS1002 digital oscilloscope (Tektronix Inc., Beaverton, OR, USA). Action potentials were amplified (1000-fold) between 0.1 and 1.0 kHz with a Grass A.C. preamplifier (Model P15, W. Warwick, RI. USA) and averaged over four sweeps. Neural sensitivity was tracked by monitoring the N_1 action potential from a descending intensity series. The N_1 component of the action potential was identified based on its shape and latency relative to stimulus onset. The approximate response amplitude of the N_1 component at the lowest stimulus needed to stimulate the nerve was 1 mV as measured at the output of the preamplifier. Neural sensitivity for a particular frequency was the lowest stimulus intensity in dB SPL needed to elicit an N_1 above background noise.

4.6. *Cytocochleogram*

OHCs are among the most sensitive cell types to noise-injury therefore cytocochleograms of missing OHCs were constructed for each animal's right ear. This was conducted at the end of the study (4 weeks post-noise exposure) on the same animals that received CAP testing. Under high-dose anesthesia (xylazine/ketamine 13/87 mg/kg, im) each animal was decapitated and within 60 s cochleae were fixed by round-window perilymphatic perfusion with 1 mL of periodate-lysine-paraformaldehyde (PLP). The cochleae were then post-fixed for 24 h at 22 °C in PLP. Following fixation the cochlea was washed with 0.1 M phosphate buffered saline then stained with 2% OsO_4 in water for 2 h and finally washed again with 70% ethanol. The cochlear neurosensory epithelium was micro-dissected in 70% ethanol then mounted in glycerin on microscope slides. A 40× objective mounted on a Carl Zeiss upright light microscope was used to visualize and count OHCs. OHCs were counted as present when the cell nucleus could be visualized. The degree of cellular damage to surviving cells was not determined. The Müller-rat frequency-place map was used to estimate frequency loci as a function of length along the cochlear spiral (Müller, 1991). This map reflects the logarithmic-tonotopic scale of a rat's cochlea where high frequency receptive OHCs are located at the base while low frequency receptive OHCs are located at the apex. A cytocochleogram showing the percentage of OHC loss as a function of distance from the apex of the cochlea was plotted for each animal. The results were then averaged across each group of subjects for between-group comparisons.

4.7. *Statistical analyses*

All statistical analyses were conducted with Prism 5 version 5.03 (GraphPad Software, Inc., La Jolla, CA, USA). The DPOAE data was analyzed for within-group effects. A 16×4 repeated measures analysis of variance (ANOVA) was calculated for DPOAE levels where 16 frequencies within 8–24 kHz were compared across four time points (baseline, 1 day, 1 week and 4 weeks post-noise exposure) within each group (control, noise-only, CAE-only and CAE+noise). Dunnett's post-hoc analyses were performed to determine statistically significant differences between DPOAE levels obtained at baseline compared with those obtained at subsequent time points;

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1 day, 1 week and 4 weeks post-noise exposure. The frequency range between 8 and 24 kHz was chosen for statistical analysis because it was the largest area affected by the 8 kHz OBN and it is 1 1/2-octave above the center frequency (8 kHz) of the OBN. This high frequency shift is common among humans and animals exposed to damaging noise and represent the interaction between the maximum displacement of the basilar membrane and the Helmholtz resonance of the external auditory meatus (Johnstone et al., 1986).

The CAP data were analyzed for between-group effects. Therefore, CAP recordings of neural sensitivity in dB SPL were subjected to an 11×4 two-way ANOVA where 11 frequencies (~1/2 octave steps) within 2–40 kHz were compared across the four treatment groups (control, noise-only, CAE-only and CAE+noise). Lastly, the OHC-count data was also analyzed for between-group effects. A 29×4 two-way ANOVA was calculated where the percent of missing OHCs at 29 serial locations (0.31 mm increments along the entire epithelium) within 9 mm of the neurosensory epithelium was compared between the four treatment groups. Data from CAP and OHC-counts were treated with Dunnett's post-hoc testing to determine significant differences relative to the control group.

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