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Hearing Preservation in Guinea Pigs with Long-Standing Endolymphatic Hydrops

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Abstract

Hypothesis—Interruption of the excitotoxic and inflammatory pathways implicated in endolymphatic hydrops (ELH)-associated hearing loss (HL) should afford hearing protection at the neuronal level.

Background—Previous work in our lab in the mouse model of ELH shows that DMSO, an anti-inflammatory solvent, can slow the progression of HL before neuronal degeneration occurs. Riluzole, a glutamate release inhibitor, may provide synergistic benefit. This study was designed to quantify the effects of DMSO and riluzole in a long-term model.

Methods—Guinea pigs with surgically-induced ELH were sorted into three groups: riluzole +DMSO (G1), DMSO alone (G2) and untreated controls (G3). Animals in Groups 1 and 2 received daily injections of the study drug(s). All animals underwent ABR evaluation every 4 weeks until 24 weeks, when they were sacrificed. Cochleae were preserved; spiral ganglion density was quantified. Animals without hydrops were excluded from the study as surgical failures.

Results—Animals from all groups developed unilateral HL. At the end of the experiment, HL was significantly lower in Group 1 relative to Group 3 ($p=0.049$) and trended toward lower in Group 2 relative to Group 3 ($p=0.097$). Groups 1 and 2 were not different ($p=0.311$). At the cellular level, there is no evidence of neuronal degeneration in either treated group, while there is significant neuronal degeneration in the untreated group.

Conclusion—These results confirm the hearing protection observed with DMSO in short-term studies. However, unlike the previous study which showed no additive benefit to riluzole, the combined treatment group in this study showed a hearing-protective effect at 24 weeks. This indicates a potential additive benefit conferred by riluzole toward long-term hearing protection. The study also finds evidence of statistically-significant neuronal protection with both treatment groups. Overall, study provides additional evidence that DMSO and riluzole may preserve or slow the long-term progression of ELH-associated HL.

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INTRODUCTION

Although Ménière's disease (MD) was first defined 150 years ago, its etiology has remained enigmatic. While scientific advances, including the identification of endolymphatic hydrops (ELH) as a histopathologic correlate(1,2)and the development of a reliable animal model, (3,4)have enabled better understanding of its mechanisms, treatments to prevent hearing loss are still unavailable.

Numerous studies have suggested that damage to spiral ganglion neurons as the primary pathologic event leading to hearing loss. (5–7)Evidence suggests that a biochemical process causes ultra-structural damage to the neurons(8), triggering hearing loss and eventually spiral ganglion degeneration.(9) Particular attention has focused on the role of glutamate in the mediation of cochlear damage.

Glutamate is the chief neurotransmitter at the inner hair cell (IHC)-SGN interface, (10)with activity at the *N*-methyl *D*-aspartate (NMDA), kainate and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) receptors.(11)In excitotoxic conditions, excess glutamate is thought to over-stimulate post-synaptic receptors, allowing a massive ion influx into spiral ganglion neurons. It is thought that this influx results in cellular stress by activating the pro-apoptotic caspase cascade. (12)

Controlled infusion of AMPA (direct stimulation of the AMPA receptor to mimic glutamate activity), into the cochlea of adult guinea pigs produced marked ABR threshold elevations(13)and morphological changes similar to those seen in ELH-associated hearing loss(8,14,15)

Evidence suggests that inflammation and oxidative stress may play a role in the pathogenic sequence of glutamate excitotoxicity. Glutamate is known to stimulate NMDA-associated neuronal nitric oxide synthase (nNOS),leading to an increase in nitric oxide (NO) production. At high levels, NO causes the formation of apoptosis-inducing superoxide anions and peroxynitrate by reversible inhibition of oxidative respiration. (16)Immunohistochemical analysis also shows high expression of oxidative stress markers in the spiral ganglia of guinea pigs with induced ELH.(17)Researchers have also noted elevated systemic levels of Hsp70, a marker of oxidative stress, in patients with confirmed Meniere's Disease.(18)

Evidence from studies of noise-induced hearing loss (NIHL) suggests that riluzole blockade (or release inhibition) confers hearing protection. Riluzole (a glutamate release inhibitor) reduces or prevents NIHL in guinea pigs.(19)Carbamathione, which down-regulates the NMDA receptor itself, confers significant protection in models of NIHL.(14) Kynurenic acid, a broad-spectrum glutamate antagonist, provides protection against NIHL.(11)

Based on this evidence from other models of hearing loss, and to see if antagonizing the glutamate excitotoxic pathway could slow the development of ELH-associated hearing loss, we designed an experiment to evaluate the effects of riluzole and DMSO (an anti-inflammatory, anti-oxidant solvent) in a mouse model of short-term ELH-related HL.(20)In that report, animals receiving DMSO with riluzole or DMSO alone had significantly better hearing than untreated controls. However, while the addition of riluzole appeared to confer some additional hearing protection (over DMSO alone), the difference was not statistically significant.

While this study provided compelling evidence that DMSO can ameliorate short-term progression of hearing loss, it provided less information about the role of riluzole. Although the negative result could be true (that is, riluzole is an ineffective treatment), confounders

may have obscured its protective effect. In particular, the mouse is thought to have a relatively impermeable blood-brain (BBB) and blood-labyrinthine barrier (BLB) relative to other rodents.(21)Moreover, the drug doses used were derived from studies of NIHL in guinea pigs(19); mice may require a different dose.

This study attempts to test whether blockade of the glutamate excitotoxic pathway slows or prevents hearing loss in a different model. Since its initial description, surgical induction of ELH in the guinea pig has been the gold standard animal model for MD research. Using guinea pigs helps address specific concerns germane to mice and the previous study. We hypothesize that blockade of the glutamate excitotoxic pathway should confer functional hearing protection.

The design of this experiment enables us to collect data on the histological changes associated with ELH and our treatments. In the mouse model study, even untreated hydropic animals did not have degenerative histological changes on light microscopy at P30(the time limit of the experiments); we inferred that the observed hearing protection was thought to be due to a slowing of ultrastructural cell damage.(20)However, at six months post-operatively, guinea pigs with induced ELH have significant spiral ganglion degeneration; histological degeneration can be evaluated.(5)We hypothesize that treated animals will have significantly less spiral ganglion degeneration.

MATERIALS AND METHODS

Following right-sided induction of ELH, animals were assigned to one of three treatment groups: riluzole+DMSO (Group 1), DMSO alone (Group 2) or no treatment (Group 3). Animals in Groups 1 and 2 received daily drug injections, Group 3 animals did not.

Animals were maintained for six months post-operatively. All animals underwent electrophysiological testing (auditory brainstem response) at regular intervals. After six months, animals were sacrificed and bilateral cochleae preserved for histological analysis.

Animals

The Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University (CWRU) approved the care and use of the animals used in this study, Protocol #2008-0059.

Albino female guinea pigs with an initial weight of 250g–300g were obtained from Charles River Laboratories (Wilmington, MA). All animals were housed in approved cages through the Animal Resource Center (ARC) at CWRU. Standard care was provided per ARC protocol.

Induction of ELH

ELH was induced using a modified version of the classic surgery described by Kimura and Schuknecht.(3) Following the previously-described posterior fossa approach,(22)the right endolymphatic duct was obliterated and packed. A detailed description of surgical induction can be found elsewhere.(23)

Drug Preparation

Riluzole (2-amino-6-[trifluoromethoxy]benthothiazole) was purchased from Sigma-Aldrich Inc. (St. Louis, MO) and shipped in 500-mg quantities (500 mg/vial); 21.4 mL of 99.9% DMSO (Sigma-Aldrich Inc.) was directly added to the vial to make the master stock at 23.36 mg/mL.

Treatment groups

Group 1 and 2 animals received daily intraperitoneal injections, dosed by weight, of the appropriate treatment. Injections were started on post-operative day 1. Animals were weighed weekly to ensure correct drug dosing.

Group 1 animals were treated with 8mg/kg of riluzole, a dose that is known to be safe in guinea pigs.⁽¹⁹⁾ Because riluzole was dissolved in DMSO at 23.36mg/mL, the dry weight dose of 8mg/kg translates to a solution dose of 0.34mL/kg. Example: A 300g guinea pig in Group 1 would receive 0.1mL of the combined solution, or 2.4mg riluzole and 0.1mL of DMSO, each day.

Group 2 animals were given pure DMSO at 0.34mL/kg, the same volume per weight dose that was used in Group 1 animals. This dose was chosen to ensure that Group 1 and Group 2 animals received equivalent doses of DMSO.

Animals selected for the control group (Group 3) were not treated.

Electrophysiological Testing

Auditory-evoked brainstem response (ABR) testing was conducted as previously described.⁽²⁴⁾ Testing was carried out using a Tucker Davis Technology (Alachua, FL) System 3 machine. Frequencies (0.5, 2, 4, 8, 16, and 32 kHz) were presented for at least 500 sweeps to the left and right ears subsequently through high frequency transducers in a closed system. ABR thresholds were obtained by reducing the stimulus intensity from 90 dB SPL in 10 dB steps until the lowest intensity that could evoke a reproducible ABR pattern was detected. In the event of a completely deaf ear, where the maximum 90 dB SPL would not evoke any electrical response, the threshold was recorded to be 100 dB SPL. Testing was done pre-operatively then at 2, 4, 8, 16, 20 and 24 weeks post-operatively.

Average Threshold Deviation

Since the ABR protocol (described above) covered 7 different frequencies at each timepoint, we developed a single value encompassing hearing performance across all 7 frequencies to facilitate data analysis. This snapshot variable was labeled the Average Threshold Deviation (ATD). At each time point, for each frequency, the left ABR threshold (from the control ear) was subtracted from the right ABR threshold (deviation = R-L). A value less than or equal to zero represents preserved hearing, while positive values represent hearing loss. The specific deviations for the 7 frequencies were then averaged to obtain the single ATD for that particular time point.

Histology

Following euthanasia, temporal bones were harvested from both ears and preserved for histological analysis. The cochlea was perfused with phosphate buffered 2.5% glutaraldehyde through the round window after removal of the stapes from the oval window. Whole temporal bones were stored for at least 24 hours in 2.5% glutaraldehyde and subsequently decalcified in 0.35 M ethylenediaminetetraacetic acid (EDTA). For histological examination, temporal bones were embedded in paraffin and cut into 5 µm sections, then stained with hematoxylin and eosin (HE).

Spiral Ganglion Density Measurement

Spiral ganglion cells were counted in accordance with a previously-described method.^(9,25) Because previous work has shown that at the 4–6 month time point, spiral ganglion cell loss outpaces inner hair cell loss, cellular counts were focused on the ganglion cell

populations.⁹ Briefly, three non-adjacent sections were selected from each cochlea. Using an upright light microscope and 10x objective, photographs were taken of Rosenthal's canal at the apical and basal turns. With the assistance of Metamorph (Molecular Devices, Downingtown, PA), the operator used the bony borders of Rosenthal's canal to manually define regions of interest (ROI), then counted SGNs within each ROI. Metamorph automatically measured the ROI area in μm^2 . SGN counts and ROI areas from all three sections were exported to Excel (Microsoft, Redmond, WA) and used to calculate an average SGN density (expressed in cells/1000 μm^2).

The apical turn of the cochlea is generally less well preserved compared to the basal turn and because of that it was not possible to obtain accurate cell counts in the apical turns of multiple animals. To minimize possible error, all analysis focused on basal SGN densities.

After counts were complete, a spiral ganglion neuron degeneration index (SGNI) was calculated by dividing SGN density from the right ear by SGN density from the left ear ($\text{SGNI} = \text{R/L}$). In this index, a value less than one would indicate greater SGN degeneration in the right (operative) ear relative to left (control).

Exclusion criteria

Animals with pre-operative hearing loss $>10\text{dB}$ in either ear were excluded from the study. Animals without histologically-confirmed hydrops were excluded from the study.

Statistics

All data was collected and initially analyzed in Microsoft Excel (Redmond, WA), then exported for statistical analysis to the program R (R Foundation, Vienna, Austria).

A mixed-model ANOVA for repeated measures was used to test if the treatment group affected the progression of hearing loss over the entire duration of the experiment.

To determine if there were differences in average threshold deviations at Week 24, one-tailed Wilcoxon tests was used, with the assumption that a treatment would be better than no treatment.

To analyze the spiral ganglion neuron indices, one-sample Wilcoxon tests were used.

A p-value less than 0.05 was considered significant for all tests.

RESULTS

A total of 10 animals from the combined-treatment group, 9 animals from the DMSO-only group and 8 animals from the untreated group passed the exclusion criteria and were included for final analysis.

The progression of hearing loss was compared between groups across all 24 weeks of the experiment (see Fig 1 for a graphical representation of this data). Hearing loss in the guinea pig model of ELH is characterized by progressive fluctuating hearing loss over time and variations between subjects at similar timepoints are common. Because data was collected through longitudinal measurements from the same animals, standard linear regression techniques were not appropriate for this comparison. Consequently, a mixed model 2-way ANOVA for repeated measures was used to answer whether treatment group affects the overall progression of hearing loss. A p-value <0.05 indicates a significant difference.

When testing whether the line for the combined-treatment animals is different than for untreated animals, $p=0.085$. For DMSO-only animals against untreated animals, $p=0.041$. Between the combined-treatment and DMSO-only treatment groups, $p=0.58$.

ATDs at Week 24 (the experimental endpoint) from all included animals, as well as the respective group means, can be found in Table 1. All three groups demonstrated significant hearing deterioration at Week 24. To determine if there was a statistical difference in magnitude of loss between groups, Wilcoxon rank-sum tests were used to test specific hypotheses (Table 2).

Histological analysis was performed and spiral ganglion degeneration indices calculated to measure neuronal degeneration in the basal turns. After animal sacrifice, cochleas were preserved for histology. Unfortunately, during the preservation and sectioning process, not all cochleas were adequately preserved. Some animals were excluded from histological analysis because poor cochlear preservation and/or significant artifact made accurate neuron counts impossible. Animals were included in the histological analysis are as follows: 8 for riluzole+DMSO (combined), 6 for DMSO-alone and 8 for the untreated group.

Table 3 shows the specific indices for all animals included. Since the SGNI is the ratio of SGN count of the right ear (hydropic) to the left ear (non-hydropic), an index of 1 indicates equivalent right and left spiral ganglion densities; values less than 1 indicate right-sided degeneration. An otoprotective effect would ideally result in a SGNI equal to 1.

The null hypothesis was accepted if the mean SGNI is equal to 1 (no significant neuronal degeneration was observed), whereas the null hypothesis was rejected if the mean SGNI is different than 1 (significant neuronal degeneration was observed). The data shows that the mean SGNI of the combined treatment group and DMSO-alone group were statistically equivalent to 1 ($P=1.0$ and 0.437 , respectively), suggesting no spiral ganglion degeneration. Analysis of the untreated group showed that the mean SGNI was different and less than 1 ($p\text{-value}=0.004$). The fact that a one-sample Wilcoxon test was used and that the mean SGNI in the untreated group was 0.880 (the index was inferior to 1), this supported the presence of neuronal degeneration.

Representative samples from the riluzole+DMSO and untreated groups can be seen in Figure 2.

DISCUSSION

While the etiology of ELH-associated hearing loss remains unclear, evidence from animal models and human studies implicates glutamate excitotoxicity as the principle pathological insult. This group previously analyzed the efficacy of riluzole, a glutamate release inhibitor, and DMSO, an anti-inflammatory, anti-oxidant solvent, in a mouse model of short-term ELH related hearing loss.⁽²⁰⁾ This study serves as a follow-up, examining the effects of the same treatment regimen in guinea pigs with long-term surgically-induced hydrops.

Electrophysiology (ABR)

As expected, all animals in this study developed progressive hearing loss that worsened with time. A mixed-model 2-way ANOVA was used to evaluate whether the qualitative difference in hearing profiles (see Fig. 1) was a statistically-significant observation. The analysis shows that there is a significant difference in magnitude of hearing loss over time between untreated animals and DMSO-only animals ($p=0.041$), and suggests a trend toward a difference between untreated animals and combined-treatment animals ($p=0.085$). This

suggests that, when treated with DMSO, guinea pigs with induced ELH develop less hearing loss than untreated controls.

Since this is a longitudinal analysis factoring each time-point into the equation of the statistical analysis resulting in an overall analysis of all ATD at all different time-points, data can be affected by various factors at each timepoint. Unpredictable fluctuation in the hearing threshold at each individual timepoint may well account for the absent overall difference in hearing between the combined treatment and untreated group. Another explanation is the theoretical non-synergistic interaction between DMSO and riluzole that remains to be elucidated.

One would expect that a potential benefit of a drug could only be asserted after sufficient time had elapsed to allow for the manifestation of the cellular injury. Therefore, conceivably the combined-treatment arm targeting the neurotoxic pathway would have demonstrated an otoprotective effect only at the later time-points in the study. The otoprotective effects of the combined-treatment group would have been attenuated in the overall analysis of the ATD.

Therefore, a separate analysis of the ATD was conducted at the latest time-point (24 weeks).

We chose to test that the respective comparative groups are inferior as oppose to just “equal or different” not only to demonstrate a difference but also a benefit (smaller ATD signifies better hearing protection).

We then examined whether there was a difference in hearing at the experimental endpoint (Week 24). On average, the untreated animals experienced 26.4 dB of hearing loss across all seven tested frequencies. The DMSO-treated animals experienced only 18.4 dB of loss at Week 24 ($p=0.097$ vs. untreated), while the combined-treatment animals experienced 14.2 dB of loss ($p=0.049$ vs. untreated).

This indicates that the combined-treatment group shows evidence of significant hearing protection at Week 24.

However, in both analyses, there is no significant difference between treatment groups. At 24 weeks, the small observed difference – the DMSO-only threshold is 4.2dB higher – is not significant ($p=0.311$).

Overall, these two forms of analysis show that both treatments are able to alter the course of hearing loss in this model. Since there is no difference (or suggestion of difference) between the combined and DMSO-alone treatment groups, this data suggests that DMSO independently confers a degree of hearing protection.

Histology (spiral ganglion degeneration)

Researchers have suggested that ultrastructural injury to spiral ganglion neurons is responsible for observable hearing loss prior to development of gross damage on light microscopy.(8)This ultrastructural damage eventually culminates in the spiral ganglion cell death.(5)Given this background, it is logical to hypothesize that an effective protection regimen should confer protection against ultrastructural damage and, in the long term, slow or prevent neuronal degeneration.

In this study, spiral ganglion densities were quantified through the basal turns of animals from all three treatment groups. (Fig 2.) In the untreated group, which represents the natural course of disease, the degree of degeneration was consistent with the published literature on guinea pigs with surgically-induced ELH.(5)

There is evidence for significant cellular degeneration in the untreated group. For both treated groups, however, there is no evidence of significant cellular degeneration. One-sample Wilcoxon tests show that the riluzole+DMSO and DMSO-alone animals showed no significant degeneration of spiral ganglion neurons compared to untreated animals.

Along with evidence showing significant hearing protection in the combined treatment group at 24 weeks (and a trend towards protection for the DMSO-alone group) and the hearing protection seen over all 24 weeks for the DMSO-alone group, this evidence supports that assertion that DMSO, alone or in combination with riluzole, offers a neuronal protective effect.

The efficacy of DMSO

At both the electrophysiological and histological level, this study finds evidence that DMSO is an active agent that independently confers protection against ELH-associated hearing loss.

Previous studies have attributed DMSO's neuroprotective abilities to its anti-inflammatory, anti-oxidant, and calcium channel blocking properties.(26–28)These anti-inflammatory and anti-oxidant properties may help mitigate the consequences of nitric oxide overproduction (and subsequent free radical production) in ELH.(29)

Additionally, studies from the central nervous system have shown that DMSO suppresses induced currents through both the AMPA and NMDA receptors, and attenuates calcium responses to glutamate.(27)Blocking this ion influx may consequently prevent activation of a pro-apoptotic cascade,(12)which is thought to be a key mechanism through which glutamate causes excitotoxicity.

DMSO's anti-inflammatory and anti-oxidant properties may also provide protection by addressing ELH related dysfunction within the spiral ligament. In guinea pigs with surgically-induced ELH, evidence of cellular stress (30)in type I fibrocytes of the spiral ligament was seen as early as post-operative day one. (31,32)

The efficacy of riluzole

At the end of the previous study, it was suggested that blood-brain barrier impermeability may hinder the efficacy of systemically-administered riluzole. Although a previous study confirmed that riluzole crosses the murine BLB(33), those researchers administered doses in excess of 30mg/kg, far above the toxic limit.(19)

However, published data shows that an 8mg/kg systemic dose of riluzole can confer hearing protection against acoustic trauma, meaning that effective BBB/BLB penetration occurs in the guinea pig.(19) Consequently, a goal of this paper was to examine whether that non-toxic dose is effective at preventing ELH-associated hearing loss in an animal with a more-penetrable blood-brain barrier. This study suggests a potential benefit.

One possible explanation is found in the differential pharmacological parameters that affect riluzole availability and clearance. While Hockly et al(33)were able to achieve BLB penetration at high doses of riluzole in mice, marked decreases in brain and serum riluzole levels were observed within the first six hours. The authors also noted that the high clearance rate of laboratory mice would make achieving steady-state brain concentrations through IP delivery impractical.

Although Hockly et al(33) showed limited bioavailability of riluzole in mice, it is likely that riluzole metabolism in guinea pigs is different.

ELH is thought to be different than acute insults such as acoustic trauma (19), with chronic over-release of glutamate causing progressive, cumulative damage. This suggests that pharmacological treatment of ELH-associated hearing loss requires long-term anti-glutamate action. Theoretically, a once-daily injection would not be able to achieve steady-state concentrations of riluzole. However, the synergistic benefit seen in the data could be explained by a residual cellular effect that offers additional protection beyond the duration of its systemic availability. More work on the pharmacokinetics and pharmacodynamics of riluzole in the guinea pig model is needed.

When analyzing the overall hearing protection over the complete experiment, the combined treatment did not seem to offer an additive protective effect compared to treatment with DMSO alone, except when examined at the 24 week endpoint. Because DMSO is able to block the NMDA and AMPA receptors (27), both drugs are theoretically interfering with the same pathway. Because the end effects of release inhibition and receptor blockade are essentially the same, the downstream receptor activity of DMSO may mask the upstream anti-release effects of riluzole. The ideal experiment would include riluzole delivered alone. Unfortunately riluzole is highly lipophilic and can only be accurately dosed and delivered IP after being dissolved with a strong detergent. The only laboratory safe non-toxic agent available to accomplish this aim is DMSO, hence the combined arm of the experiments as outlined in a related publication (20).

However, this potential interaction would be more consequential in short term hearing protection. In the long-term, the cumulative effect of chronic excitotoxic injury may overcome the isolated benefits of DMSO.

Future directions

We believe future modifications will be necessary to enhance the delivery of riluzole to the target cells of the cochlea and to isolate/identify its specific effects.

One possibility is an intracochlear pump, which would be able to deliver a defined concentration of riluzole. Multiple studies have confirmed the efficacy of this delivery method which allows for precise, reproducible dosing. Alternatively, oral riluzole – delivered in food or drinking water – may be an effective method.(33,34)Although inexact dosing adds an element of unpredictability, this method allows frequent drug delivery without requiring an invasive pump.

Lastly, it will be necessary to study riluzole independently of DMSO, in a non-active solvent.

CONCLUSION

This study shows that DMSO can confer pharmacologic protection against long-term hearing loss in a guinea pig model of ELH, confirming results from a previous short-term study of DMSO in mice. This study is also able to show augmentation to long term hearing outcomes with the addition of riluzole to DMSO which represents a new finding not seen in our previous report of short term hearing protection in the mouse model. As discussed above, additional studies with specific modifications will be necessary to more clearly identify the effects of riluzole.

This study reinforces the notion that glutamate or some variant of related excitotoxicity is an important factor in the pathology of ELH-associated hearing loss. Although the data does not show any benefit to blocking glutamate release specifically, it does show functional and histological benefits to interfering with the downstream excitotoxic pathway. The results of

this study strongly suggest that future research into potential therapies for ELH-associated hearing loss should focus on blocking or attenuating the effects of excitotoxicity.

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Figure 1.

Hearing performance over the duration of the experiment. This plot shows the relationship between time and average threshold deviation (ATD), and allows comparison between the three treatment groups. The untreated group represents the natural history of the disease. An ANOVA for repeated measures shows the untreated group has significantly different progression of hearing loss than the DMSO-alone group ($p=0.041$), and suggests a trend toward a difference between the combined-treatment and untreated groups ($p=0.085$). This analysis takes into consideration the overall change in ATD. Initial ATDs are not zero because animals were not excluded for frequency-specific threshold deviations ≤ 10 dB; the resulting ATDs were non-zero values. However, there is no significant difference in ATDs between groups at Week 0. A one-tailed Wilcoxon test was used to analyze the differences in the mean ATD at 24 weeks. The combined-treatment group showed a significant difference from the untreated group when ATD was analyzed at 24 weeks ($p=0.049$). A trend was observed in the difference between the DMSO-alone group and the untreated group ($p=0.096$).

Figure 2.

A/B: Images of Rosenthal's canal, captured from the basal turn of the right cochlea in guinea pigs from Group 1 (A) and Group 3 (B). The region of interest, within which cell density was calculated, is outlined in red. Arrows in panel B point to probable regions of spiral ganglion degeneration. Animals from the combined-treatment and DMSO-alone groups have significantly less degeneration than untreated animals. Staining with hematoxylin and eosin.

Table 1
Average Threshold Deviations at Week 24

Average Threshold Deviations (ATD) at Week 24 for all animals. ABR thresholds were determined at seven frequencies for each time point, and an R – L deviation was calculated to quantify hearing loss; the ATD is the average value of the seven frequency-specific deviations. A higher ATD signifies greater magnitude of hearing loss.

	Combined	DMSO-alone	Untreated
Week 24 Average Threshold Deviations (dB)	27.143	11.429	11.429
	2.857	37.143	22.857
	-4.286	1.429	14.286
	10.000	8.571	30.000
	28.571	28.571	41.429
	15.714	0.000	38.571
	14.286	34.286	10.000
	11.429	11.429	42.857
	11.429	32.857	
	27.143		
Mean:	14.429	18.413	26.429
St.Dev:	10.784	14.733	13.681

Table 2
Comparisons between Week 24 Average Threshold Deviations

P-values generated by specific one-tailed Wilcoxon signed-rank tests, to determine if there was a significant difference in overall hearing performance at Week 24. Group 1 = riluzole+DMSO (combined). Group 2 = DMSO-alone. Group 3 = untreated.

Hypothesis	P-value
Group 1 < Group 2	0.311
Group 2 < Group 3	0.096
Group 1 < Group 3	0.049

Table 3
Spiral Ganglion Neuron Indices at Week 24

Spiral ganglion neuron (SGN) degeneration indices for all included animals, sorted by group. Comparison of SGN indices showed statistically significant preservation of neurons in the basal turns of the cochleae from treated animals. An SGNI =1 denotes the absence of neuronal degeneration on the hydropic (induced) side. The data shows that the mean SGNI of the combined treatment group and DMSO-alone group were statistically equivalent to 1 ($P= 1.0$ and 0.437 , respectively), suggesting no spiral ganglion degeneration. Analysis of the untreated group showed that the mean SGNI was different and less than 1 ($P= 0.004$) supporting significant neuronal degeneration.

	Combined	DMSO-alone	Untreated
SGNI	1.027	0.945	0.844
	0.865	0.914	0.786
	0.868	0.904	0.932
	1.236	0.932	0.923
	0.843	1.330	0.945
	1.370	0.951	0.872
	1.044		0.834
	0.918		0.905
Mean:	1.021	0.996	0.880
St.Dev:	0.192	0.165	0.056
P-value	1.000	0.437	0.004