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Damage and functional recovery of the Atlantic cod (*Gadus morhua*) inner ear hair cells following local injection of gentamicin

Karine Faucher*
Øyvind Aas-Hansen§
Borge Damsgård§
Einar Laukli*
Niels Christian Stenkleiv*

*ENT Department, Institute of Clinical Medicine, University of Tromsø, Norway

§Norwegian Institute of Fisheries and Aquaculture Research, Tromsø, Norway

Key Words

Fish hearing
AEP
SEM
Gentamicin
Inner ear hair cells
Regeneration

Abbreviations

SEM: Scanning electron microscopy
AEP: Auditory evoked potentials
NaCl: Sodium chloride
CO₂: Carbon dioxide
χ²: Chi-square

Introduction

As in birds, fish inner ear hair cells have the ability to regenerate after damage by noise or ototoxic drugs (Lombarte et al, 1993; Scholik & Yan, 2002; Smith et al, 2006). This capacity for regeneration has led to increased interest in fish models for investigations on hair cell regeneration with the goal of a possible translation into mammalian hair cell research. Aminoglycoside antibiotics are commonly used for damage of sensory hair cells in the fish lateral line system (Kaus, 1987; Song et al, 1995; Harris et al, 2003; Santos et al, 2006) and inner ear (Matsuura et al, 1971; Yan et al, 1991; Lombarte et al, 1993). In the past, intramuscular (Yan et al, 1991; Lombarte et al, 1993) or intravenous (Faucher et al, 2008) gentamicin injections have been used to study sensory hair cell regeneration in fish. However, systemic administration of aminoglycosides may be accompanied by unwanted side-effects, like nephrotoxicity and subsequent mortality of study animals. Matsuura et al (1971) used intrasaccular kanamycin and streptomycin injections into the goldfish (*Carassius auratus*) inner ear, and our group has recently demonstrated that local injection of gentamicin directly into the inner ear sacculus is an efficient method to obtain inner ear damage without undue side effects in the

Abstract

This study addresses the ultrastructural and functional damage and subsequent recovery of the inner ear in the Atlantic cod following intrasaccular gentamicin injection. Inner ear damage was assessed using SEM and measurements of AEP following 250-Hz pure-tone stimuli. Data from gentamicin-treated fish were compared with control (no injection) and sham (injection of saline) fish. Control fish had normal response thresholds associated with well-developed hair cell bundles in their macula sacculi. Sham fish had higher response thresholds compared with control fish during the first week post-intervention, but response thresholds were subsequently normal. Treated fish displayed significant inner ear damage associated with an increased average AEP threshold on the third day following treatment. Thereafter, inner ear tissue displayed signs of progressive regeneration until it was comparable to controls from the 14th day. Response thresholds were similar to those of control fish from the 17th day following treatment. These observations suggest that the macula sacculi of Atlantic cod can regenerate towards a near-complete functional and ultrastructural recovery within 17–21 days following ototoxic gentamicin treatment.

Atlantic cod (Faucher et al, 2008). Until now, most studies showing effect of aminoglycoside antibiotics on fish inner ear hair cells have considered either the functional aspects (Matsuura et al, 1971) or the ultrastructural damage and recovery (Yan et al, 1991; Lombarte et al, 1993; Schönleber & Anken, 2004) following an ototoxic intervention. The time-course of ultrastructural and functional recovery in fish inner ears following local aminoglycoside injection remains, however, uncertain. To investigate these issues, we examined fish response thresholds using the AEP protocol in combination with inner ear electron microscopy. The AEP protocol is routinely used in humans to provide objective assessment of response thresholds and for evaluation of brainstem integrity (Hecox & Galambos, 1974; Starr & Achor, 1975). More recently, it has been adapted for use in fish to standardize hearing investigations (Kenyon et al, 1998; Wysocki et al, 2007). AEP responses are short latency voltage changes that are evoked by acoustic stimuli and recorded with electrodes located a short distance from generators in the central nervous system (Corwin et al, 1982; Kenyon et al, 1998). In the present study, the Atlantic cod was chosen for its acute hearing and sensitivity to sound pressure (Hawkins, 1972a; Chapman & Hawkins, 1973). In addition, a number of electrophysiological (Enger & Andersen, 1967; Sand & Enger, 1973)

and behavioural hearing studies, based on conditioning and food rewards (Chapman & Hawkins, 1973; Schuijf, 1975; Schuijf & Buwalda, 1975; Schuijf & Hawkins, 1983), have already been performed on this species. From those studies it appears that the lowest response thresholds for pure tones are obtained in the frequency range from 200 to 300 Hz (Hawkins, 1972b; Chapman & Hawkins, 1973; Sand & Enger, 1973). The aim of the present study was to investigate possible changes in Atlantic cod inner ear ultrastructure (scanning electron microscopy, SEM) and functional response threshold at 250 Hz (auditory evoked potentials, AEP) over a time course following intrasaccular gentamicin injection.

Materials and Methods

Animal origin and fish tagging

The experiment took place in October–November 2007. One hundred and twenty hatchery reared Atlantic cod (*Gadus morhua*, L.) of about 680 ± 104 g and 39 ± 2 cm standard length, were obtained from the Aquaculture Research Station in Tromsø, Norway. They were housed in a 13.5 m^3 tank supplied with aerated and filtered seawater, at natural temperature (about 6.5°C) and photoperiod. All fish were initially anaesthetized by immersion in sea water containing metacaine (55 mg l^{-1}) for 5 minutes, and fitted with coloured floy tags (FLOY TAG Inc., USA) for subsequent identification.

Intrasaccular gentamicin or saline solution injections

Just after tagging, gentamicin (40 mg ml^{-1} Gensumycin injection solution, Aventis Pharma) or saline solution (10 g l^{-1} NaCl) was injected into both inner ear sacculi of the fish. Injections were done in the same manner as described in a previous publication (Faucher et al, 2008). Intraluminal saccular injections of 0.05 ml of saline (sham fish) or gentamicin (treated fish) were performed in the anaesthetized fish fixed in a polystyrene gutter. The sacculi were entered from a lateral access route, using a metal trephination device. A thin needle ($\text{Ø} = 0.40 \text{ mm}$) was inserted through this device, on both sides successively. Correct positioning of the needle inside the inner ear sacculi was ascertained in every fish using X-ray imaging. The volume and dose of injected gentamicin corresponded to our previous study (Faucher et al, 2008).

The Atlantic cod were divided into three groups with 40 fish in each: control (no injection), sham (injection of saline), and treated fish (injection of gentamicin). Fish swimming behaviour was observed to be normal and no mortality from gentamicin-induced nephrotoxicity was observed throughout this study.

AEP recording setup

To determine the average hearing capacity of fish from each group at regular intervals, 10 fish were randomly chosen within each group for each session of AEP measurements. These were anaesthetized with seawater containing metacaine (0.55 mg l^{-1}) until being unresponsive to a light pinch in the tail. They were then secured in the experimental tank, which consisted of a ~ 40 -litre flexible plastic bag located within a custom-made, soundproof wooden box ($1 \times 1 \times 2 \text{ m}$). The bag was mounted on a steel framework suspended by wires hanging from the roof of the laboratory through holes in the roof of the wooden box. Maintenance anaesthesia was administered by supplying half-

dose metacaine at a flow rate of $\sim 200 \text{ ml min}^{-1}$ through a rubber tube inserted into the fish's mouth. Under this regime, animals had slight opercular movements, but were unable to initiate gross muscle contractions. The animals were continuously observed during the ongoing experiment using a video camera. Free-field airborne stimuli were delivered using a Brüel & Kjør Sound Source 4224 loudspeaker (Brüel & Kjør Corp., Denmark) mounted 1.5 m above the water surface level. Our AEP protocol was identical to the one used by Kenyon et al (1998) except that our fish were completely immersed in seawater, and had all electrodes placed subcutaneously. AEP responses were recorded in the 32-ms post-stimulus range using three subcutaneous stainless steel electrodes on the fish head, a filter setting of 30–3000 Hz, and a gain of 100 000. The tips of the two active electrodes were placed subcutaneously on the midline of the skull in the anterior and the posterior part of the midline furrow, approximately 3 cm apart. The ground electrode was inserted into muscle tissue about 2 cm lateral (left) of the midline furrow. The distance from the fish body to the air-water interface was more than 5 cm in all directions. Sound stimuli were a series of 2000 sinusoidal 250 Hz alternating polarity tone bursts, with five cycles (rise / fall two periods, plateau one period) duration at a presentation rate of 22.3 per second. The stimuli were adjusted using the Bio-Logic AEP (Bio-logic Systems Corp., USA) software. After enhancement in a Navigator Pro preamplifier (Bio-logic Systems Corp., USA), responses were averaged and visually assessed using the Bio-Logic AEP software on a separate computer. The time latencies for responses were graphically estimated. The stimulus level was gradually reduced by sound level decrements of 5 dB, until responses were no longer visible. The lowest stimulus level for which a reproducible AEP trace could be obtained was considered to be the response threshold of that animal. For lowest stimulus levels, when approaching the response threshold, the AEP was recorded twice. For evaluation of the stimulus spectral content (sound frequency) and peak level (amplitude), a Brüel & Kjør 8103 hydrophone (Brüel & Kjør Corp., Denmark) was submerged in the experimental tank, adjacent to the ear region of the animal. The hydrophone was calibrated using a Brüel & Kjør 4229 hydrophone calibrator (Brüel & Kjør Corp., Denmark). The amplified sound stimuli were then analysed using the Avisoft SASLab Pro v. 3.5 software (Avisoft Bioacoustics, Germany).

After treatment, AEP recording sessions were performed once a week for sham and control fish, and three times a week for gentamicin treated fish. After each AEP recording session, two fish from each group were sacrificed for later investigation of their inner ears using SEM.

Inner ear SEM observations

Two fish from each group were anaesthetized with metacaine (75 mg l^{-1}), and sacrificed to isolate inner ear sensory maculae. The roof of the fish skull was delicately removed in order to expose the inner ear. Fixative solution (4% glutaraldehyde in sodium cacodylate buffer, 0.4 M, pH 7.2) was injected into the semicircular canals of the inner ear of both sides of the head. Whole heads were then immediately fixed in 4% glutaraldehyde (Merck) in sodium cacodylate buffer (0.4 M, pH 7.2). After one night of primary fixation, the sensory maculae from the sacculi of each inner ear were carefully removed and fixed again in 4% glutaraldehyde in sodium cacodylate buffer (0.4 M, pH 7.2) for

24 hours. They were then post-fixed with 2% osmium tetroxide (Carl Roth, Germany), and then dehydrated in graded acetone. Sensory maculae were critical point-dried using liquid CO₂ (BALTEC CPD 030), mounted on brass supports, and sputter coated with gold (Cressington Sputter Coat) for observation using scanning electron microscopy (JEOL JSM-5410LV operating at 20 kV).

Data processing and statistical analyses

Average response thresholds were obtained at days 4, 11, 18 for control fish, at days 6, 13, 20 for sham fish, and at days 3, 5, 7, 10, 12, 14, 17, 19, 21 following treatment for treated fish, and compared using the non-parametric Mann-Whitney and Kruskal-Wallis tests. In order to examine the effect of intrasaccular injection of saline or gentamicin on inner ear sensory hair cells, the ultrastructural status of the inner ear sacculi was compared between groups. SEM pictures of local population samples were taken at three different locations in the area close to the wide indentation in the ventral margin of the macula sacculi (Faucher et al, 2008). Micrographs at 1500 × magnification were taken at each sample location to show an area of 5000 μm². Hair cell bundles were classified in three groups depending of their tissue state: (1) normal with well-developed hair bundles, (2) damaged with shorter hair bundles, stereocilia fused and sometimes just the cuticular plate retained, and (3) abnormal-looking hair cells characterized by smaller hair bundles in which the kinocilium was lacking and the stereocilia were shorter. Counts of visible hair bundles (normal, damaged, and abnormal) were performed to assess the hair cell population. Results were converted to density in the form of hair cell number μm⁻². Counts of abnormal hair cells were converted into prevalence (%) relative to total number of hair bundles. Likewise, micrographs at 4000 × magnification were sampled at each location to measure kinocilium length (μm) of hair bundles. Averages of densities and kinocilium measures were calculated for each group. A normality test of Shapiro-Wilk performed on average densities confirmed the normal distribution of data (p=0.344). Intergroup analysis was performed using an analysis of variance (ANOVA), followed by a parametric multiple comparison test (Tukey). Averaged kinocilium lengths were compared between groups using the non-parametric Kruskal-Wallis and Mann-Whitney tests. Prevalence of abnormal hair cells were compared between groups using the chi-square (χ²) test. All statistical tests were conducted with the Xlstat-Pro 6.0 statistical analysis software (Addinsoft, France). The level of significance was set at p < 0.05.

This study was approved by The Animal Care Committee of Norway (FDU 07/29678).

Results

After treatment (intrasaccular injection of saline or gentamicin), all the fish presented a normal swimming behaviour. No gentamicin-induced symptoms (bloated abdomen or rectal haemorrhages) were observed.

AEP recordings

AEP recordings were successfully performed in all the fish that were tested. The responses consisted of a complex of relatively narrow waves (Figure 1). The onset latency of the main complex of the AEP was around 11 ms at higher stimulus levels. Latencies

increased as stimulus levels decreased. As the stimulus levels approached threshold, AEP waveforms were less reproducible, and amplitude decreased.

After treatment, fish response thresholds were recorded once a week for control and sham fish, and three times a week for treated fish. Throughout the three-week experimental period, response thresholds of control fish were unchanged (116.8 ± 4.6 dB, n=30, H=1.16, p=0.561, Figure 1 A, and 2). As their average response thresholds were constant during the whole duration of experiment, those values were averaged for comparison with other groups. In contrast, the average response threshold that was recorded in sham fish during the first week (123.1 ± 5.9 dB, n=10, Figure 1 B) was significantly higher than average response thresholds from the second week (118.2 ± 5.2 dB, n=10, U=76.00, p=0.025), and the third week (115.9 ± 4.8 dB, n=10, U=90.50, p=0.001, Figure 2). During the first week, sham fish (123.1 ± 5.9 dB, n=10) had a higher average response threshold than control fish (116.5 ± 3.1 dB, n=10, U=12.00, p=0.004). During the second week, no significant difference was seen in average response thresholds between sham (118.2 ± 5.2 dB, n=10) and control fish (118.0 ± 5.7 dB, n=10, U=47.50, p=0.850). Average response thresholds were also similar during the third week following treatment between sham (115.9 ± 2.5 dB, n=10) and control fish (116.0 ± 4.8 dB, n=10, U=43.00, p=0.597). When comparing the average response thresholds between control (116.8 ± 4.6 dB, n=30) and sham fish (119.1 ± 5.5 dB, n=30) throughout the experimental period, no significant difference was observed (U=330.00, p=0.076, Figure 2).

Three days following gentamicin injection, treated fish had a significantly higher average response threshold (133.2 ± 11.1 dB, n=10) than control fish (116.8 ± 4.8 dB, n=10, U=273.00, p < 0.0001, Figure 1 C and 2). This average response threshold in treated fish after three days was not significantly different from the average response threshold recorded in sham fish during the first week of the experiment (123.1 ± 5.9 dB, n=10, U=74.00, p=0.070). Average response thresholds of treated fish then progressively decreased. From the 17th day, average response thresholds of these fish (117.0 ± 3.4 dB, n=30) were not significantly different from control fish (116.8 ± 4.8 dB, n=10, U=409.00, p=0.544, Figure 1 D, and 2).

SEM observations of inner ear

Sensory maculae from the inner ear sacculi of control, sham, and treated fish were observed using SEM. Ultrastructural differences at the level of hair cell bundles were seen between groups (Figure 3). The ANOVA on hair cell densities showed significant differences between control, sham, and treated fish (F_{5, 111} = 14.64, p < 0.0001, n=116). In control fish (Figure 3 A), like sham fish (Figure 3 B), the maculae had sensory cells with well-developed hair bundles, with no observed inner ear damage. The average densities of hair cells were not significantly different between control fish (0.025 ± 0.006 hair cells μm⁻², n=36) and sham fish (0.026 ± 0.004 hair cells μm⁻², n=33, t=1.05, p=0.899, Figure 4 A). In particular, if the average response threshold in sham fish was different from control fish during the first week of recording, the average hair cell density of these fish (0.024 ± 0.003 hair cells μm⁻², n=12) was not significantly different from the average hair cell density in control fish (0.023 ± 0.008 hair cells μm⁻², n=12, t=0.78, p=0.999) during

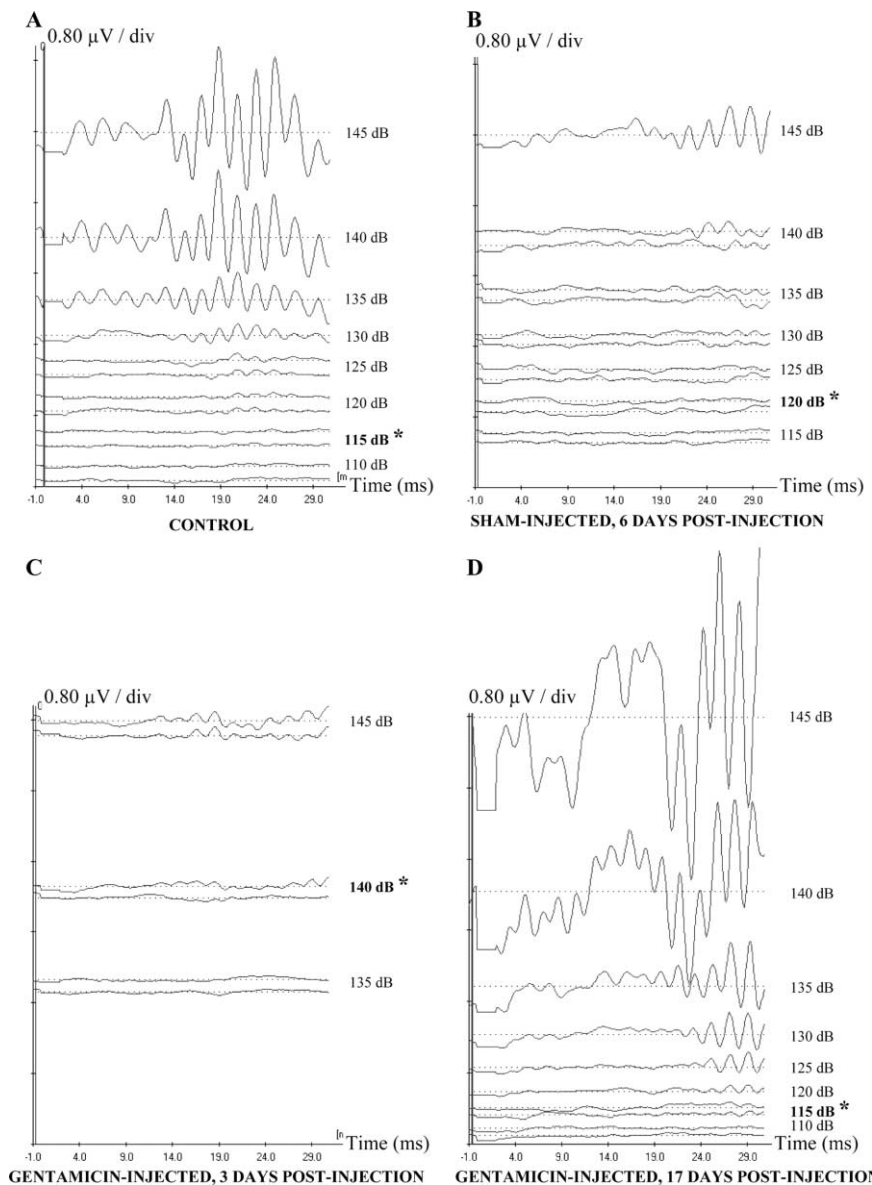


Figure 1. AEP recordings obtained in the cod *Gadus morhua* (L.), in response to tone bursts at 250 Hz. AEP waveforms consisted of a series of narrow waves with an onset latency of the major complex around 11 ms at higher stimulus levels. When the response threshold approached, the AEP was recorded twice. (A) Control cod, (B) Sham cod, (C) After intrasacculus gentamicin injection, higher response thresholds were observed, (D) From the 17th day after treatment, fish showed evidence of functional recovery. The asterisks show the response thresholds. Stimulus levels are expressed in dB (re 1 μ Pa).

the first week after treatment. During the three weeks of the experimental period, the prevalence of abnormal hair cell bundles was not significantly different between control fish (1.58%, $n=36$) and sham fish (1.29%, $n=33$, $\chi^2=1.25$, $p=0.264$). The only difference seen between control and sham fish concerned the average length of kinocilia. The average kinocilia length of sham fish ($4.71 \pm 0.89 \mu\text{m}$, $n=457$) was slightly shorter than in control fish ($4.78 \pm 0.75 \mu\text{m}$, $n=487$, $U=120490.00$, $p=0.028$, Figure 4 B). However, this slight difference between sham and control fish cannot be related to the higher auditory thresholds of sham fish observed during the first week of

experiment. During this first week, the average kinocilia length of sham fish ($4.98 \pm 0.95 \mu\text{m}$, $n=160$) was not significantly different from the average kinocilia length of control fish ($4.80 \pm 0.78 \mu\text{m}$, $n=133$, $U=9625.00$, $p=0.111$).

Three days following local gentamicin injection, fish had maculae that were significantly altered at the level of hair bundles. SEM observations showed hair bundles that were definitely disorganized, widespread loss of hair cell bundles, fusion of some stereocilia, and some areas were entirely deprived of hair cells (Figure 3 C). Some hair cells had lost hair bundles and retained only the cuticular plates. The average inner-ear hair

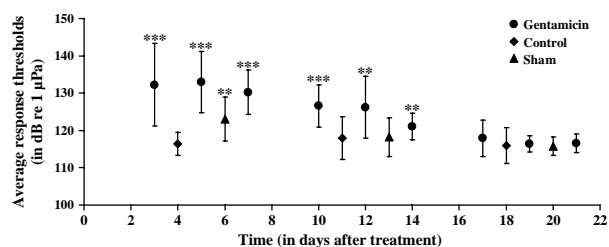


Figure 2. Average response thresholds recorded in each group of fish (control, sham, intrasaccular gentamicin injection, $n = 10$ individuals for each measure) throughout the 21 days of the experimental period. From 3 to 14 days after treatment, gentamicin-exposed fish had elevated response thresholds. From the 17th day, the average response thresholds of treated fish were similar to control fish. Vertical bars represent standard deviation of mean. Asterisks show data that were significantly different from the average of response thresholds of control group ($p < 0.0001$: *** and $p < 0.01$: **).

cell density of these fish (0.015 ± 0.002 hair cells μm^{-2} , $n = 12$) was significantly lower than what was measured in control fish (0.025 ± 0.006 hair cells μm^{-2} , $n = 36$, $t = 6.50$, $p < 0.0001$), and sham fish (0.026 ± 0.004 hair cells μm^{-2} , $n = 33$, $t = 7.18$, $p < 0.0001$, Figure 4 A). The maculae of these treated fish had a significantly higher prevalence of abnormal hair bundles (13.42%, $n = 12$) than those of control fish (1.58%, $n = 36$, $\chi^2 = 285.75$, $p < 0.0001$, Figure 4 A). In addition, at three days after treatment, the average kinocilia length was significantly lower (2.17 ± 0.77 μm , $n = 106$) than in control fish (4.78 ± 0.75 μm , $n = 487$, $U = 397.50$, $p < 0.0001$, Figure 4 B).

At ten days following treatment, the ultrastructural alterations of hair bundles were similar to the third day after treatment. Hair bundles that were observed using SEM were either totally absent, or disorganized and some areas were depleted of hair bundles with mere retention of the cuticular plates (Figure 3 D). The average hair cell density was significantly lower in treated fish (0.018 ± 0.004 hair cells μm^{-2} , $n = 12$) than in control fish (0.025 ± 0.006 hair cells μm^{-2} , $n = 36$, $t = 4.54$, $p = 0.000$, Figure 4 A). Like three days after treatment, the prevalence of abnormal hair bundles was significantly higher in treated (13.28%, $n = 12$) than in control fish (1.58%, $n = 36$, $\chi^2 = 300.50$, $p < 0.0001$, Figure 4 A) at ten days. The average kinocilia length was still lower in treated (2.61 ± 0.87 μm , $n = 144$) than in control fish (4.78 ± 0.75 μm , $n = 487$, $U = 2543.00$, $p < 0.0001$, Figure 4 B).

Fourteen days following treatment, SEM observations of the maculae showed a tendency toward better organization of hair bundles. No areas without hair bundles were seen. Furthermore, some very short hair bundles could be observed (Figure 3 E). At that stage, the average hair cell density of treated fish (0.022 ± 0.003 hair cells μm^{-2} , $n = 12$) was not significantly different from that of control fish (0.025 ± 0.006 hair cells μm^{-2} , $n = 36$, $t = 2.03$, $p = 0.331$, Figure 4 A). The prevalence of abnormal hair bundles in these fish (5.99%, $n = 12$), however, remained significantly higher than in control fish (1.58%, $n = 36$, $\chi^2 = 65.42$, $p < 0.0001$, Figure 4 A). Likewise, average kinocilia length of hair bundles, at fourteen days following treatment (3.80 ± 1.12

μm , $n = 172$), was still significantly lower than in control fish (4.78 ± 0.75 μm , $n = 487$, $U = 18470.00$, $p < 0.0001$, Figure 4 B).

At 21 days following local injection of gentamicin, hair bundles that were observed using SEM were comparable to those seen in control fish (Figure 3 F). The average hair cell density in treated fish (0.023 ± 0.004 hair cells μm^{-2} , $n = 12$) was not significantly different from control fish (0.025 ± 0.006 hair cells μm^{-2} , $n = 36$, $t = 1.43$, $p = 0.706$, Figure 4 A). The prevalence of abnormal hair bundles at this stage (2.17%, $n = 12$) was also not significantly different from control fish (1.58%, $n = 36$, $\chi^2 = 0.65$, $p = 0.419$, Figure 4 A). Only the average kinocilia length of treated fish hair bundles (4.53 ± 0.73 μm , $n = 176$) was still significantly lower compared to control fish (4.78 ± 0.75 μm , $n = 487$, $U = 34874.50$, $p = 0.000$, Figure 4 B).

Discussion

This study presents ultrastructural and functional damage, and subsequent near-total recovery in the Atlantic cod inner ear following an intrasaccular gentamicin injection using scanning electron microscopy (SEM) and auditory evoked potential (AEP) responses following a 250-Hz stimulus.

It is relevant to note that this study does not demonstrate directly whether there is change at the level of the entire sensory hair cell; nevertheless, hair bundle changes were used as a correlate of whole cell changes. For technical reasons, hair cell bundles from controls, sham, and treated fish were not examined on the same day. However, this did not decrease the strength of the study, given the great difference in tissue state between each fish group. Indeed, if controls and sham fish always presented normal hair bundles (except for slightly shorter hair bundles in sham fish) during the whole duration of the experiment, from the 3rd to the 14th day following gentamicin treatment, treated fish showed hair cell bundles that were greatly damaged. We demonstrated that gentamicin led to hair cell bundle damage: fusion of stereocilia and areas without any hair bundles. Not only has this kind of hair cell bundle alteration previously been described in the fish and lizard inner ear (Presson & Popper, 1990; Yan et al, 1991; Lombarte et al, 1993; Avallone et al, 2008) but also on cod inner ear (Faucher et al, 2008). To evaluate hair bundle damage, for each sample, kinocilia were assessed at three different areas, all of them located in the central part close to the wide indentation in the ventral margin of the macula sacculi. This was to avoid confounding with the known difference in kinocilia length between the periphery and the centre of the inner ear macula sacculi in the Atlantic cod (Dale, 1976). This fact and the observation of greater inter-group than intra-group kinocilia length differences corroborated the data obtained here. The toxicity of gentamicin can be related to its cationic nature. Indeed, it has been found that, in the bullfrog's sacculus, gentamicin interfered with transduction through voltage-dependent blockage of transduction channels (Kroese et al, 1989) located at the apical membrane surface of hair cells (Corey & Hudspeth, 1983), probably near the tips of stereocilia (Hudspeth, 1982). This voltage-dependent blockage occurs when a molecule of aminoglycoside from the extracellular solution interacts with a site in the transduction channel, and thereby

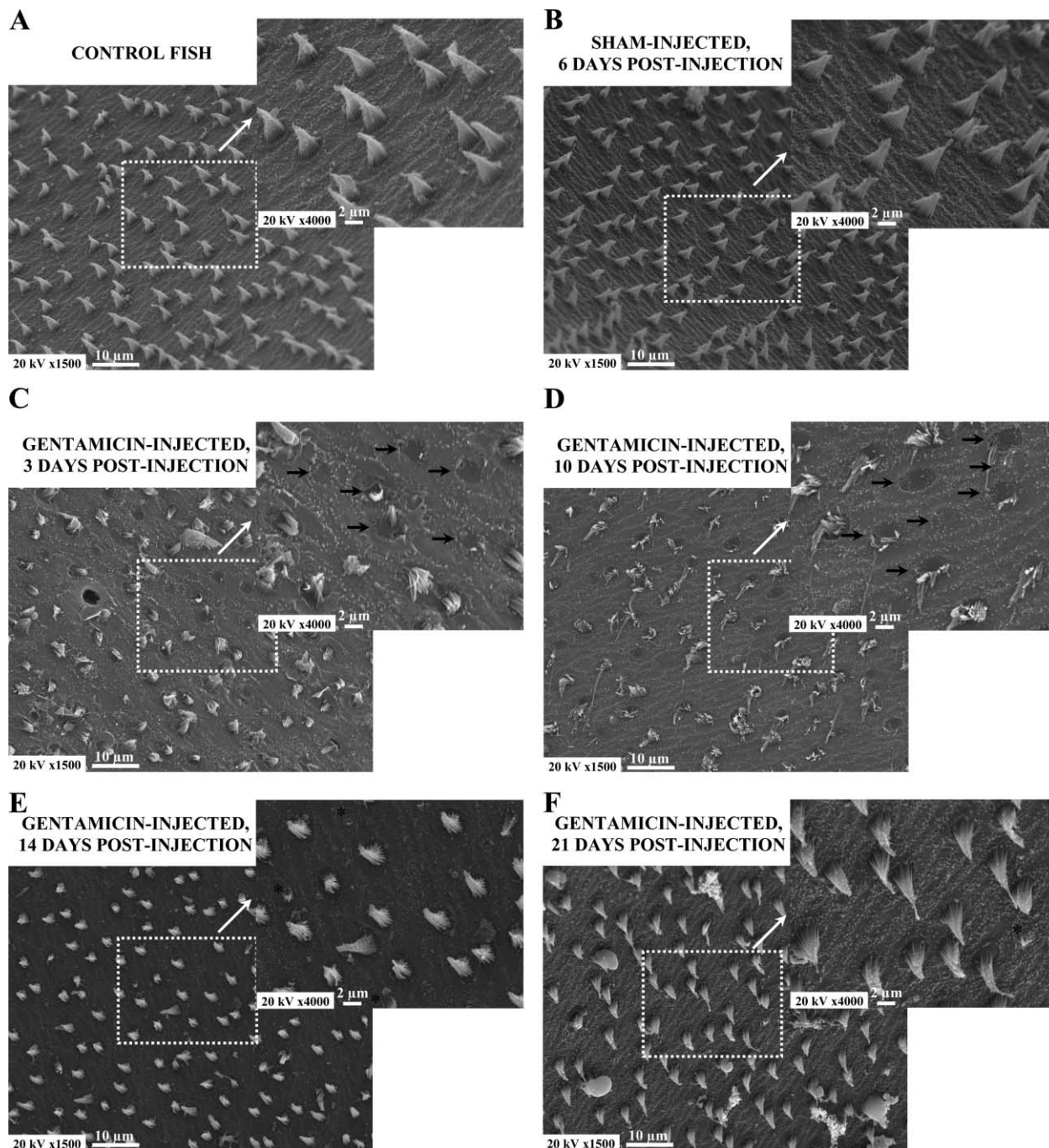


Figure 3. Sensory maculae of inner ear sacculi from the Atlantic cod observed using scanning electron microscopy. (A) Control fish: intact inner ear, (B) Sham fish: no visible damage observed, (C) Three days after intrasaccular gentamicin injection: hair bundles were disorganized and some areas were totally deprived of cilia, (D) Ten days after treatment, hair bundles were highly damaged: very shortened hair bundles and areas without cilia, (E) Fourteen days after treatment, hair bundles seemed to be less damaged, some short hair bundles could be seen, (F) Twenty-one days after treatment, hair bundles were similar to those observed in control fish. The black arrows show cells deprived of cilia. Asterisks show short hair cells. Inserts show higher magnification of hair cell bundles.

obstructs the flow of cations through the pore (Hudspeth & Kroese, 1983). Gentamicin results in fusion of the stereocilia, and at later stages, the stereocilia remnants disappear and hair cells may degenerate entirely (Wersäll et al, 1973).

Control fish, which received no injection, had normal AEP responses and maculae sacculi tissue with well-developed hair cell bundles. Response thresholds of sham fish were higher than in control fish during the first week post-intervention. However,

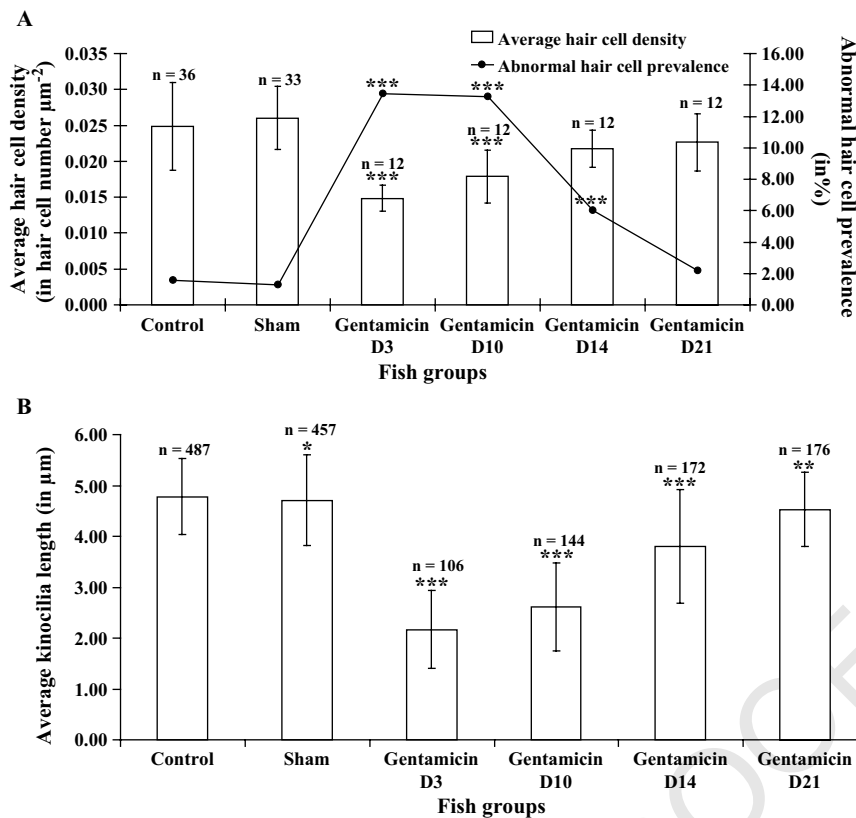


Figure 4. Quantitative hair cell analysis. (A) Average hair cell density (number μm^{-2}) and prevalence of abnormal hair cells in control, sham, and fish that had received intrasaccular gentamicin injection. Just after gentamicin treatment (three days), fish had a significantly lower average hair cell density. It progressively increased with time until it was not significantly different from control fish, from the 17th day on. The prevalence of abnormal hair cells was much higher in gentamicin-treated fish compared to controls. It progressively decreased with time, being similar to controls at the end of the experimental period. Numbers of SEM pictures (n) from which hair cell densities were calculated are mentioned for each value. (B) Average kinocilia length of hair bundles in control, sham, and gentamicin injection fish. At three days following gentamicin treatment, kinocilia were significantly shorter in treated fish. Then, cilia grew progressively longer with time, although they were still slightly shorter than controls at the end of the experimental period. Vertical bars represent standard deviation of mean. Asterisks show data that were significantly different from control group ($p < 0.0001$: ***; $p < 0.01$: **, and $p < 0.05$: *). Numbers of kinocilia measured (n) are mentioned for each value.

sham fish subsequently had normal hearing, and their maculae were similar to control fish, except for the average kinocilia length which remained slightly shorter than in control fish. Three days after intrasaccular gentamicin injection, fish had higher response thresholds compared to control fish, which decreased until the 17th day following treatment. This threshold difference was associated with hair cell damage: disorganized hair bundles and areas that were entirely deprived of hair cells. Indeed, compared with control fish, hair cell density in the macula sacculi of treated fish was lower until the 14th day following treatment, the prevalence of abnormal hair bundles was higher until the 21st day and the average kinocilia length remained shorter throughout the experimental period.

The average hair cell density measured in the inner ear sacculi of the control (0.025 ± 0.006 hair cells μm^{-2}) and sham fish (0.026 ± 0.004 hair cells μm^{-2}) corroborated the average of 0.025 hair cells μm^{-2} previously found by Dale (1976) and Faucher et al (2008) in the same species. However, sham fish showed signs of slightly altered hearing during the first week of experiment. We

cannot exclude that the injection procedure in itself may have led to very slight damage of sensory hair cells, not visible at the ultrastructural level, resulting in short-term functional alterations. An alternative explanation could be that the cod physiological saline may have temporarily altered the chemical composition of the intrasaccular fluid, leading to changes in the sacculus electrophysiological properties, and a subsequent threshold shift. Very slight alterations of inner ear have previously been observed after injection of physiological saline in the inner ear of the mouse (Nakagawa et al, 2003). After intrasaccular injection of gentamicin in the cod, the functional and ultrastructural damages observed were pronounced. Three days after treatment, fish that had received gentamicin had an upwards threshold shift of 14%. In the same period, their average hair cell density decreased by 40%, prevalence of abnormal hair bundles increased nine times, and the average kinocilia length was halved, in comparison with control fish.

An extension of the AEP measurement window could have yielded additional information in gentamicin-treated fish, as

latencies are expected to increase after damage. However, the existing data convincingly suggest the recovery of normal responses, and their thresholds, in the late phase of the study period.

The present study clearly demonstrates that gentamicin induced significant inner ear ultrastructural impairment associated with increased AEP threshold from the third day following treatment. Sensory inner ear tissue then progressively regenerated until it was indistinguishable from that of control fish at 14 days post-treatment. This was also associated with AEP threshold recovery at the 17th day following the gentamicin intervention. This indicates that after gentamicin-induced ototoxic impairment, the macula sacculi of the Atlantic cod have the capacity for functional recovery of hearing which is associated with near-total ultrastructural recovery of saccular hair cells.

In the present study, the maximal gentamicin effects were observed relatively early after the intervention. The ototoxic effects of aminoglycosides in fish have usually been studied following systemic (intramuscular) administration (Yan et al, 1991; Lombarte et al, 1993). In addition to the nephrotoxicity that can be induced by this mode of administration (Dulon et al, 1988), systemic administration is characterized by slow pharmacokinetics. For instance, with a daily intramuscular injection of gentamicin on four successive days in the oscar (*Astronotus ocellatus*), at a dose of 20 mg kg⁻¹ day⁻¹, maximal tissue damage was observed between 10 and 15 days following the onset of the experiment (Yan et al, 1991; Lombarte et al, 1993). In comparison, Matsuura et al (1971) demonstrated a reduction of saccular potentials about 3 or 4 minutes after intrasaccular gentamicin administration in the goldfish (*Carassius auratus*). This method of gentamicin administration is thus regarded to be the most suitable to observe damage and recovery of inner ear hair cells and hearing function in fish.

The regeneration of macula sacculi sensory tissue seems to occur earlier (14 days following treatment for hair cell density) than full recovery of response thresholds (17 days following treatment). The prevalence of abnormal hair cell bundles and kinocilia length needed more time to be comparable to normal fish. This could indicate that tissue regeneration is not a fast process, and that functional normalization can occur although hair bundle ultrastructure is not yet normal. Hearing recovery preceding morphological regeneration of the inner ear sacculi has already been described in the goldfish (*Carassius auratus*) after damage by noise exposure (Smith et al, 2006). In the cod, as in the goldfish, results hence suggest that a full population of inner ear hair cells is not necessary for normal hearing.

After treatment, some hair cells lost their hair bundles while their cuticular plates were retained. As suggested by previous studies in the bullfrog (*Rana catesbiana*) (Gale et al, 2002) and in the lizard (*Podarcis sicula*) sacculi (Avallone et al, 2008), these cells might participate at hair cell renewal without requiring mitosis thanks to a self-repair ability. On the other hand, after gentamicin treatment in the oscar inner ear, the number of such cuticular plates in areas where damage was extensive seemed not to be sufficient to account for the normal complement of ciliary bundles after recovery (Lombarte et al, 1993). This author suggests that these cells may either contribute to the ciliary bundle restoration, or could represent late-dying hair cells. We can hypothesize that this self-repair capacity may be accom-

panied by the proliferation of precursor cells for new sensory hair cells triggered by gentamicin damage. Indeed, as already observed in fish lateral line neuromasts (Rouse & Pickles, 1991) and in the lizard inner ear (Avallone et al, 2008), some hair cells with small hair bundles, commonly named immature hair cells, have been seen during regeneration.

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