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The effect of anti-inflammatory drugs on hair cell regeneration in the zebrafish lateral line

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THE EFFECT OF ANTI-INFLAMMATORY DRUGS ON HAIR CELL REGENERATION IN THE ZEBRAFISH LATERAL LINE

by

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Abstract: Hair cell death and regeneration on the three distal-most neuromasts in the posterior lateral line of zebrafish larvae were investigated after neomycin treatment. Treatment with ibuprofen, dexamethasone and salicylate examined the role of a normal inflammatory response on hair cell regeneration. Regeneration was observed 48 hours post-treatment indicating these reagents have no effect on the regeneration process.

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The Use of a Zebrafish Model

Zebrafish eggs undergo external fertilization which allows for *in vivo* examination during any stage of embryonic development. Due to an optically transparent appearance and small size, zebrafish larvae serve as a beneficial model for experimentation. Fluorescent dyes and stains can easily be applied to large groups of fish simultaneously allowing for quick examination without dissection. In addition, zebrafish larvae develop quickly and exhibit high fecundity once they reach adulthood (Chiu, Cunningham, Raible, Rubel, and Ou, 2008; Hertog, 2005; Whitfield, 2002).

The zebrafish functions as a useful model for the study. Although zebrafish do not have outer or middle ears, inner ears resemble those typical of vertebrates in development and anatomy. Hair cells within the lateral line compare structurally, functionally and molecularly to those of the mammalian inner ear (Chiu et al., 2008; Ma, Rubel, and Raible, 2008; Nicolson, 2005; Whitfield, 2002).

The Zebrafish Lateral Line

In zebrafish, mechanosensory organs responsible for detecting sound and movement can be found in the inner ear and the lateral line organ. The zebrafish lateral line is a sensory system composed of specialized sense organs located in a reproducible pattern (Ledent, 2002). These specialized sense organs, known as neuromasts, contain a sensory epithelium consisting of mechanosensory hair cells and non-sensory supporting cells. Hair cells within each neuromast lie in a rosette formation surrounded by supporting cells and corresponding afferent central axons that project to the hindbrain (Pujol-Martin and Lopez-Schier, 2013). The neuromasts cover the zebrafish head and body. Those found on the head form the anterior lateral line system and

those on the body, caudal fin and tail make up the posterior lateral line system (Harris et al., 2003; Ledent, 2002; Nicolson, 2005). Larval zebrafish have a few superficial neuromasts which mature into numerous complex sensory hair cell systems (Pujol-Martin and Lopez-Schier, 2013; Whitfield, 2002).

Lateral line hair cells extend into a gelatinous cupula that moves in response to water flow. Zebrafish use the lateral line sense organs for detecting changes in water current and low frequency vibrations, both of which aid in schooling and safety from predators (Buck, Winter, Redfern, and Whitfield, 2012; Harris et al., 2003; Nicolson, 2005; Owens et al., 2009). Each neuromast detects sensory information from a specific position along the animal's body (Pujol-Martin and Lopez-Schier, 2013).

The lateral line forms early in embryonic development. Neuromasts first appear in the head and then migrate towards the tail. Around 20 hours post fertilization (hpf) the posterior lateral line consists of seven to eight cellular rosettes stretching from the head to the tail. As the animal develops into adulthood, these rosettes evolve into multiple neuromasts grouped in lines on both sides of the body (Ledent, 2002; Pujol-Martin and Lopez-Schier, 2013). This complex structure continues to develop throughout life. However, from 5 five days post fertilization (dpf) until adulthood, the posterior lateral line does not undergo any appreciable changes in sensitivity to mechanical stimuli. Therefore zebrafish can be used for histological and functional experiments by 5 dpf (Buck et al., 2012).

Aminoglycoside Antibiotics and Ototoxicity

Aminoglycosides are a class of antibiotics that are highly effective against gram-negative bacteria. Commonly this class of drugs is used to treat tuberculosis, complications of cystic fibrosis and other serious bacterial infections. Due to their broad antibacterial spectrum, they

have been utilized clinically since the 1940s (reviewed in Forge and Schacht, 2000; Owens, et al., 2009). Aminoglycosides can also have adverse side effects on the inner ear. Although the exact structural mechanisms of aminoglycoside ototoxicity remain unclear, evidence suggests involvement of formation of free radicals and apoptotic cell death pathways. While different aminoglycosides show varying degrees of ototoxity, all induce hair cell death (Harris, et al., 2003; Owens, et al., 2009). In particular, neomycin has been classified as highly toxic to cochlear hair cells. Aminoglycoside toxicity targets the Organ of Corti and destroys its structures. Hair cell death occurs in a base-to-apex pattern, resulting in a high frequency hearing loss. Outer hair cells are most affected, but with continued exposure, inner hair cells are also damaged. In addition, the stria vascularis becomes thinner with a decrease in marginal cells. Despite these adverse effects, aminoglycosides continue to be the most commonly used antibiotics worldwide due to their high effectiveness and low cost (reviewed by Forge and Schacht, 2000; Schacht and Hawkins, 2006).

Hair Cell Death and Regeneration

Vertebrates possess mechanosensory hair cells that are essential for normal hearing and detection of head movements and orientation. Hair cells in the vertebrate inner ear, as well as those of the lateral line organs of fish, are susceptible to damage from aminoglycoside ototoxicity. In mammals, once such damage occurs hair cells do not regenerate. As stated by Warchol (2011) “The ability to regenerate hair cells was lost during the evolution of the mammalian ear, for reasons that remain unknown” (p. 72).

In contrast, nonmammalian vertebrates (e.g., fish, amphibians, reptiles and birds) possess the ability to regenerate hair cells after damage. The exact mechanisms behind regeneration

remain incompletely understood, but evidence suggests both mitosis of supporting cells and direct transdifferentation. Direct transdifferentiation involves the process of postmitotic supporting cells differentiating into hair cells without mitosis (Roberson, Alosi, and Cotanche, 2004; Warchol, 2011).

The aminoglycoside antibiotic neomycin is effective at inducing hair cell death in the lateral line of zebrafish. Chiu et al. (2008) used neomycin to screen for ototoxicity in hair cells in the lateral line of zebrafish and concluded that neomycin has a toxic, dose-dependent effect on the hair cells. Similarly, Harris et al. (2003) reported significant hair cell loss following neomycin treatments, and the overall decrease in hair cell survival increased with increasing neomycin concentrations. This hair cell damage secondary to neomycin exposure occurs rapidly and consistently (Buck et al., 2012).

Numerous studies have demonstrated regeneration of hair cells in the zebrafish lateral line after neomycin exposure. According to Harris et al. (2003) lateral line hair cells of larval zebrafish regenerate after aminoglycoside damage as early as two days following aminoglycoside exposure. The findings of Harris et al. (2003) support the notion that mitosis mediates hair cell regeneration. Ma et al. (2008) obtained similar findings to Harris et al. (2003) using neomycin to induce hair cell death in zebrafish larvae. Following treatment, increases in proliferation of supporting cells were observed as early as 12 hours after exposure. One-third of the initial hair cell population was restored by 24 hours post-treatment and 80% by 48 hours after neomycin treatment. Similar studies support the zebrafish larvae ability to regenerate hair cells in the lateral line after neomycin-induced damage (reviewed in Coffin et al., 2010; Mackenzie and Raible, 2012; Owens et al., 2009).

Immune System Regulation of the Regeneration Process

Tissue damage quickly evokes a sequence of cellular responses in order to stimulate repair. Wounded tissues sense physical and chemical changes that result from injury initiating cellular migration near the lesion site. In mammals, this involves a complex yet rapid process that begins with damage recognition. Different types of tissue damage produce unique signal combinations, and several mechanisms work together to alert nearby tissues that damage has occurred. Such mechanisms involve, but are not limited to, changes in calcium (Ca^{2+}), hydrogen peroxide (H_2O_2), and adenosine triphosphate (ATP). Cordeiro and Jacinto (2013) report that *in vivo* and *in vitro* vertebrate and invertebrate model systems utilize Ca^{2+} as a damage signal that mediates instantaneous cellular effects. Injury causes an influx of intracellular Ca^{2+}. H_2O_2 are a type of reactive oxygen species (ROS) that serve as signaling molecules that lead to cell attraction, migration, adhesion and immune cell activation. Lastly, ATP plays a vital role in signaling the immune system after injury. Typically cells contain high concentrations of ATP compared to the extracellular concentration. Evidence demonstrates an active release of ATP in response to cellular damage ultimately activates the immune system (Cordeiro and Jacinto, 2013).

Current Study

Recent evidence suggests that an inflammatory response might contribute to the regeneration of hair cells in the zebrafish lateral line. According to a study by d'Alencon et al. (2010), damage to the lateral line neuromast cells can induce an acute inflammatory response. Specifically, d'Alencon et al. administered known anti-inflammatory drugs to test for potential inhibition of leukocyte infiltration of the neuromast and concluded that damage of hair cells creates an acute inflammatory reaction within minutes. While it appears zebrafish possess this

inflammatory response in hair cell regeneration, further study is warranted. The current study investigated the effect of a normal inflammatory response on the hair cell regeneration process within neuromasts on the posterior lateral line of larval zebrafish.

MATERIALS AND METHODS

Animals

Zebrafish (*Danio rerio*) larvae wild-type AB were obtained from the zebrafish facility at Washington University in St. Louis. The embryos were maintained until six days post-fertilization (dpf) when the experiments were conducted. All experiments were approved by Washington University Institutional Animal Research Committee.

Drug Treatments

Zebrafish larvae were divided into groups of ~50, and incubated for two hours in one of the following anti-inflammatory drugs: 10 μM Ibuprofen (IBP), 1.0 μM IBP, 0.1 μM IBP, 100 μM Dexamethasone (DEX), 10 μM DEX, 1.0 μM DEX, 0.1 μM DEX, 100 μM Sodium Salicylate (SAL), 10 μM SAL, and 1.0 μM SAL. Age-matched controls (N = 50) were incubated in normal fish water and received no treatment. After this initial two hour pre-incubation in anti-inflammatory drugs, fish were transferred to water that contained the same drug/concentration, but also contained the ototoxic antibiotic neomycin (50 μM). After 30 min in neomycin, fish were rinsed 3x, returned to water that contained the anti-inflammatory drugs alone, and allowed to survive for 48 hr. At this point, fish were fixed and processed for immunohistochemical labeling of hair cells (see below). Additional studies examined whether treatment with any of the anti-inflammatory drugs was otoprotective. In those experiments, fish were pre-treated for 2 hr in anti-inflammatory drugs, followed by 30 min in 50 μM neomycin (with anti-inflammatory

drugs), and then allowed to survive for an additional 2 hr. Finally, to assess whether the anti-inflammatory drugs or dimethyl sulfoxide (DMSO) were ototoxic, approximately 10 zebrafish larvae were treated with 50 μM of each of one of these reagents for 2 hrs. Fish were then euthanized and fixed (as above) and processed for visualization of hair cells.

Fixation and Immunohistochemistry Procedures

For hair cell counts, all specimens were euthanized by treatment in 10 μg/ml tricane (MS-222) and fixed overnight in 4% paraformaldehyde at 4º C. After fixation, specimens were thoroughly rinsed in phosphate buffered saline (PBS, 5 min).

Fish were placed for 2 hrs in a blocking solution composed of 5% normal horse serum (NHS), 95% PBS with 1% Triton X-100 detergent and 1% (DMSO), in order to block nonspecific epitopes and increase permeability across membranes. After each specimen was blocked, the fish were incubated for 24 hrs at room temperature in a primary antibody solution that was made in PBS with 2% NHS and 1% Triton X-100 detergent. Hair cells were labeled with HCS-1 (Hair Cell Soma-1; diluted 1:200), a mouse monoclonal antibody that targets otoferlin, a molecule that is highly abundant in hair cells. The next day, the larval zebrafish were rinsed in PBS 5 times over 25 min and were then incubated for 2 hrs at room temperature in a secondary antibody solution. The secondary antibody solution was composed of PBS with Alexa Fluor 488 anti-mouse IgG (1:500), Phalloidin Alexa Fluor 546 (1:40), and 4',6-diamidino-2-phenylindole (DAPI 1:500). Alexa Fluor 488 anti-mouse IgG fluorescently labeled the hair cells green, Phalloidin Alexa Fluor 546 labeled actin filaments red, and DAPI fluorescently labeled the nucleus of every cell blue. Each specimen was rinsed in PBS 5 times over 25 min. After carrying out the immunohistochemical labeling protocol, the zebrafish larvae were mounted on

microscope slides in glycerol/PBS (9:1) with their tail surfaces flat and facing upwards to ensure an accurate view of the posterior lateral line neuromasts.

Data Quantification and Imaging

To quantify the extent of the hair cell regeneration within neuromasts of the posterior lateral line, a Carl Zeiss LSM 700 Confocal Microscope with a 40x objective lens was used to count the total numbers of remaining hair cells per neuromast in the three most posterior neuromasts, P9, P10, P11 (Fig. 1).

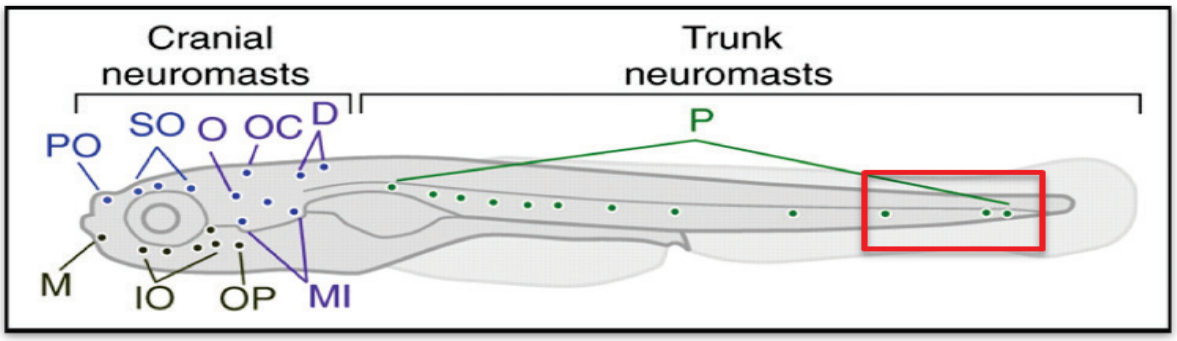


Fig. 1 The stereotyped locations of lateral line neuromasts in zebrafish larvae (6 dpf). The box indicates assumed positions of P9, P10, and P11. (Used with permission from WJ Van Trump and MJ McHenry J Exp Biol 211: 2105-2115 (2008).

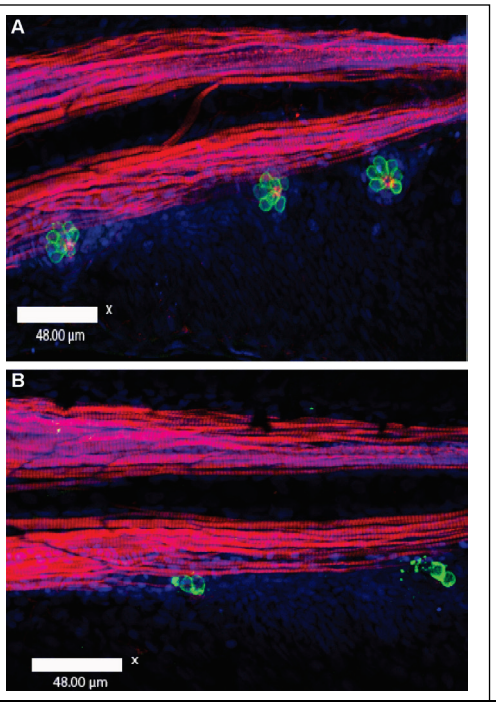
Average numbers of hair cells per neuromast were calculated for each specimen and used to compute an overall mean number of hair cells per neuromast per condition. When quantifying the surviving number of hair cells in each neuromast, a hair cell was counted only if it exhibited a complete membrane and preserved its structure. Hair cell counts from fish treated with anti-inflammatory drugs were compared to those obtained from control fish. All data were entered into a Microsoft Excel spreadsheet and statistical analyses were performed.

Statistical Analysis

The average number and standard deviation of hair cells per neuromast were calculated for each condition using Microsoft Excel. Data were then further analyzed and graphed using GraphPad Prism software, version 6.0d. To assess the ototoxic and otoprotective effects of the anti-inflammatory drugs, a one-way analysis of variance (ANOVA) was performed to determine statistical differences between the control and experimental data for each treatment group. Then, to examine interactions between anti-inflammatory drug and dose, a two-way ANOVA with Tukey's multiple comparisons tests was performed. Differences were considered significant at $p < 0.05$.

RESULTS

Neomycin Toxicity on the Zebrafish Lateral Line



To verify uniform ototoxic effects of neomycin on zebrafish lateral line neuromasts, zebrafish larvae (at 6 dpf) were treated for 30 minutes in 50µM neomycin. Approximately 15 fish per group were fixed at two hours post-treatment. Microscope examination of the immunolabeled fish indicated a rapid destruction of hair cells in the three most posterior neuromasts (P9, P10, P11) of the posterior lateral line. Neomycin treatment resulted in a

Fig. 2 Neomycin lesion of posterior lateral line hair cells. Zebrafish larvae (6 dpf) fluorescently labeled with Alexa Fluor 488, Phalloidin Alexa Fluor 546 and DAPI. **A.** Neuromasts P9, P10, and P11 from an untreated (control) zebrafish **B.** Neuromasts from a zebrafish fixed 2 hrs after 30 minute treatment in 50 µM neomycin.

statistically significant decrease in average number of hair cells per neuromasts in P9, P10, and P11 as compared to the control (untreated) groups. Neuromasts in untreated fish contained ~7 hair cells, while neuromasts in neomycin-treated fish contained ~1 hair cell. A one-way ANOVA established that this decrease was statistically significant in all three groups ($p < 0.0001$), when compared to the respective control group.

Treatment with Ibuprofen, Dexamethasone, Salicylate, or Dimethyl Sulfoxide (DMSO) Does Not Affect Lateral Line Hair Cells

To determine whether treatment with IBP, DEX, SAL or DMSO had a negative effect on hair cell survival, zebrafish larvae were treated for two hours with a 50μM solution of one of these reagents. The average number of hair cells per neuromast in the treated fish did not differ from that in the controls, indicating these drugs do not have an ototoxic effect on the lateral line.

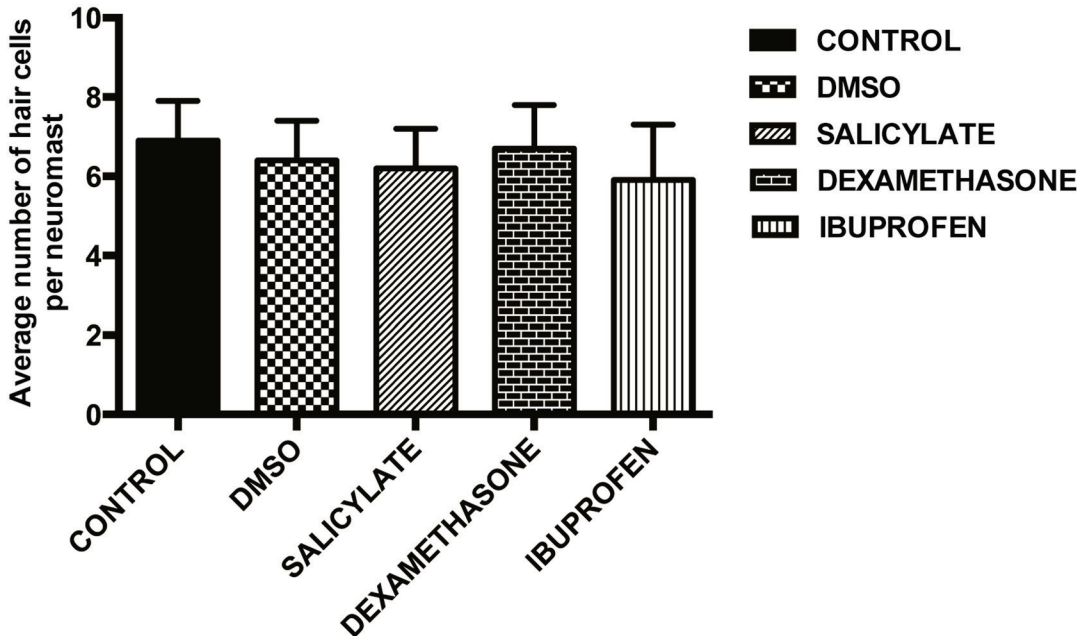


Fig. 3 Drug effect on mean number of hair cells per neuromast. The average number of hair cells per neuromast was not statistically significant compared to controls, indicating these drugs do not have an ototoxic effect on hair cell survival in the lateral line of zebrafish. Statistical significance between groups was analyzed using one-way ANOVA with Dunnett's multiple comparisons test (P = 0.3). Error bars show ± one standard deviation.

Specifically, neuromasts of all fish contained ~6 hair cells (Fig. 3). A one-way ANOVA with Dunnett's multiple comparisons test established that the remaining number of hair cells was not statistically significant in all four groups ($p = 0.3$) compared to the control group.

Ibuprofen, Dexamethasone, and Salicylate are not Otoprotective

Since the objective of these studies was to determine whether anti-inflammatory drugs had an effect on regeneration, it was first necessary to demonstrate that all fish received an identical ototoxic lesion. To examine whether treatment with any of the anti-inflammatory drugs was otoprotective, fish were pre-treated for 2 hr in anti-inflammatory drugs, followed by 30 min in 50 μM neomycin (with anti-inflammatory drugs), and then allowed to survive for an additional 2 hrs. At this point all specimens were euthanized, fixed and processed for quantification of hair cells. Control fish were maintained in parallel; they were treated for 30

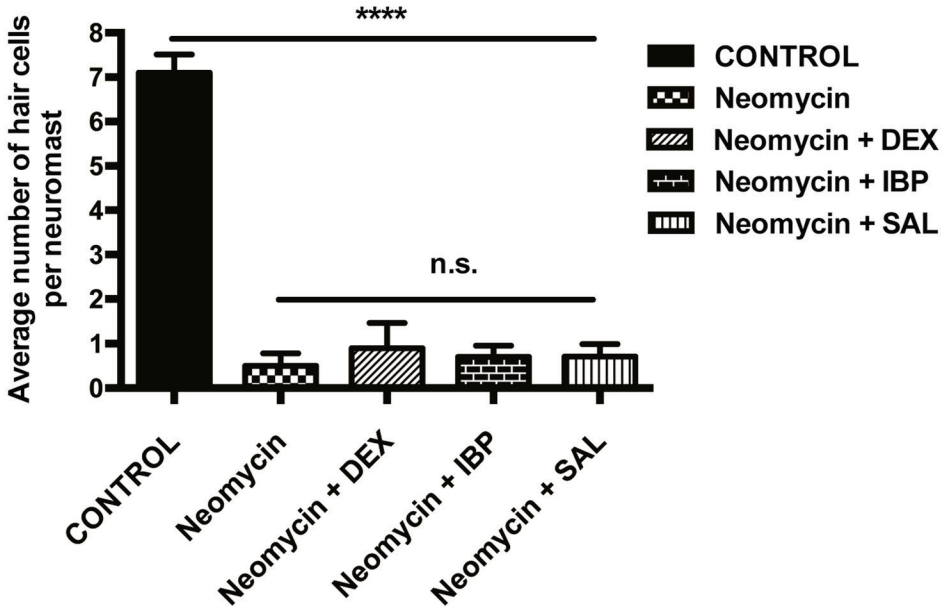


Fig. 4 Non-otoprotective effect of anti-inflammatory drugs. Average number of hair cells per neuromast 2 hours post exposure to the ototoxin neomycin indicates a statistically significant decrease compared to controls. Treatment with anti-inflammatory drugs did not reverse the neomycin effect and was not otoprotective. Statistical significance between groups was analyzed using one-way ANOVA with Tukey's multiple comparisons test. Statistical significance is indicated by asterisks. ****$p < 0.0001$. n.s. = not significant ($p = 0.08$). Error bars show ± one standard deviation.

min in 50 µM neomycin alone, and were not treated with anti-inflammatory drugs. Quantification of hair cells in these specimens revealed that both the control group and treatment groups contained approximately one-hair cell per neuromast (Fig. 4). A one-way ANOVA with Tukey's multiple comparisons tests established that the number of surviving hair cells was not statistically significant across the three groups ($p = 0.08$), compared to the control group. This finding indicates that these anti-inflammatory drugs do not protect hair cells from neomycin ototoxicity.

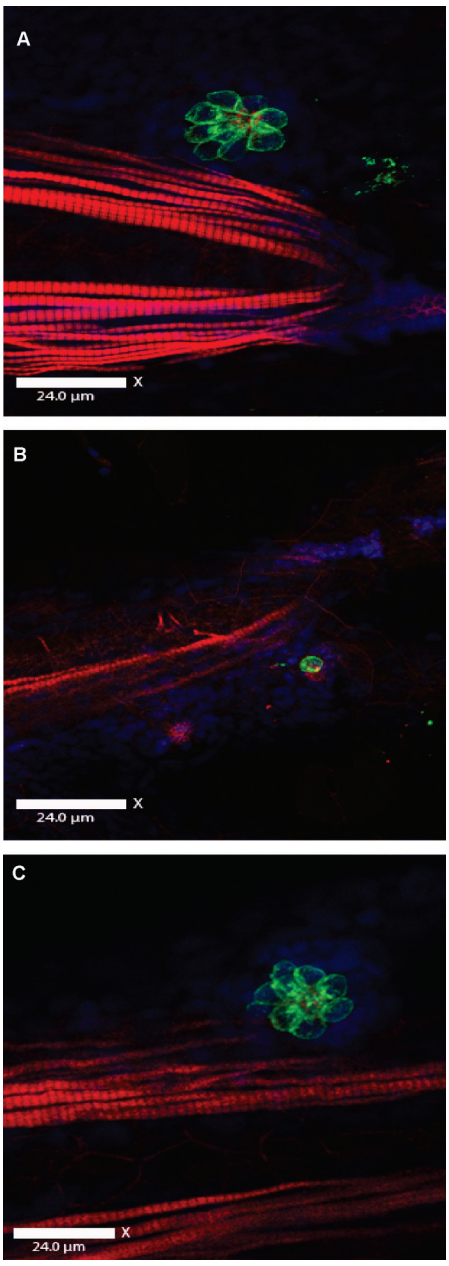


Fig. 5 Hair cell regeneration after neomycin lesion of posterior lateral line hair cells. Zebrafish larvae (6 dpf) fluorescently labeled with Alexa Fluor 488, Phalloidin Alexa Fluor 546 and DAPI. **A.** Control (untreated) zebrafish **B.** Neuromast from a zebrafish fixed 2 hrs after 30 minute treatment in 50 µM neomycin. **C.** Neuromast from a zebrafish fixed 48 hrs after 30 minute treatment in 50 µM neomycin.

Hair Cell Regeneration after Neomycin Toxicity

As noted above, we found that treatment for 30 min with 50 µm neomycin reduced the number of lateral line hair cells, from approximately 7 hair cells per neuromast to less than one. However, fish that were allowed to survive for 48 hr after ototoxic injury showed considerable regeneration of hair cells. Specifically, after 48 hours, average hair cell count averaged 6 hair cells per neuromast. A two-way ANOVA established that this increase in hair cells was statistically significant ($p < 0.0001$). When comparing the mean number of regenerated hair cells to that of the controls, we found no statistically significant differences for

the DEX and SAL treatment groups. However in the IBP group, a statistically significant difference ($p < 0.05$) was seen between the mean number of hair cells per neuromast in the control fish compared to those 48 hours after neomycin treatment. Finally, although significant regeneration was evident at 48 hr after neomycin treatment, hair cell numbers did not recover to their original values. These data demonstrate that hair cells in P9, P10, and P11 neuromasts of the zebrafish lateral line can regenerate after neomycin treatment (Fig. 5).

Anti-Inflammatory Drugs and Hair Cell Regeneration after Neomycin Toxicity

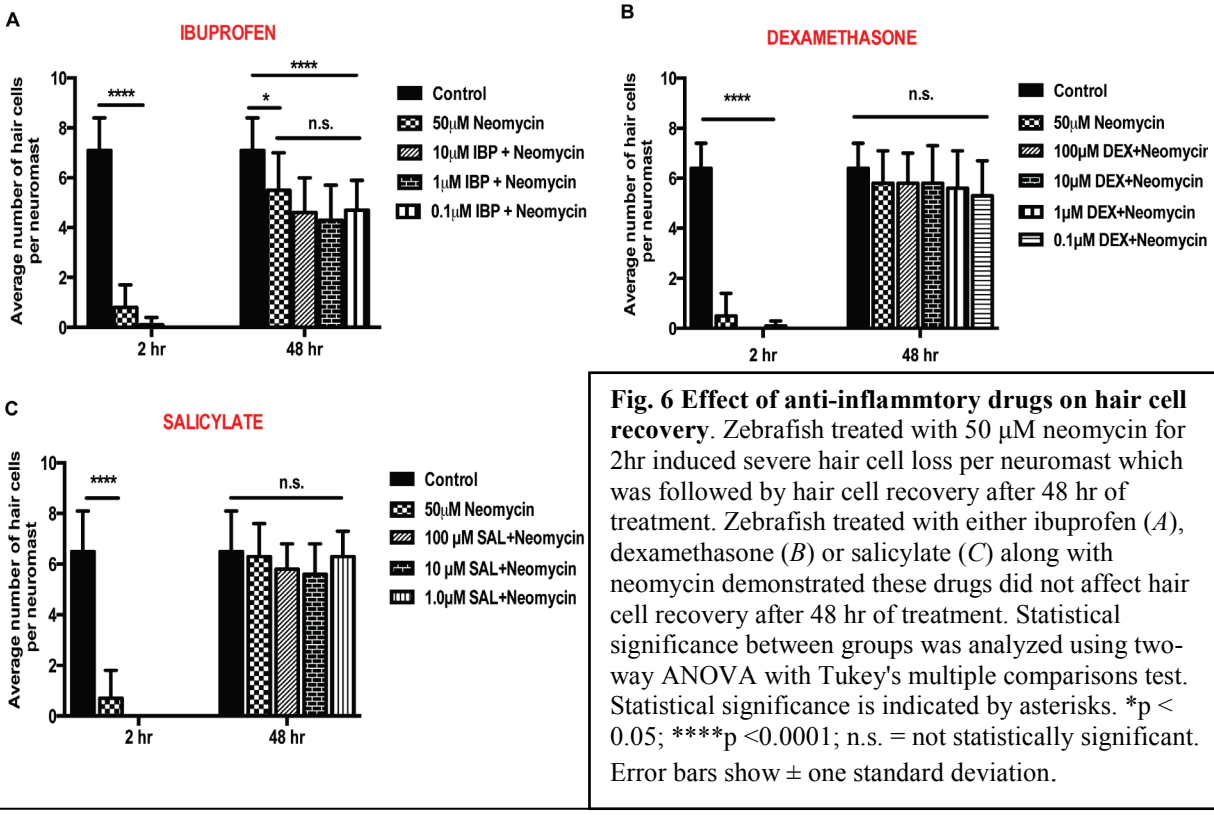


Fig. 6 Effect of anti-inflammtory drugs on hair cell recovery. Zebrafish treated with 50 μM neomycin for 2hr induced severe hair cell loss per neuromast which was followed by hair cell recovery after 48 hr of treatment. Zebrafish treated with either ibuprofen (*A*), dexamethasone (*B*) or salicylate (*C*) along with neomycin demonstrated these drugs did not affect hair cell recovery after 48 hr of treatment. Statistical significance between groups was analyzed using two-way ANOVA with Tukey's multiple comparisons test. Statistical significance is indicated by asterisks. *$p < 0.05$; ****$p < 0.0001$; n.s. = not statistically significant. Error bars show ± one standard deviation.

To determine whether immunosuppression affected the process of hair cell regeneration, we next examined the effects of anti-inflammatory drugs on hair cell recovery. Zebrafish were incubated for two hours in one of the following anti-inflammatory drugs: 10 μM IBP, 1.0 μM

IBP, 0.1 μM IBP, 100 μM DEX, 10 μM DEX, 1.0 μM DEX, 0.1 μM DEX, 100 μM SAL, 10 μM SAL, and 1.0 μM SAL. Fish were then transferred to water that contained the same drug/concentration, but also contained the ototoxic antibiotic neomycin (50 μM), and incubated in these solutions for 30 minutes. Following neomycin treatment, fish were rinsed and returned to solutions that contained only the anti-inflammatory drugs, and allowed to survive for 48 hr. Specimens were then euthanized, fixed and processed for visualization and quantification of hair cells. Treatment IBP, DEX, and SAL (at any concentration) did not have a statistically significant effect on hair cell regeneration. Fish that were allowed to recover for 48 hours post-treatment contained approximately 5.5 hair cells per neuromast, while the controls contained approximately 6 hair cells per neuromast (Figs. 6). A two-way ANOVA with Tukey's multiple comparisons tests revealed no significant differences in hair cell numbers among fish treated with varying doses of anti-inflammatory drugs, nor versus controls. These results indicate that immunosuppression does not affect hair cell regeneration in zebrafish lateral line neuromasts.

DISCUSSION

Neomycin is Ototoxic to the Posterior Lateral Line

The goals of this study were to assess the effect of anti-inflammatory drugs on hair cell survival and regeneration in the posterior lateral line of zebrafish following ototoxic drugs. Resulting data indicated that treatment for 30 minutes with 50 μM of neomycin resulted in the death of nearly all hair cells in the three distal-most neuromasts of the posterior lateral line. These results are similar to those of Buck et al. (2012) who reported neomycin produces an extensive hair cell lesion throughout the zebrafish lateral line. Buck and colleagues treated zebrafish for 1 hr with varying doses of neomycin (10 μM, 50 μM, 100 μM, 200 μM, 300 μM)

and found that the number of hair cells significantly decreased after neomycin treatment at all doses. Similarly, Chiu et al., (2008) reported neomycin killed hair cells on the zebrafish lateral line. Through fluorescence microscopy, Chiu and colleagues assessed hair cell survival in the posterior lateral line after 1 hr treatment with varying doses of neomycin (0 μM, 50 μM, 100 μM, 200 μM, 400 μM). Results suggested that neomycin has a dose-dependent effect on hair cell loss.

The current experiment used a single dose of neomycin (50 μM), which killed nearly all hair cells in the posterior later line. After treatment, neuromasts P9, P10, and P11 each contained ~1 hair cell prior to regeneration, which was a statistically significant decrease when compared to control fish. Therefore, the 50 μM dose successfully created an extensive hair cell lesion on the zebrafish lateral line without being dangerously toxic.

Ibuprofen, Dexamethasone, Salicylate, and DMSO are not Ototoxic to the Posterior Lateral Line

Treatment for two hours with 50μM solution of IBP, DEX, SAL or DMSO demonstrated that these reagents do not cause any measurable loss of lateral line hair cells. Mean number of hair cells per neuromast in fish treated with any of these compounds did not differ from those in the controls. Uribe et al., (2013) found similar results for DMSO and lateral line hair cells and reported that treatment for 1 hr in a 0.5% DMSO solution did not cause lateral line hair cell loss or morphological changes in zebrafish larvae. Notably, no prior studies have examined possible ototoxic effects of treatment with ibuprofen or dexamethasone.

Salicylate has previously been classified as an ototoxic drug. Although the exact mechanisms remain unknown, large doses of salicylate can cause reversible hearing loss and

tinnitus in mammals (Schacht and Hawkins, 2006). Almeida- Silva et al., (2011) described the effects of salicylate on the mammalian inner ear as structural and functional changes that result in reduced electromotility of outer hair cells without loss of cells. Reversibility of salicylate-induced damage has been noted 24-72 hrs after termination of dosage. The current study likewise suggests that salicylate does not directly kill hair cells. It is possible that salicylate toxicity in zebrafish could target other structures than hair cells. Additionally, a higher dosage of salicylate may affect the lateral line.

Anti-inflammatory drugs are not otoprotective

This study also analyzed whether treatment with any of the anti-inflammatory drugs was otoprotective. Resulting data indicated that pre-treating zebrafish larvae for 2 hr with IBP, DEX, or SAL does not protect hair cells on the posterior lateral line from neomycin ototoxicity. Prior studies have indicated otoprotective properties of both DEX and SAL against aminoglycoside toxicity. For example, Rybak and Whitworth (2005) suggested that aminoglycosides interact with iron molecules to form reactive oxygen species (ROS), which contribute to the ototoxic effect of aminoglycosides. Salicylates act both as antioxidants and weak iron chelators, and can influence the expression of genes related to cell survival and death. For this reason, salicylates have been suggested to protect against aminoglycoside toxicity. They protect the cochlea from oxidative stress and free radicals that lead to cell death (Ryback and Whitworth, 2005; Xie, Talaska, and Schacht, 2011). Both animal and human studies have demonstrated the effectiveness of salicylate protection against aminoglycoside ototoxicity. Sha and Schacht (1999) treated guinea pigs with gentamicin, another aminoglycoside antibiotic, plus salicylate. Post-treatment examination of the inner ear structures of the guinea pigs confirmed protection of auditory sensory cells confirming salicylate effectively protected against

aminoglycoside-induced hearing loss (Sha and Schacht, 1999). Chen et al., (2007) showed similar effects of salicylate in humans by administering salicylate in the form of aspirin (acetyl salicylate) to patients receiving gentamicin for acute infections. Results showed a reduction of aminoglycoside-induced hearing loss in the experimental group as compared to placebo-controls (Chen et al., 2007).

Dexamethasome is a corticosteroid that reduces free radical formation and controls immune responses caused by tissue damage. Himeno et al., (2002) demonstrated protective properties of DEX against aminoglycoside-induced hair cell death by administering DEX (1 ng/ml) directly to the inner ear of guinea pigs prior to aminoglycoside treatments (400 mg/kg kanamycin or 40 mg/kg ethacrynic acid). Post-treatment evaluation revealed DEX successfully reduced aminoglycoside-induced ototoxicity.

The current study found no protective effect of DEX or SAL against neomycin ototoxicity. It is possible that the protective properties of both drugs differ across animal models. Additionally, various types and doses of aminoglycosides can interact uniquely with the assumed otoprotective agents. Finally, the mode of drug administration might alter the interaction with cellular structures.

Anti-Inflammatory Drugs have No Effect on Neuromast Hair Cells Regeneration on the Posterior Lateral Line

After verifying a uniform lesion of hair cells in the zebrafish lateral line, the regenerative abilities after neomycin damage were investigated. Hair cell regeneration following neomycin treatment was observed after 48 hrs of recovery, and reached approximately 90% of the control hair cell population. These results are consistent with previous studies of hair cell regeneration

following neomycin injury. Ma et al., (2008) treated 5-6 dpf larval zebrafish for 1 hr in a 400 μM neomycin solution. At 48 hrs post-treatment, lateral line neuromasts had regenerated ~80% of their hair cells. Similarly, Mackenzie and Raible (2012) treated 5 dpf larval zebrafish for 30 min with varying concentrations of neomycin (100 μM and 200 μM) and observed regenerated lateral line hair cells at 48 hrs after neomycin treatment reached approximately 85% of the initial hair cell count.

Finally, we examined if treatment with anti-inflammatory drugs had any impact on the extent of hair cell regeneration. These experiments were motivated by observations that inflammatory cells play a critical role in other types of tissue repair (Cordeiro and Jacinto, 2013; Richardson et al., 2013). When fish were pre-treated with varying doses of anti-inflammatory drugs, significant regeneration was still observed after 48 hrs recovery (Fig. 7). Posterior lateral line hair cells regenerated to levels similar to original values in fish treated with DEX or SAL. For the IBP group, although significant regeneration was evident at 48 hr after neomycin treatment, hair cell numbers did not recover to their original values. This difference was likely due to the variability in that experimental group. This experiment utilized a large sample size with multiple variables. Therefore, slight variation is expected. We conclude that immunosuppression does not affect hair cell regeneration in zebrafish lateral line neruomasts. Although there is an inflammatory response after hair cell injury (d'Alencon et al., 2010), it is likely that the recruited immune cells simply assist in the clearance of cellular debris, and do not play a vital role in the regeneration process.

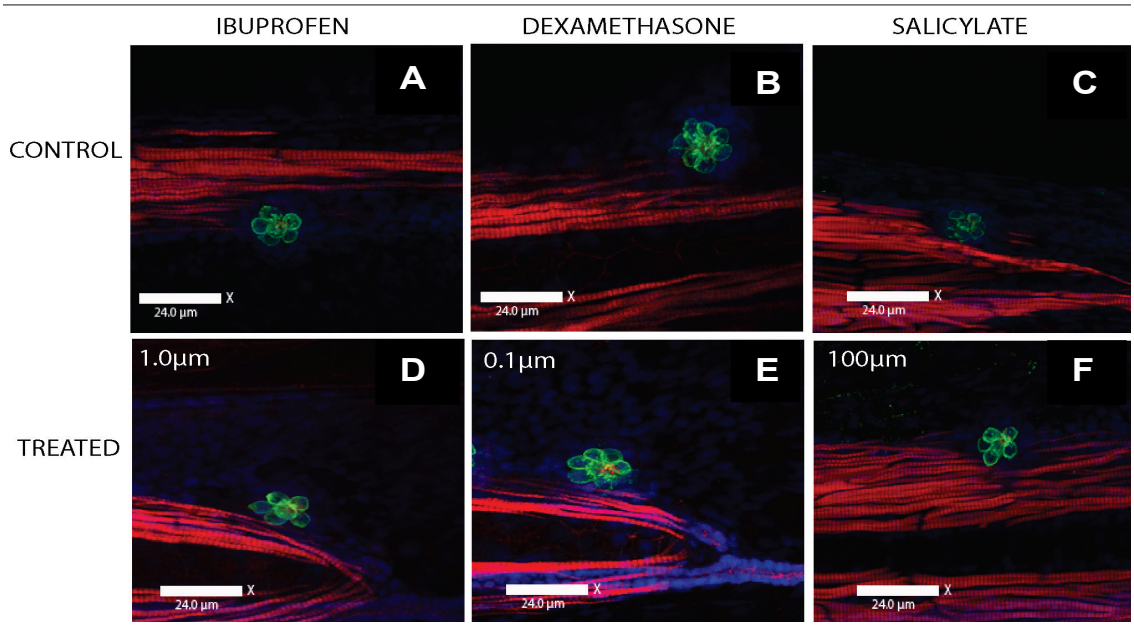


Fig. 7 Posterior lateral line hair cell regeneration following treatment with neomycin and anti-inflammatory drugs. A-C. Lateral line neuromasts from zebrafish fixed 48 hrs following 30 min treatment in 50µM neomycin. **D.** Lateral line neuromasts from zebrafish fixed 48 hrs following 30 min treatment in 50µM neomycin and 1.0 µM IBP. **E.** Lateral line neuromasts from zebrafish fixed 48 hrs following 30 min treatment in 50µM neomycin and 0.1 µM DEX. **F.** Lateral line neuromasts from zebrafish fixed 48 hrs following 30 min treatment in 50µM neomycin and 100 µM SAL.

CONCLUSION

The current study analyzed the impact of anti-inflammatory drugs on hair cell regeneration in the posterior lateral line of zebrafish larvae. First, it was established that a 50 µM dose of neomycin for 30 minutes was sufficient to kill nearly all hair cells in the three distal-most neuromasts on the posterior lateral line. Additionally, a 2 hr treatment with 50 µM of IBP, DEX, SAL or DMSO did not have a negative effect on hair cell survival indicating these drugs are not ototoxic in fish. Treatment with these anti-inflammatory drugs did not protect hair cells from neomycin toxicity. Lastly, hair cell regeneration in the posterior lateral line was observed at 48 hrs post neomycin treatment. Treatment with anti-inflammatory drugs neither enhanced

nor inhibited the regeneration, indicating that immunosuppression does not affect hair cell regeneration in the zebrafish lateral line neuromasts. These findings suggest that inflammatory responses do not play a crucial role in the process of hair cell regeneration in the zebrafish lateral line.

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