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14. ABSTRACT The current study identified optimal protective D-methionine (D-met) dose and delayed time response from steady state and impulse noise-induced hearing loss (NIHL) in groups of <i>Chinchillas laniger</i> (n = 10/group). The first year determined the dose-response curves and optimal D-met dosing for protection from NIHL with D-met (BID) delivered for 48 hours before and 48 hours after steady state noise or impulse noise using either 0 (saline placebo), 25, 50, 100, or 200 mg/kg ip D-met per dose. The second year determined the latest time D-met may be administered for efficient protection in groups receiving an intraperitoneal D-met (200 mg/kg/dose) every 12 hours for 48 hours, totaling 5 ip injections, beginning 7, 9, 12, 18, 34, 36, or 48 hours after steady state or impulse noise. The third year further defined maximum time delay for D-met post-noise rescue protection from permanent NIHL and determined if 5 additional D-met doses for another 48 hours can improve post-noise rescue protection at the first rescue time with suboptimal protection. D-met otoprotection was assessed by auditory brainstem response (ABR) analysis and outer hair cell (OHC) quantification. ABR analyses identified optimal protective dose at 50 mg/kg/dose in steady state noise-exposed groups and 100 mg/kg/dose in impulse noise-exposed groups. ABR analyses also identified time-dependent D-met protection from steady state and impulse noise exposures when D-met administration began as late as 24 hours post-noise exposure. Additional D-met administration significantly recovered hearing protection in steady state and impulse noise-exposed treatment groups at a 28 hour rescue paradigm. Histological cytochrome analysis confirmed electrophysiological findings. Overall, results will help to identify critical dosing and timing parameters required for current and future D-met protection clinical trials and enable D-met to proceed to FDA approved clinical trials.					
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INTRODUCTION:

In military settings, frequently noise exposure exceeds the capacity of any physical hearing protectors to prevent permanent NIHL. Consequently, an oral antioxidant that could prevent permanent NIHL could allow more military personnel to be redeployed, maintain the auditory capabilities needed in combat situations, and keep our military personnel from having a permanent disability. Currently, no pharmacologic protective agents exist in the clinical arena but the need to develop one is great. We have developed an antioxidant, D-methionine (D-met), which has already shown promise in preventing NIHL in animals whether started before or after the noise exposure. In clinical trials for other applications it has shown no side effects. Its safety data and formulation have already been approved by the FDA for clinical trials to prevent radiation-induced oral mucositis. In both human and animal studies we have demonstrated excellent otoprotection from radiation induced oral mucositis and cisplatin induced hearing loss.

In these studies we performed additional animal experiments using steady state and impact noise exposure conditions to prepare for D-met NIHL protection clinical trials in humans. We performed the dose response curves needed to determine the lowest effective D-met dose that can use in clinical trials. We also determined how long after a noise exposure we can first initiate D-met protection and still provide significant rescue from NIHL. Further, we determined if additional D-met dosing can extend the delay interval for rescue from NIHL.

BODY:

Year 1 Purpose: To determine the dose-response curves and optimal D-met dosing for protection from noise induced hearing loss (NIHL) with ip D-met (BID) delivered for 48 hours before and 48 hours after steady state noise and after impulse noise using either 0 (saline placebo), 25, 50, 100, or 200 mg/kg ip D-met per dose.

Year 2 Purpose: To determine maximum time delay for effective D-met post-noise rescue protection from permanent NIHL. Because military personnel cannot always anticipate noise exposures, the second set of experiments determined the maximum time delay between noise cessation and D-met initiation for post-noise rescue protection from permanent NIHL after steady state or impulse noise exposures. For these experiments, D-met administration started at time delays of 7, 9, 12, 18, 24, 36, 48, or 72 hours after completion of the noise exposure and then another 48 hours BID for a total of 5 ip D-met injections. The control group received the placebo starting 7 hours after noise cessation.

Year 3 Purpose: To complete data analysis from Year 2 and to further define maximum time delay for D-met post-noise rescue protection from permanent NIHL and to determine if additional D-met dosing for another 48 hours totaling 9 D-met injections can improve post-noise rescue protection at the first rescue time with suboptimal protection.

Methods:

Year 1 Methods: Each animal group (5 groups for steady state noise and 5 groups for impulse noise) comprised 10 male *Chinchillas laniger*. Auditory brainstem response thresholds (ABRs) were measured at baseline, prior to any drug or noise exposure, and again 21 days after noise exposure cessation using tone-burst stimuli centered at the frequencies of 2, 4, 6, 8, 14 and 20 kHz. Steady state noise exposure comprised a 105 dB SPL narrow band of noise centered at 4 kHz. Impulse noise will comprised simulated M-16 weapon fire at 155 dB peSPL 150 impulses at 2/s. After the 21 day post noise ABR, each animal was sacrificed and the cochleae were harvested for and outer hair cell (OHC) counts.

Year 2 Methods: Eighteen groups (9 for steady state noise and 9 for impulse noise) of 10 male *Chinchillas laniger* each were used for the time delay intervals listed in the SOW. Each animal in the experimental groups received 200 mg/kg D-met starting at the initial delay interval and continuing twice per day at 12 hour intervals

for an additional 48 hours. Two noise-exposed control groups, one with steady state noise and one with impulse noise, were tested with placebo administration only. ABR thresholds were measured at baseline, prior to any drug or noise exposure, and again 21 days after noise exposure cessation using tone-burst stimuli centered at the frequencies of 2, 4, 6, 8, 14 and 20 kHz. Steady state noise exposure will comprised a 105 dB SPL narrow band of noise centered at 4 kHz. Impulse noise comprised simulated M-16 weapon fire at 155 dB peSPL 150 impulses at 2/s. After the 21 day post noise ABR, each animal was sacrificed and the cochleae were harvested for OHC counts.

Year 3 Methods: Two additional time delays for each exposure type were for testing at intervals, determined from Year 2 experiments, between the last effective time delay and the first ineffective time delay using a time interval halving strategy (28 and 32 hour rescue). Then one group of animals for each noise exposure type was tested at the first time delay providing less than maximal protection (28 hours rescue) with an additional 48 hours of D-met administration to determine if post-noise rescue protection from permanent NIHL can be improved at that time delay by additional D-met administration. Auditory brainstem response thresholds (ABRs) were measured at baseline, prior to any drug or noise exposure, and again 21 days after cessation of the noise exposure using tone-burst stimuli centered at the frequencies of 2, 4, 6, 8, 14 and 20 kHz. Steady state noise exposure comprised a 105 dB SPL narrow band of noise centered at 4 kHz. Impulse noise comprised simulated M-16 weapon fire at 155 dB peSPL 150 impulses at 2/s. After the 21 day post noise ABR, each animal was sacrificed and the cochleae were harvested for OHC hair cell counts.

Results:

Year 1 Results:

Steady state noise-induced threshold shift results at 21 day post-noise identifies a dose response curve that indicated 50 mg/kg/dose as the optimal otoprotective dose (Figure 1). Statistically significant protection was observed for all D-met-treated animals at the 0.05 level. However, the 50 mg/kg/dose group was noticeably optimal. Interestingly, sub-optimal protection was identified at 100 and 200 mg/kg/dose; indicating that optimal dose between 50 and 100 mg/kg/dose is possible. An additional dose response assessment at 75 mg/kg/dose is therefore merited for future studies to further define lowest maximally effective D-met protective dose from steady-state noise.

Steady state noise-exposed OHC counts in chinchillas 21 days after a 6-hour 105 dB SPL noise exposure measured significantly ($p \leq 0.01$) increased remaining OHCs, coinciding with electrophysiological measurements (Figure 2). OHC were markedly increased in D-met groups compared to saline control groups, particularly in the 25 and 50 mg/kg/dose D-met-treated groups.

D-met-treated groups also demonstrated dose-dependent impulse noise-induced threshold shift reduction at 21 day post-noise exposure (Figure 3). Significant protection was observed for the 100 mg/kg/day D-met-treated animals at $p \leq 0.05$ (8 and 20 kHz) and at $p \leq 0.01$ (14 kHz). Significant protection was observed for the 200 mg/kg/day D-met-treated animals at $p \leq 0.05$ (8 and 20 kHz). The 100 mg/kg/dose D-met-treated animal group was the only group to achieve significance at $p \leq 0.01$; thus, the 100 mg/kg/dose appears to be the optimal tested otoprotective D-met dose against impulse NIHL.

D-met did not significantly increase remaining OHC percentages (group effect = 0.14) in D-met-treated animals exposed to impulse noise relative to controls (Figure 4). However, when controls were compared only to the optimally protective from the electrophysiological study (100 mg/kg/dose), we measured a slight group effect ($p = 0.059$) and Tukey's post-hoc analysis indicated a significant OHC increase in the 6 and 8 kHz regions at the 0.05 level.

Year 2 Results:

D-methionine protected from steady state noise-induced hearing loss when administration began up to 36 hours after impulse noise exposure (Figure 5). The 7, 9, 12, 18, and 24 hour D-met rescue groups measured significantly reduced ABR threshold shifts at the 0.01 level at all frequencies. The 36 hour D-met rescue group measured significantly reduced ABR threshold shifts at 4, 8, 14, and 20 kHz. The 48 hour D-met rescue group also measured a significant ABR threshold shift reduction at 4 kHz at the 0.05 level. We determined almost no D-met protection with a 48 hour rescue dosing regimen and therefore did not test the 72 hour time point to prevent animal pain, suffering, and sacrifice.

D-met-treated rescue groups also demonstrated significant remaining OHC percentage increases (Figure 6). The percentage OHCs present in the D-met treatment groups were significantly greater than the saline control group at all frequencies tested except in the 7 hour D-met treatment group at 6 kHz. The optimal rescue group, 12-hour D-met rescue from steady state noise exposure, also demonstrated reduced missing OHCs compared to the control group.

D-methionine demonstrated protection from impulse noise-induced hearing loss when administration began up to 36 hours after impulse noise exposure (Figure 7). The 9, 12, 18, and 24 hour D-met rescue groups measured significantly reduced ABR threshold shifts at all frequencies compared to saline controls at the 0.01 level. The 36 hour D-met rescue group also measured significant ABR threshold shift reductions at the 0.01 level at 6, 8, 14 and 20 kHz. The 7 hour D-met rescue group measured significant ABR threshold shift reductions at the 0.05 level at 2 and 4 kHz. Further, the 36 hour D-met rescue group also measured significant ABR threshold shift reductions at the 0.05 level at 2 and 4 kHz.

Excepting four frequency regions across 7, 36, and 48 rescue paradigms, the remaining D-methionine-treated rescue interval groups exposed to impulse noise exposure measured significant missing OHC reductions compared to saline-injected controls at the 0.05 or 0.01 level (Figure 8).

Year 3 Results:

We first measured D-met rescue in steady state and impulse noise-exposed animals administered D-met 28 or 32 hours post noise exposure. We determined suboptimal D-met protection at 28 hours in the steady state and impulse noise-exposed groups. Thus, a 28 hour D-met rescue group for each noise exposure type was tested with additional D-met dosing.

We measured significant protection recovery in the 28 hour D-met rescue steady state group receiving 4 additional D-met doses (Figure 9). Impulse noise-exposed animals rescued at 28 hours and given additional D-met doses did not significantly reduce ABR threshold shifts compared to the 28 hour measured observed threshold shift reductions however more frequencies were significantly different compared to the saline control group (Figure 11).

OHC analysis measured significant missing hair cell reductions in both 5- and 9- dose D-met 28 hour rescue groups for both noise exposure types (Figures 10 and 12).

KEY RESEARCH ACCOMPLISHMENTS:

- All dosing and timing animal studies are complete for both of the noise exposure types.
- ABR and histological analyses identified optimal dose at 50 mg/kg for steady state and 100 mg/kg for impulse noise exposure.
- ABR and histological analyses identified optimal rescue time at 12 hours post-noise exposure for both noise exposure types with protection possible up to 24 or 36 hours rescue for the first D-met dose.
- ABR and histological analyses demonstrated D-met protection recovery when additional D-met doses are administered at a suboptimal rescue time.

- Developed a cytochleagram quantitative analysis technique of chinchilla cochlear hair cells.
- Developed an animal model for chinchilla cytochleagram quantitative analysis.

REPORTABLE OUTCOMES:

We have identified optimal protective D-met doses for steady state (optimal protection at 50 mg/kg/dose) and impulse (optimal protection at 100 mg/kg/dose) noise exposure. We have also extended our published 7-hour D-met rescue from steady state noise exposure (Campbell et al. 2011) to protection from steady state and impulse NIHL when D-met administration begins at least 24 hours post-noise exposure. Further, D-met protection may be recovered with additional D-met doses at suboptimal rescue times.

CONCLUSION:

D-met significant protects from steady state or impulse noise exposure via dose- and time-dependent criteria. The conclusions from the current study will help to define future clinical applications for optimal D-met protection from NIHL. These data will also enable us to proceed to FDA approved clinical trials and submit the data for publication and the FDA. A follow up study may be required to tailor an optimal dose to specific rescue times because we determined dose- and time-dependent mechanisms with a potential rescue recovery following additional D-met administrations.

REFERENCES:

Campbell, K., Claussen, A., Verhulst, S., Fox, D., Hughes, L. 2011. D-methionine (D-met) significantly rescues noise-induced hearing loss: timing studies. *Hear Res.* Dec: 282(1-2): 138-44.

APPENDICES: N/A

SUPPORTING DATA:

Dose Response D-methionine (D-met) Protection from Steady State Noise Exposure: ABR Threshold Shifts

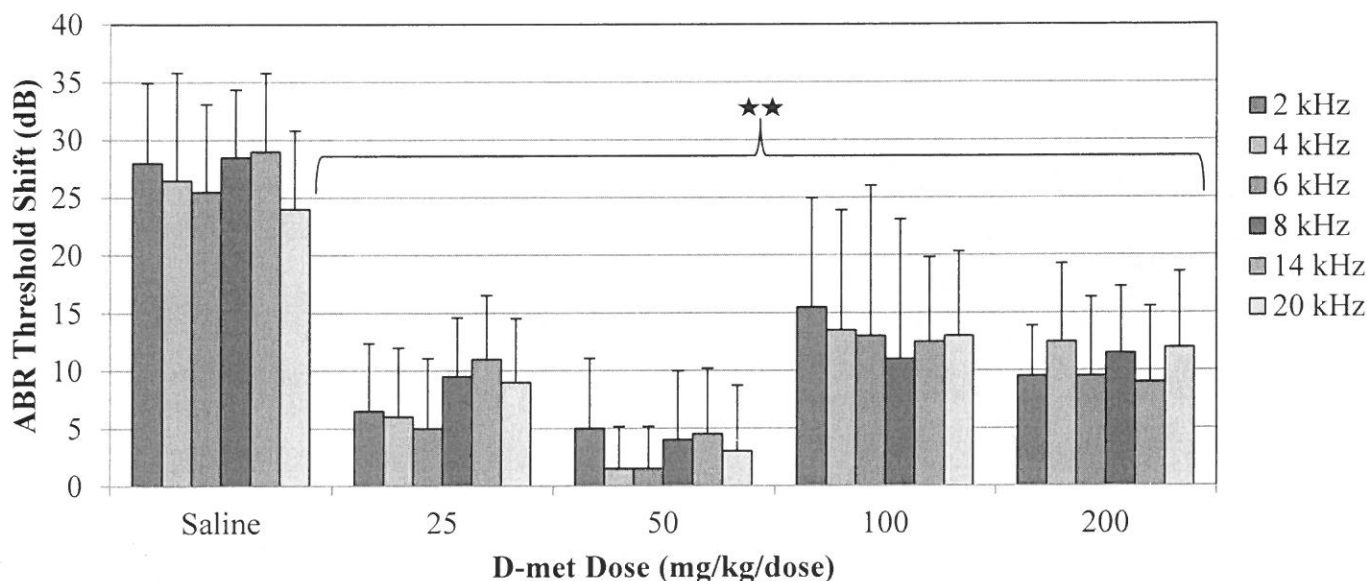


Figure 1. Steady state noise-induced ABR threshold shifts in chinchillas (n = 10/group) 21 days after a 105dB 6 hour noise exposure centered at 4 kHz. D-met has provided robust protection particularly at 50 mg/kg/dose. Statistically significant results ($p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by two stars.

Dose Response D-methionine (D-met) Protection from Steady State Noise Exposure: OHC Counts

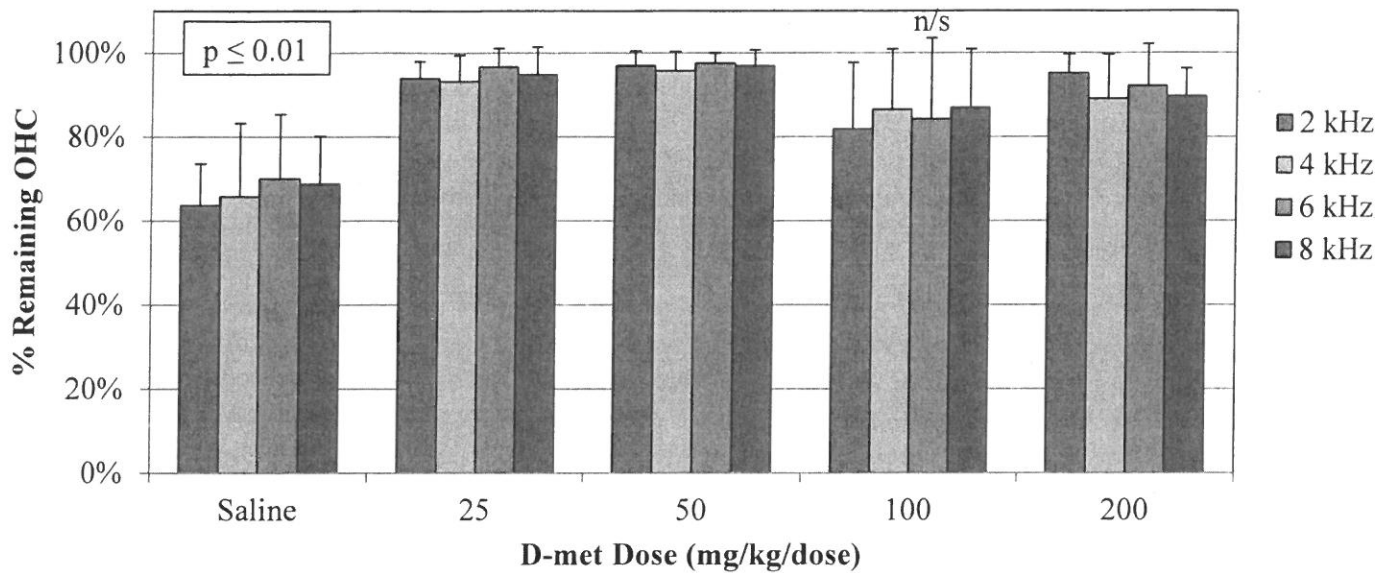


Figure 2. OHC percentages at sacrifice 21 days post-noise exposure ($n = 10/\text{group}$). Significant D-met protection occurred for all time delays at all but one frequency. One cochlea per animal was analyzed. All OHC count averages but one were significantly different from controls at the 0.01 level.

Dose Response D-methionine (D-met) Protection from Impulse Noise Exposure: ABR Threshold Shifts

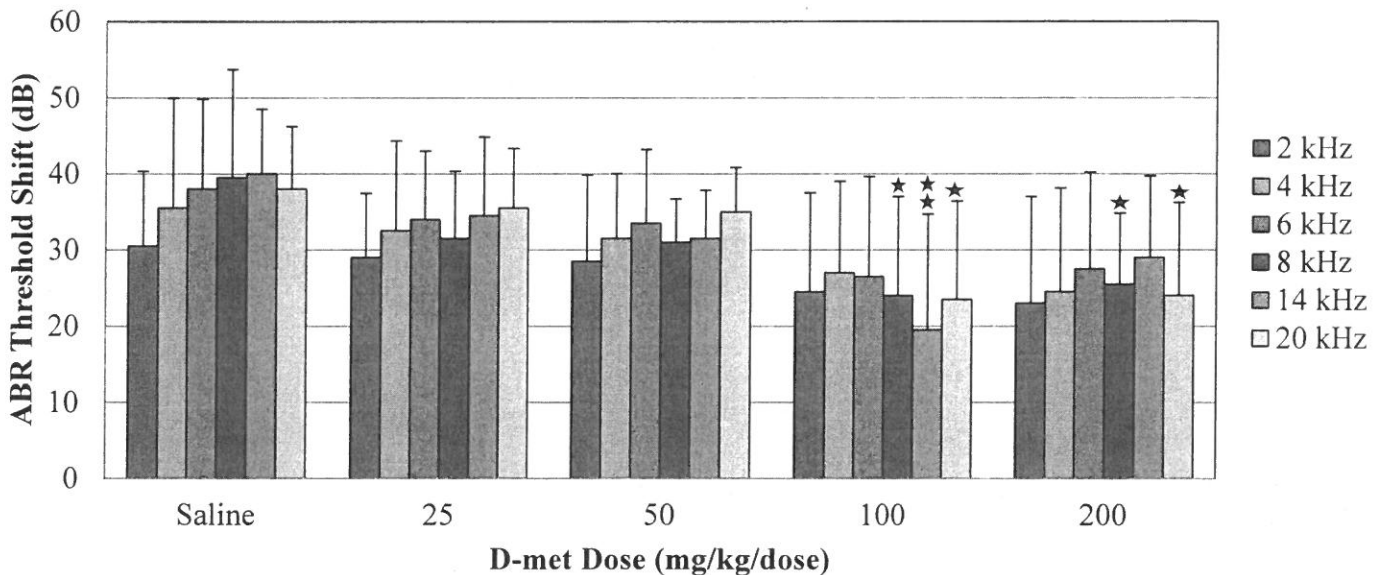


Figure 3. Impulse noise-induced ABR threshold shifts in chinchillas 21 days after exposure to simulated M-16 weapon fire at 155 dB peSPL 150 impulses at 2/s. D-met has provided optimal protection with a 100 mg/kg/dose. Results are statistically significant at $p \leq 0.05$ (one star) and $p \leq 0.01$ (two stars) by Tukey's post-hoc follow up test.

Dose Response D-methionine (D-met) Protection from Steady State Noise Exposure: OHC Counts

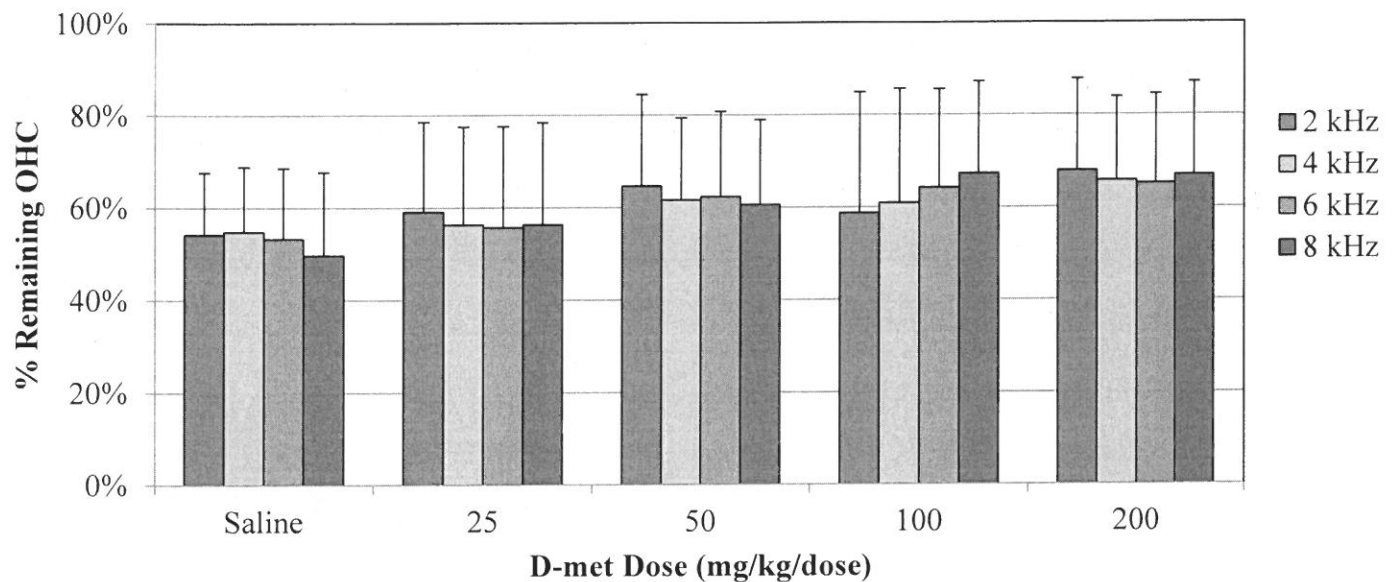


Figure 4. OHC percentages at sacrifice 21 days post-noise impulse exposure ($n = 10/\text{group}$). D-met did not significantly increase remaining OHC percentages in D-met-treated animals exposed to impulse noise relative to controls. However, when controls were compared only to the optimally protective from the electrophysiological study (100 mg/kg/dose), we measured a slight group effect ($p = 0.059$) and Tukey's post-hoc analysis indicated a significant OHC increase in the 6 and 8 kHz regions at the 0.05 level.

Rescue D-methionine (D-met) Protection from Steady State Noise Exposure: ABR Threshold Shifts

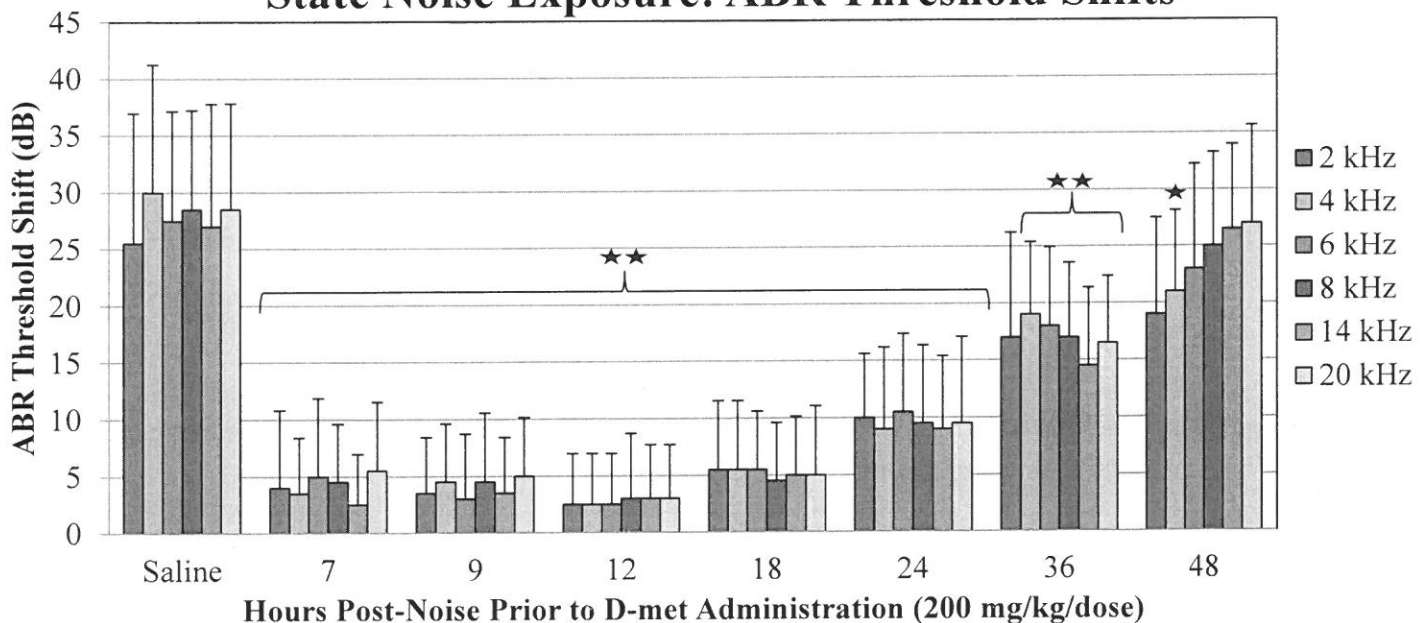


Figure 5. Steady state noise-induced ABR threshold shifts in chinchillas 21 days after a 105dB peSPL 6 hour noise exposure centered at 4 kHz ($n = 10$ per group). D-met has provided protection when administration began

as late as 24 hours post-noise exposure. Statistically significant results ($p \leq 0.05$; $p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by one or two stars, respectively.

Rescue D-methionine (D-met) Protection from Steady State Noise Exposure: OHC Counts

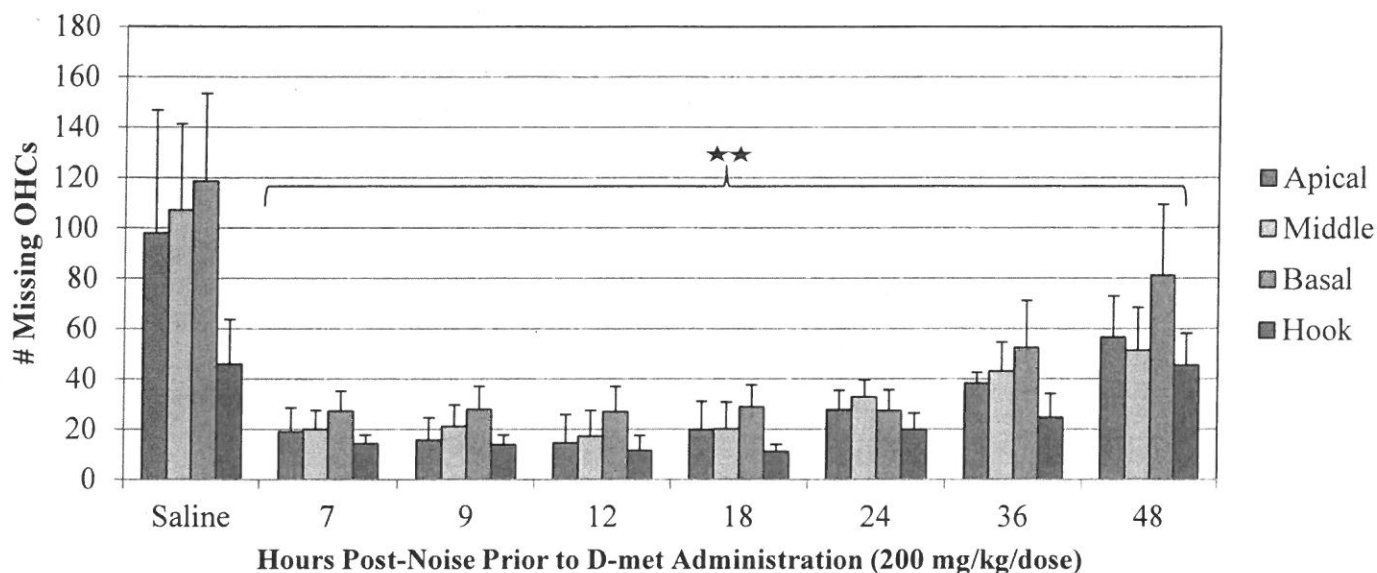


Figure 6. OHC cytochrome analysis at sacrifice 21 days post steady state noise exposure ($n = 10/\text{group}$). Significant missing OHC reductions were measured in D-met-protected groups for all time delays at all but one cochlear region. All OHC count averages but one were significantly different from controls at the 0.01 level. Statistically significant results ($p \leq 0.05$; $p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by two stars.

Rescue D-methionine (D-met) Protection from Impulse Noise Exposure: ABR Thresholds

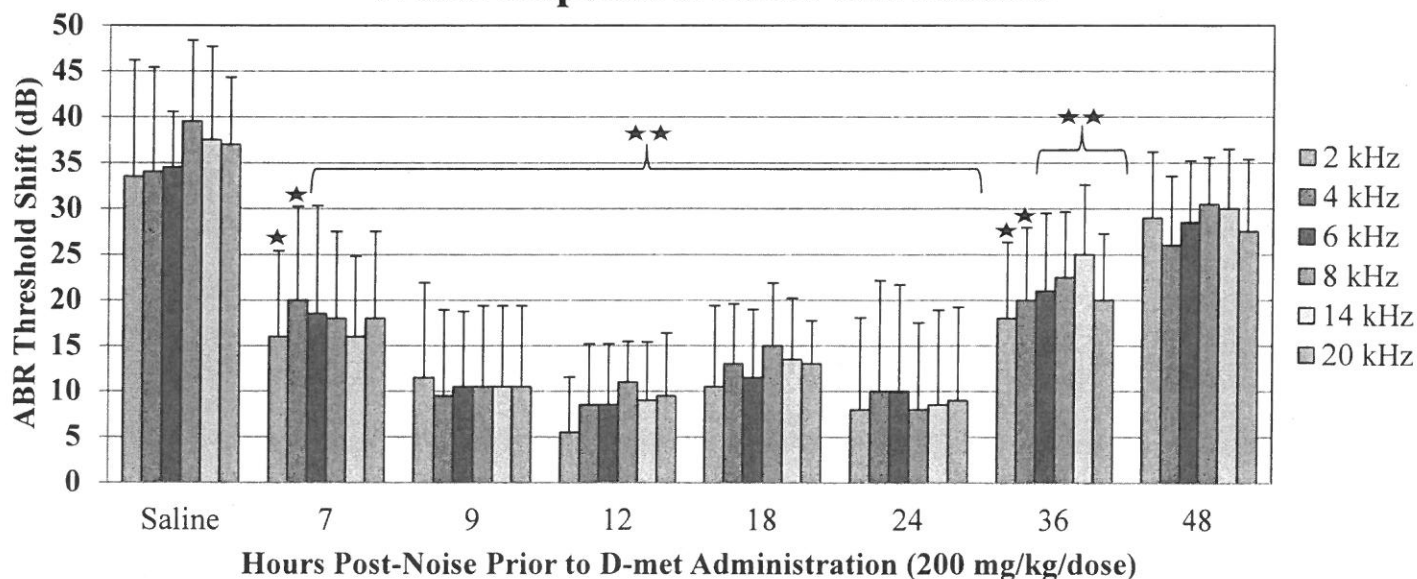


Figure 7. Impulse noise-induced ABR threshold shifts in chinchillas 21 days after simulated M-16 weapon fire at 155 dB peSPL 150 impulses at 2/s ($n = 10$ per group). D-met significantly protected from impulse noise

exposure when administration began as late as 24 hours post-noise exposure. Statistically significant results ($p \leq 0.05$; $p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by one or two stars, respectively.

Rescue D-methionine (D-met) Protection from Impulse Noise Exposure: OHC Counts

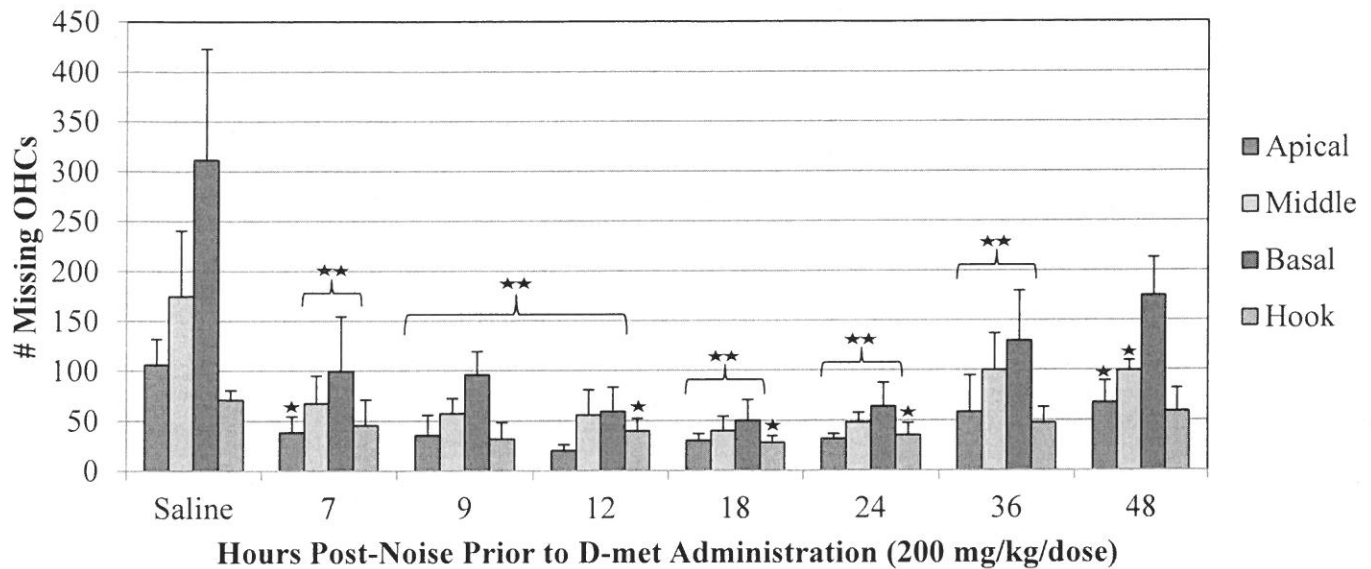


Figure 8. OHC cytochrome analysis at sacrifice 21 days post impulse noise exposure ($n = 10/\text{group}$). Significant missing OHC reductions were measured in D-met-protected groups for all time delays at all but four cochlear regions. Statistically significant results ($p \leq 0.05$; $p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by one or two stars, respectively.

Rescue D-methionine (D-met) Protection Recovery from Steady State Noise Exposure: ABR Threshold Shifts

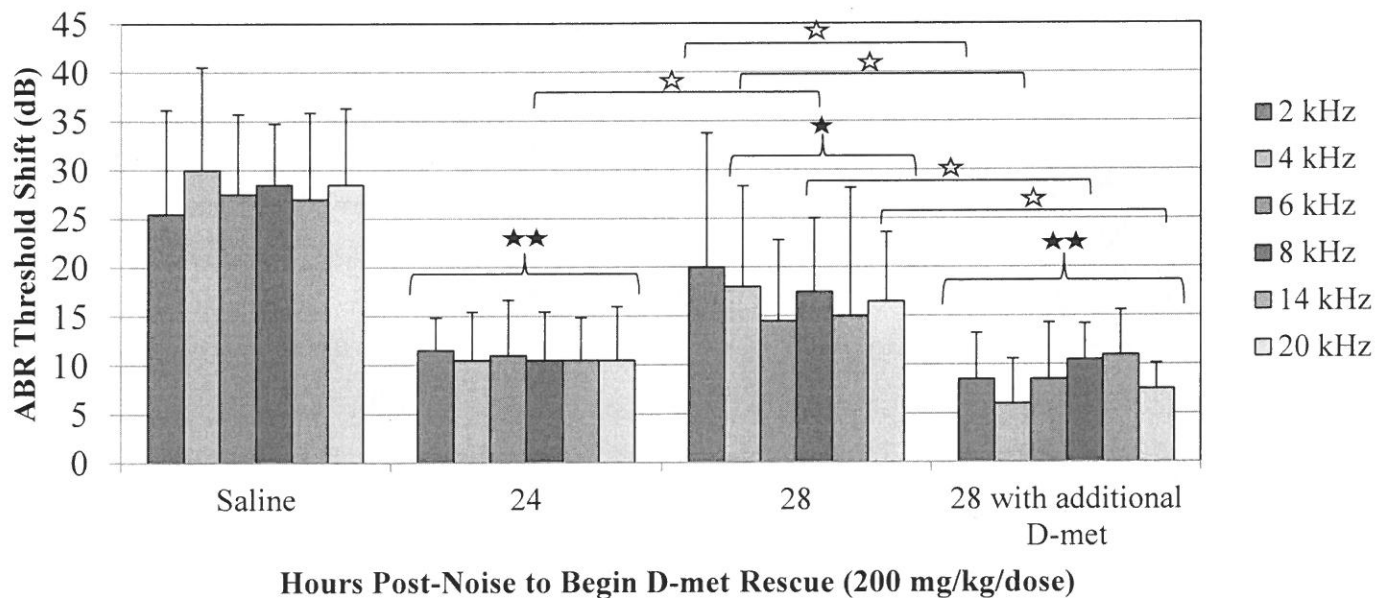


Figure 9. Steady state noise-induced ABR threshold shifts in chinchillas 21 days after a 105dB peSPL 6 hour noise exposure centered at 4 kHz ($n = 10$ per group). When 4 additional D-met doses were administered at suboptimal rescue time points, D-met protection was recovered. Statistically significant results ($p \leq 0.05$; $p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by one or two stars, respectively. Closed stars

represent significant differences compared to controls. Open stars represent significant differences between treatment groups.

Rescue D-methionine (D-met) Protection Recovery from Steady State Noise Exposure: OHC Counts

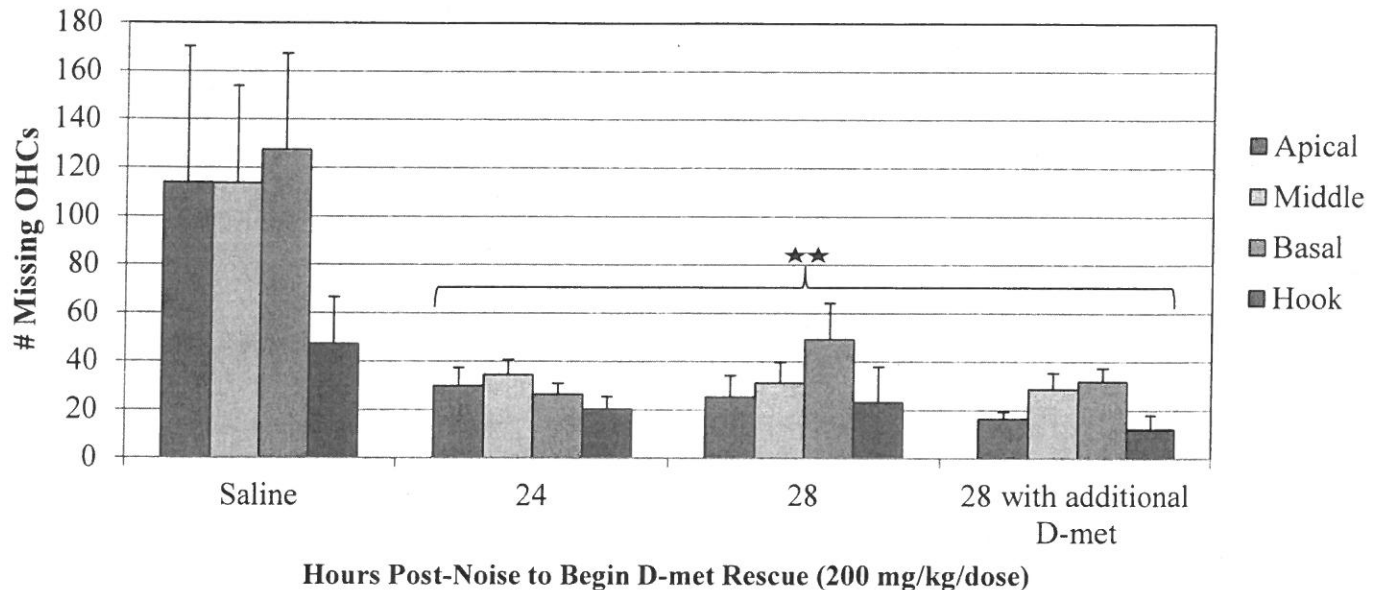


Figure 10. OHC cytochrome analysis at sacrifice 21 days post steady state noise exposure (n = 10/group). Significant missing OHC reductions were measured in all D-met-protected groups for all time delays at all cochlear regions. Statistically significant results ($p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by two stars.

Rescue D-methionine (D-met) Protection Recovery from Impulse Noise Exposure: ABR Threshold Shifts

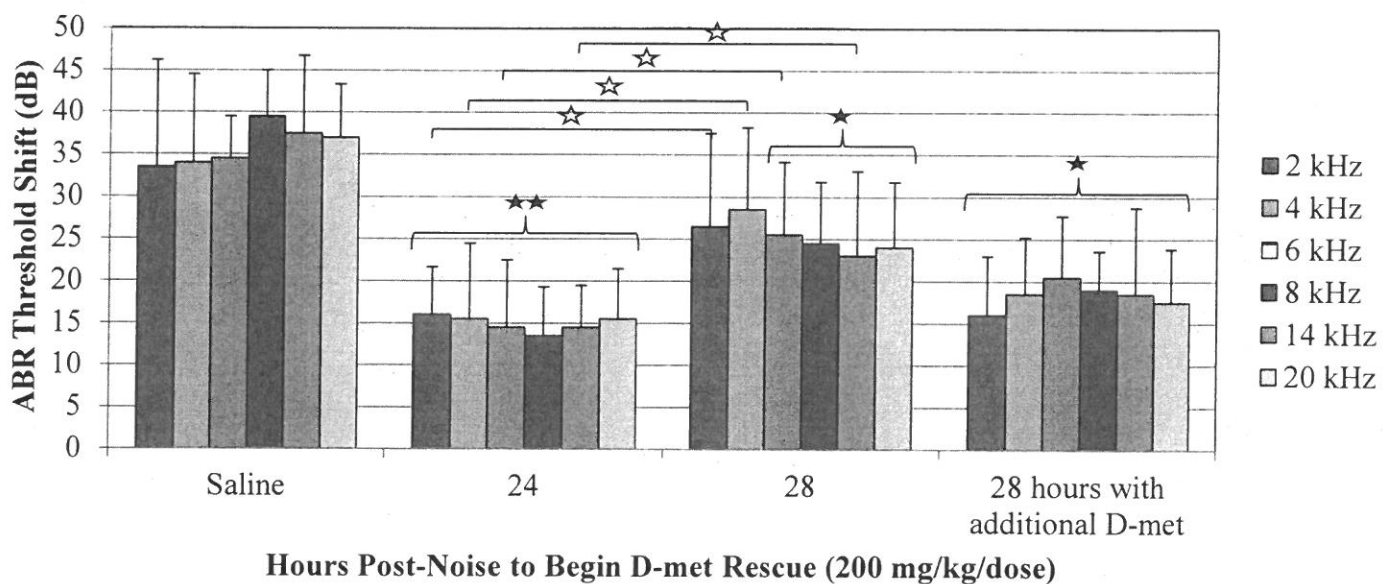


Figure 11. Impulse noise-induced ABR threshold shifts in chinchillas 21 days after simulated M-16 weapon fire at 155 dB peSPL 150 impulses at 2/s (n = 10 per group). When 4 additional D-met doses were administered at suboptimal rescue time points, D-met protection was recovered. Statistically significant results ($p \leq 0.05$; $p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by one or two stars, respectively. Closed stars

represent significant differences compared to controls. Open stars represent significant differences between treatment groups.

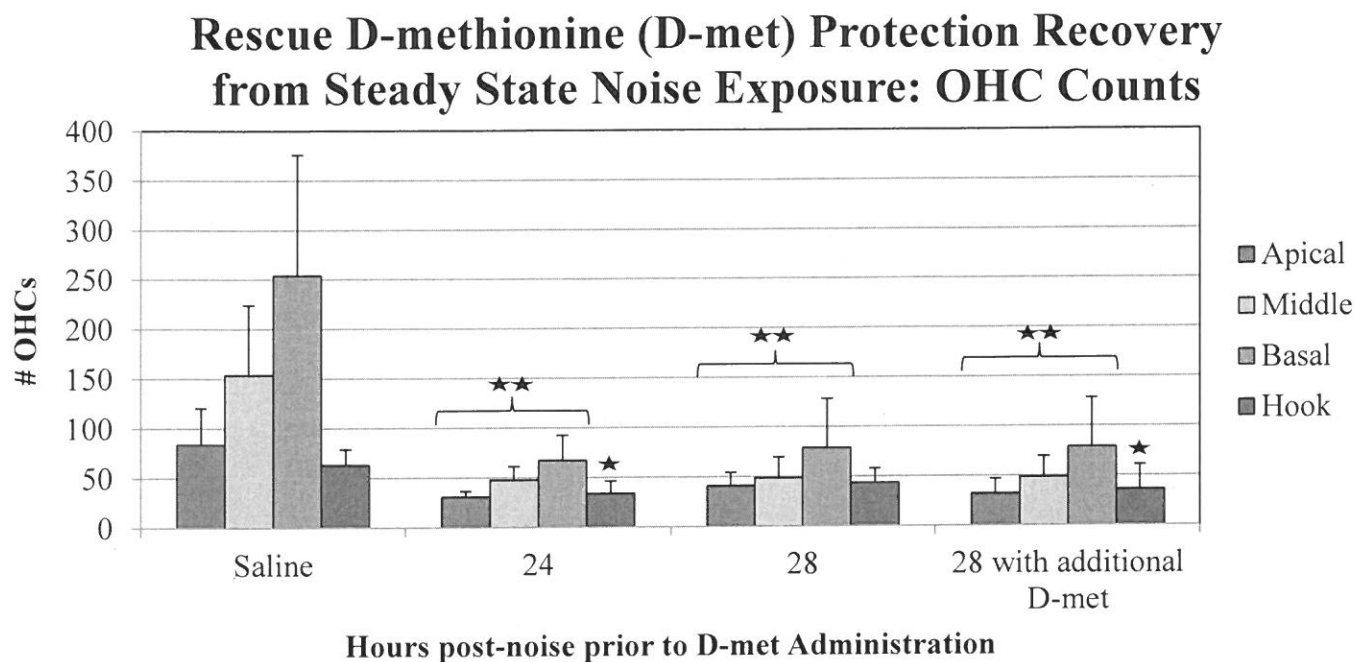


Figure 12. OHC cytochrome analysis at sacrifice 21 days post impulse noise exposure (n = 10/group). Significant missing OHC reductions were measured in all D-met-protected groups for all time delays at all but one cochlear region. Statistically significant results ($p \leq 0.05$; $p \leq 0.01$) measured by a Tukey's post-hoc follow up are indicated by one or two stars, respectively.