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RESEARCH ARTICLE

Fin reduction is a novel and unexpected teratogenic effect of amikacin-treated zebrafish embryos

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Abstract
We used zebrafish as a model to assess amikacin-induced embryotoxicity. We exposed zebrafish embryos to amikacin, using different amikacin doses (0–10 ppm), durations (12–48 h), and onsets (0, 24, 48 hpf). Amikacin-induced embryonic toxicity and reduced survival rate were found dependent on the exposure dose, duration and onset. Based on immunostaining with neuron-specific antibodies, amikacin reduced the number and size of zebrafish neuromasts. In addition, Amikacin caused pelvic, dorsal and anal fin defects in dose-dependent and duration-dependent manners. Proliferating cell nuclear antigen immunostaining revealed that amikacin-induced fin defects were not due to reduction of proliferating mesenchymal cells. TUNEL assay demonstrated that amikacin-induced fin defects might not associate with apoptosis. Therefore, further investigations are required to elucidate if other cell death pathways are involved in amikacin-induced fin defects.

Keywords: Amikacin, fin, zebrafish, ototoxicity, neuromast

Introduction

Amikacin ($C_{22}H_{43}N_5O_{13}$) is a semisynthetic aminoglycoside antibiotic. In medical applications, amikacin is commonly used to treat acute respiratory exacerbations in patients with cystic fibrosis, intra-abdominal sepsis, complicated urinary tract infections, and other infections caused by gram-negative enteric bacilli, or other gram-positive bacteria such as *Mycobacterium* (McCracken 1986; Duff 2002; Ji et al., 2006). Though amikacin is generally considered safe for human use at recommended doses, its therapeutic window is pretty narrow, an acute overdose or even a normal dose can lead to severe side effects such as hearing loss as well as liver and renal damages (Forge and Schacht, 2000; Martínez-Salgado et al., 2007; Taylor et al., 2008). Amikacin is assigned a pregnancy risk class D designation by the US Food and Drug Administration (FDA), meaning that there is positive evidence of human fetal risk but potential benefits may warrant use of the drug in pregnant women despite potential risks (Berglund et al., 1984; Lacy et al., 2010). In particular, amikacin can cross the placenta and may have potential risk for the developing embryo (McCracken, 1986; Pacifici, 2006). Thus, the developmental toxicity and the teratogenic effect of amikacin are important issues that should be investigated.

Studies using animal models have shown that continuous infusion (16 mg/kg) or intra-muscular injections of amikacin (60 mg/kg) in rabbit but little or no nephrotoxicity was observed (Brion et al., 1984). In rodents, amikacin treatment causes kidney

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dysfunctions (ex: polyuria, proteinuria, enzymuria, and decreases in urine osmolality) as well as hair cell damages (Rankin et al., 1980; Aran et al., 1995). In humans, overdose of amikacin can cause nephrotoxicity and ototoxicity (Lerner et al., 1977; Luft et al., 1981; McCracken 1986; Begg and Barclay, 1995). Although the current therapeutic dose of amikacin (15 mg/kg) is safe in adult, may increase the risk of toxicity in infant (Siddiqi et al., 2009). These observations suggest that amikacin-induced toxicities might differ depending on the species, the route, the dose, and the duration of exposure. Several alternative methods developed using cell, organ or whole embryo cultures are available for investigating the embryo toxic and/or teratogenic potential of amikacin (Schaad et al., 1988; Amacher et al., 1989; Lass et al., 1989; Kotecha and Richardson, 1994; Chang et al., 1995; Wang et al., 1996). However, it is highly disadvantageous that subtle changes during early embryonic development are difficult to observe using mammal model; thus, development of an alternative animal model is essential.

In contrast to other vertebrate models, zebrafish embryos are fertilized externally, having large amount of transparent embryos, and their early development can be easily observed that makes it an effective model for toxicological studies (Westerfield, 1995; Chen et al., 2011). To further investigate the amikacin-induced subtle defects, we conducted a series of time- and dose-response amikacin exposure experiments using zebrafish as a model. This strategy is excellent for studying amikacin-induced teratogenicity during early embryonic development.

Materials and methods

Fish embryos staging and amikacin treatment

Mature zebrafish (AB strain) was raised at the zebrafish facility of the Life Sciences Development Center, Tamkang University. The fish were maintained at 28°C with a photoperiod of 14 h light and 10 h dark, in an aquarium supplied with freshwater and aeration (Chen et al., 2009a). Embryos were produced using standard procedures and were staged according to standard criteria: hours postfertilization, hpf; or days postfertilization (dpf; Westerfield, 1995). Amikacin (25 mg/ml; Tai Yu Pharmaceutical Co., Taiwan) was diluted in sterile distilled water to the appropriate concentrations. Six exposure protocols (methods I–VI) were applied in the amikacin treatment experiments according to their exposure durations (Figure 1A). For dose titration, zebrafish embryos were collected, randomly divided into several groups (30 embryos per group), and exposed to either water (no treatment control) or water containing amikacin at selected concentrations (0–10 ppm). All embryos were cultured in 6-well cell culture plates with 5 ml solution each, and survival rates were counted at the check point. All animal experiments in this study were performed in accordance with the guideline issued by regional animal ethic committee.

Fin morphology recording

To develop a quantitative assessment of fin morphology, fins of amikacin-treated fish were compared with fins of healthy, untreated fish and subjectively classified as (i) normal: at least 90% of the fin was intact, (ii) reduced: 20–90% intact, and (iii) absent: less than 20% intact (Wang et al., 2009a, Chen et al., 2011).

Antibody labeling

Proliferating cell nuclear antigen (PCNA) (Santa Cruz, USA) and anti-acetyl tubulin (Sigma, USA) were employed to visualize proliferating cells and neuron cells, respectively, as previously described (Wang et al., 2009b). For antibody staining, embryos were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS, pH 7.0) for 4 h at room temperature. Embryos were then washed in PBS twice for 15 min each, soaked in 100% acetone at –20°C for at least 10 min, and rehydrated with 0.1% (v/v) Tween 20 in PBS (PBST) three times for 15 min each. After rehydration, the embryos were treated with PBS containing 5% goat serum albumin for blocking, and then subjected to immunofluorescence staining.

TUNEL assay

To detect cells undergoing programmed cell death, we performed terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuridinetriphosphate [dUTP] nick end labeling (TUNEL) experiment as described before (Chen et al., 2009a; Peng et al., 2010). By 96 hpf (48 h after exposure with amikacin, method IV), embryos from mock control (fish from the same population of embryos but were not treated with amikacin) and 10 ppm of amikacin were fixed overnight at 4°C in 4% paraformaldehyde and TUNEL was performed using the *In Situ* Cell Death Detection kit (Roche, Switzerland).

Cryosection, histology and images

The procedures for cryosection and hematoxylin/eosin Y (H/E) staining have been described previously (Wang et al., 2008; Chen et al., 2009b; Pai and Chen, 2010). All embryos and sections were observed under a microscope (DM 2500, Leica) equipped with Nomarski differential interference contrast optics and a fluorescent module having green fluorescent protein (GFP), red fluorescent protein (DsRed), and 4′6-diamidino-2-phenylindole·2HCl (DAPI) filter cubes (Kramer Scientific). Images of embryos were captured at specific stages with a digital carema (Eos550D, Canon).

Statistical analysis

All analyses and survival curves in this study were carried out by Matlab software (version 7.7.0). The median dosage levels (LC_{50} values) for different exposure methods were separately predicted by logistic regression analysis. The dosage, exposure time, and starting exposure time effects

on survival rate were also examined by logistic regression method. A significance level of 0.05 was used in all statistical analyses. The two-sample *t*-test with a significance level of 0.05 was employed to explain the significant difference between the treatment and control samples.

Results and discussion

Titration of amikacin dose and analysis of survival rates

To address the dosage and exposure-time effect of amikacin on the teratogenic incidence, we first treated zebrafish embryos with different dosages of amikacin (0, 2, 5, 10 ppm) and exposure-times (0–24, 0–48, 0–72, 0–96 hpf) via four experiment protocols (methods I–IV; Figure 1A) to investigate the survival rates. Figure 1B depicts the mean survival rate and its standard error (SE) at each dosage level for four exposure design groups. For example, 92.7 ± 2.4% (mean ± standard error, SE; $n = 30$, number of embryos tested in each group; $N = 5$, repeated five times) of the mock control embryos were alive at the check points of each method (I: 24 hpf; II: 48 hpf; III: 72 hpf; IV: 96 hpf); and 64.0 ± 2.4% ~ 54.7 ± 2.7% ($n = 30$, $N = 5$) of the embryos were alive after exposure to 2–10 ppm of amikacin for 48 h (method II). As shown in Figure 1B, similar phenomena that the survival rates decreased apparently when the dosage of amikacin increased were also observed for other exposure design groups (methods I, III, IV). In fact, the logistic regression analysis based on whole data reported the dosage and exposure-time effects are negative with *p*-values 0.0000 and 0.0045 respectively, indicating a significant decrease in survival rates when amikacin concentration or exposure time increases. In addition, for each exposure method, we employed the logistic regression analysis to calculate the median dosage level (LC_{50} value) at which the survival rate exactly achieves 50%. Logistic regression assumes that the survival probability p is related to dosage level x by the logit function:

$$p(x) = (1 + e^{-\beta_0 - \beta_1 x})^{-1}$$

and the median dosage level is thus given by

$$p^{-1}(0.5) = -\beta_0 / \beta_1$$

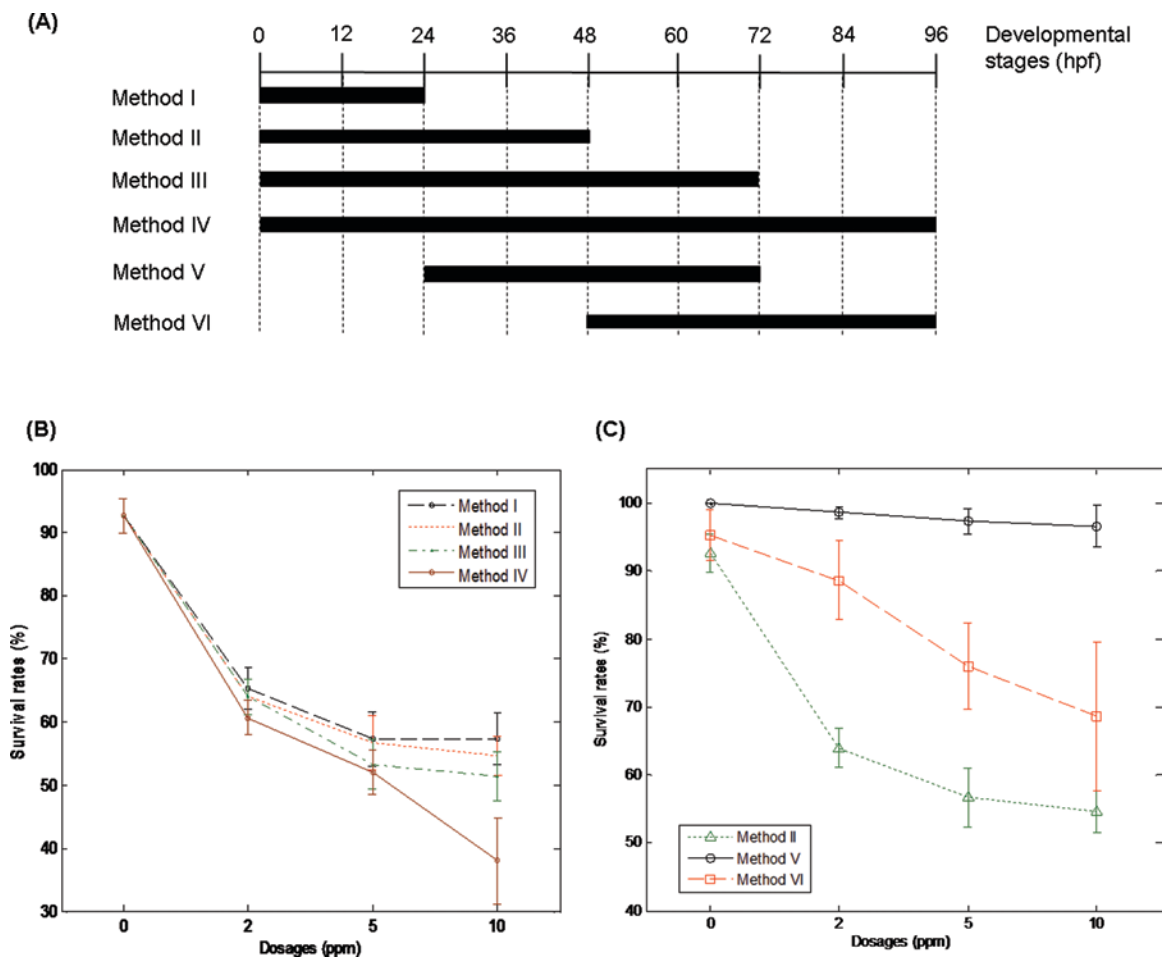

Figure 1. Exposure protocols and survival rate analysis in this study. (A) Exposure methods of amikacin treatment in this study. Six types of exposure methods were designed for the amikacin treatment experiments according to the exposure durations and onsets; I: 0–24 hpf, II: 0–48 hpf, III: 0–72 hpf, IV: 0–96 hpf, V: 24–72 hpf, and VI: 48–96 hpf. (B) Survival rates of zebrafish embryos after exposure to water (mock control) or water containing 2, 5, 10 ppm of amikacin when the exposure methods I–IV or (C) methods II, V, VI were used. The X- and Y-axes represent developmental stages and survival rates, respectively.

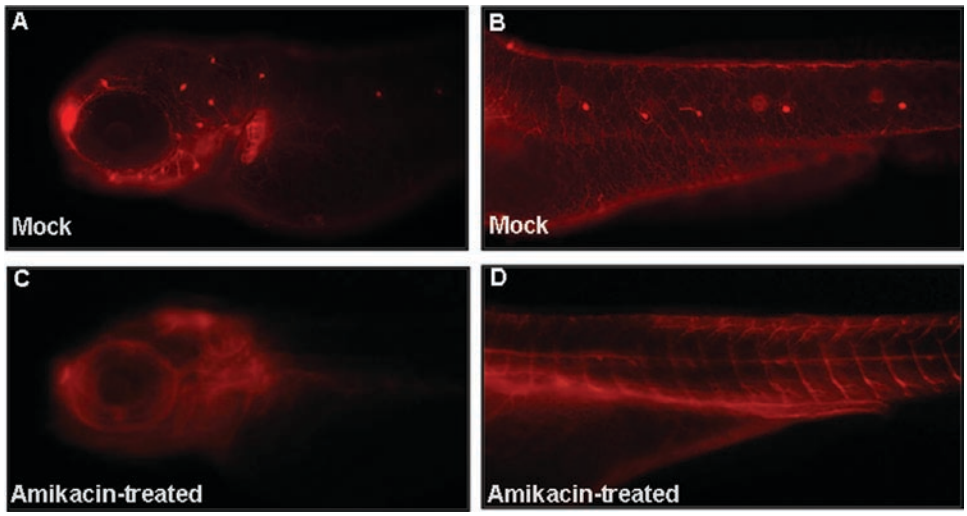

Figure 2. Zebrafish embryos were stained with anti-acetyl tubulin antibody to make neuromasts visible. Embryos were exposed to water (mock control: 0 ppm of amikacin, A, B) or water containing 10 ppm of amikacin (C, D) by methods IV. After exposure, embryos were stained with anti-acetyl tubulin antibody to label all neurons, especially neuromasts. All the photos were taken from the lateral view and were of developmental stages at 96 hpf.

The predicted LC_{50} values for exposure methods I–IV are 10.2, 9.5, 8.5, 6.6 ppm, respectively, indicating the longer exposure time induces the lower dosage to achieve half survival rate.

To investigate the effect of the starting exposure time, we further conduct two experiments (methods V, VI; Figure 1A), in which the same exposure time period (48 h) with method II remained but different starting times (24 or 48) were considered. Figure 1C presents the mean survival rate and the SE of mean at each dosage level for exposure methods II, V, VI. The corresponding predicted LC50 values are 9.5, 28.9, 13.5 ppm, respectively. The logistic regression analysis based on whole data from methods II, V, VI reported the dosage effect and the starting exposure time effects on survival rate among three exposure methods are significant (all p-values < 0.0001).

Loss of neuromasts and fin reduction are the obvious defective phenotypes of zebrafish embryos after exposure to amikacin

Previous studies have shown that hearing loss is one of the adverse effects after amikacin treatment (Taylor et al., 2008). The hair cells in the neuromasts of zebrafish share both morphological and functional similarity to those of the mammalian inner ear (Chiu et al., 2008). Here, we examined the phenotypic defects caused by amikacin using method VI. At 96-hpf, neuromasts are easily observed in the head as well as in the trunk regions of no treatment control embryo (Figure 2A and B). In contrast, no neuromasts are observed in the 10-ppm amikacin-treated embryos (Figure 2C and D), indicating that amikacin is toxic for neuromasts development. The average number of neuromasts for no treatment control and amikaicn-treated groups is 10.93 (0.07, $n = 40$; SE, sample size) and 0.42 (0.16, $n = 40$), respectively. A two sample t-test reported a significant difference between two groups (p-value < 0.0001). This result suggests that amikacin-induced ototoxicity in zebrafish is similar to that in mammals; and 10-ppm, 48 h exposure is suitable for studying amikacin-induced teratogenicity in zebrafish. Besides loss of neuromasts, we found amikacin-exposed embryos also displayed an unexpected teratogenic defect, fin-reduction phenotype, including dorsal, ventral and pelvic fins shrinkage or absence (Figure 3A vs. 3B and 3C). These fin-reduction phenotypic defects were the most obvious consequence of amikacin exposure in this animal model.

Amikacin-induced fin-reduction phenotypes are dose-dependent

Fin-reduction phenotypic changes were classified as "normal", "reduced", and "absent" according to the affected areas of fins on each embryo. The percentages of fish with normal, reduced, and absent fins after treatment with 0–10 ppm amikacin were quantified and it was found that, as the exposure dosages of amikacin increased, the percentages of fish with malformed (reduced plus absent) fins increased. At the end points (72 hpf), no embryos displayed malformed fins (reduced plus absent) after 0, 2, 5 ppm of amikacin exposure using method V (24–72 h). The percentages of malformed fins slightly increased to 12.0 ± 2.3% ($n = 30$, $N = 9$) when the dose increased to 10 ppm (Figure 3D). On the other hand, the fin-malformation rates significantly increased to 32.7 ± 8.2% ($n = 30$, $N = 9$) after 10-ppm amikacin exposure by method VI (48–96 hpf; Figure 3E), but no "fin-reduction defect" was observed when exposure protocol method II (0–48 hpf) was applied (data not shown). These observations clearly indicated that amikacin-induced fin-reduction phenotypes of zebrafish embryos depended on their exposure onset and exposure dose.

The most evident adverse effects of amikacin are ototoxicity and nephrotoxicity; here, we found a novel

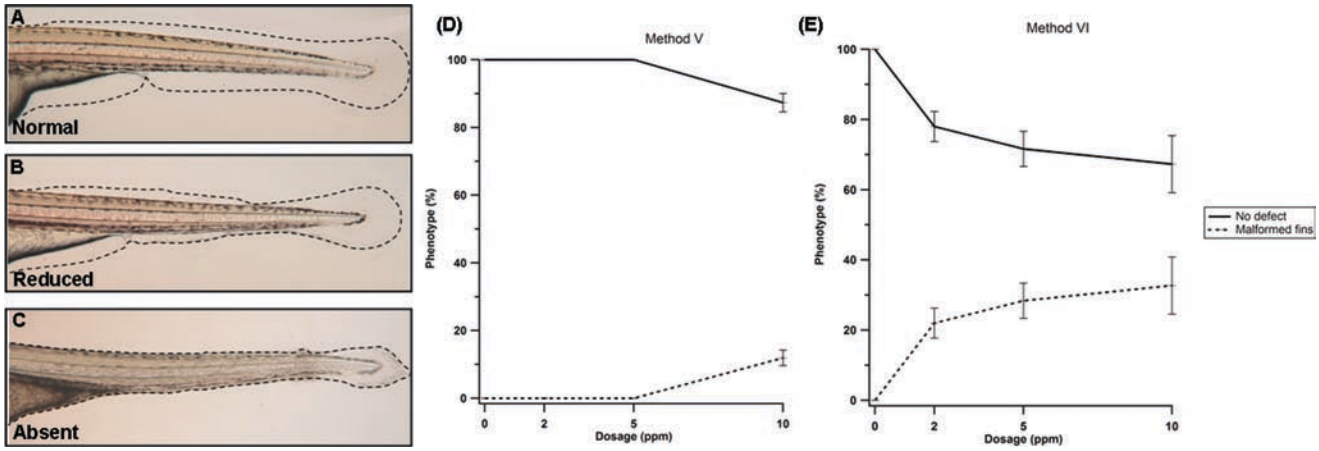

Figure 3. Amikacin-induced fin malformation phenotypes are dose-response. Phenotypic changes after amikacin exposure: (A) normal fins, control, (B) reduced fins and (C) absent fins. Dashed lines in each figure indicate the morphology of fins. (D) Phenotype percentages of zebrafish embryos after amikacin treatment (0, 2, 5, 10 ppm) were illustrated when the exposure methods V (E) and VI (F) were used. The X- and Y-axes represent concentrations of amikacin and phenotype percentages, respectively. Data are presented as means ± standard error.

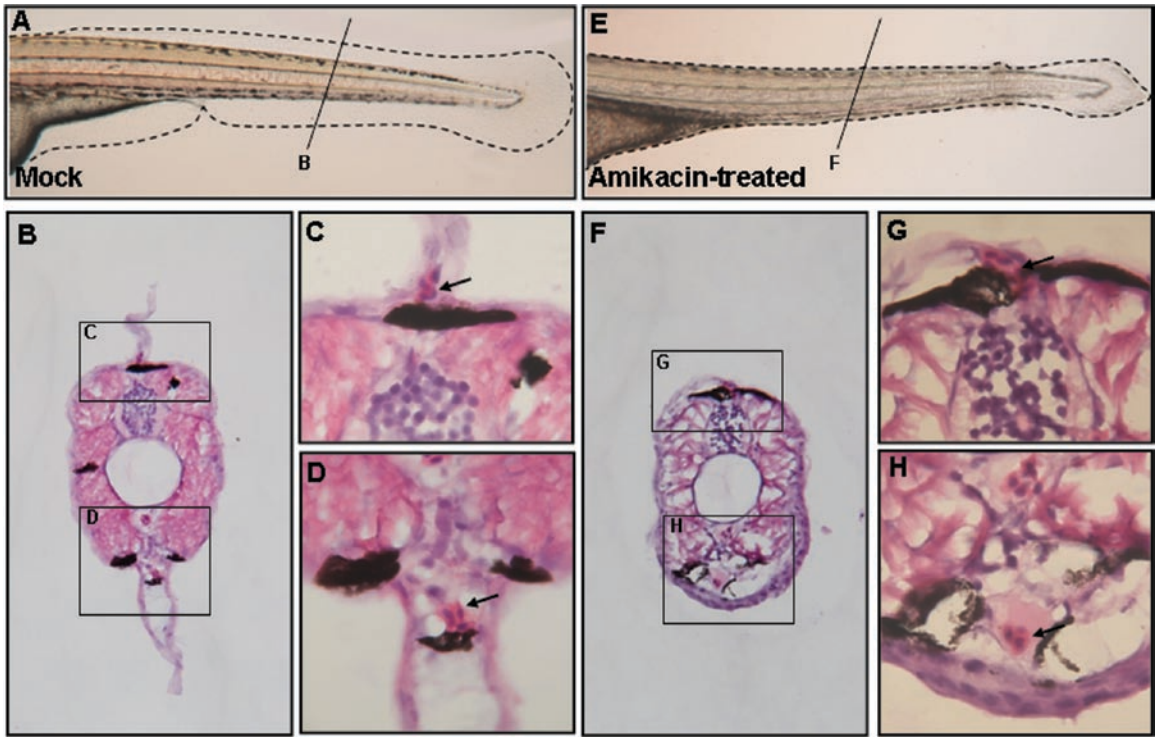

Figure 4. Histological features of amikacin-treated embryos. Transverse sections of mock (A–D) and amikacin-treated embryos (E–H) at trunk regions (96 hpf) were stained with hematoxylin/eosin Y, and their fin mesenchymal cells around the soma/fin junction were indicated by arrows.

and unexpected teratogenic defect, fin reduction, which hasn't been reported in mammal model so far. In this regard, we applied method V with another aminoglycoside antibiotic, gentamicin, to observe whether fin reduction is amikacin-specific teratogenic defect. Results showed that the percentages of malformed fins significant increased to ≈60% after 5 ppm gentamicin treatment (data not shown). These observations indicate that fin reduction might be general malformed phenotypes after aminoglycosides antibiotic treatment, but more exposure data should be accumulated.

Treatment with amikacin does not affect proliferating fin mesenchymal cells

Investigation of the possible target cells attacked by amikacin, which in turn cause fin-reduction phenotypes, was extended using cryosectioning and hematoxylin (H)/eosin Y (E) staining to visualize cell nuclei/cytoplasm in the mock control and amikacin-treated embryos. In mock control embryos, there were numerous nuclei located at the fin/soma junctions (Figure 4A, B, C, and 4D, black arrows), and distributed evenly on the fins. In the amikacin-treated embryos, the number of nuclei located on fins

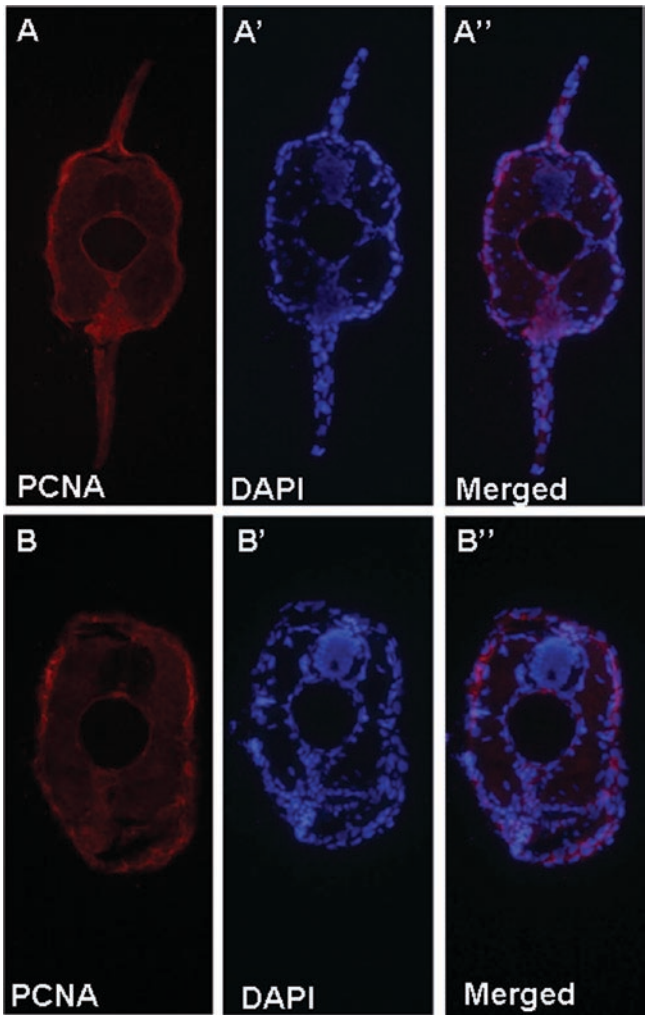

Figure 5. Effects of amikacin on proliferating fin mesenchymal cells. Embryos derived from mock control (A, A′, A″) or amikacin-treated groups (B, B′, B′) stained with antibodies against PCNA. Cryosections of A′ and B′ were counter-stained with DAPI. Panels A″ and B″ were merged figures of A, A′ and B, B′, respectively.

appeared unchanged in comparison with mock control embryos (Figure 4B, red arrows). Furthermore, mock control and amikacin-treated embryos were stained with monoclonal antibodies against PCNA, a proliferating cell marker. The results showed that the numbers of PCNA-positive cells were unchanged between mock control and amikacin-treated embryos (Figure 5A, A′, A″ vs. 5B, B′ B″). These observations clearly indicated that fin reduction is not a consequence from reduced number of proliferating fin mesenchymal cells.

Amikacin-induced cell apoptosis is the main cause for loss of neuromasts phenotype, but not for fin reduction

Experimental evidence accumulated from previous studies has shown that aminoglycoside can generate free radicals within the inner ear, that are potentially destructive to any or all cell constituents, resulting in apoptotic cell death and permanent hearing loss (Wu et al., 2002; Selimoglu 2007). We have demonstrated that number of proliferating fin mesenchymal cells are unaffected after amikacin (Figure 5); thus, our hypothesis is that amikacin-induced loss of neuromasts and reduced-fin phenotypes of zebrafish embryos may be through cell apoptosis. Next, we carried out TUNEL experiments and results showed that no apparent apoptotic signals were observed in the embryos derived from mock control group (Figure 6A). In contrast, almost all neuromasts underwent apoptosis after amikacin treatment (Figure 6B, black arrows), indicating that amikacin-induced ototoxicity may share the same mechanism between mammals and zebrafish. On the other hand, we surprisingly found that TUNEL-positive signal did not appear on fin mesenchymal cells (Figure 6B), suggesting that apoptosis might not be the main cause for reduced-fin phenotypes. In this regard, we hypothesize that amikacin treatment might activate other unknown cell death pathway, and consequently cause fin reduction.

Comparison of amikacin exposure doses among different species

For amikacin treatment in adult human, the appropriate multidose regimen is 5.0 mg/kg actual weight every

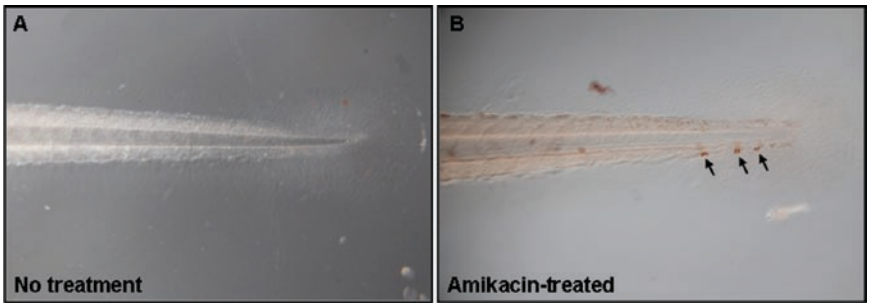

Figure 6. Effects of amikacin on cell apoptosis. TUNEL assay of embryos derived from mock control (A) or amikacin-treated groups at 72 hpf (B). Arrows in panel B indicate that neuromasts undergoing apoptosis.

8 h or 7.5 mg/kg actual weight every 12 h. The single daily dose should be 15 mg/kg ideal body weight (Duff 2002). In this study, we used single dose (10 ppm) for two days (method VI, 48–96 hpf). It is difficult to compare toxicities between species where one group is receiving the dose in the diet and the other is systemically exposed to a solution of the toxin, but we can still estimate the daily exposure of zebrafish embryos using appropriate calculations. The total amount of 2–10 ppm of amikacin in 5 ml solution is around 10–50 μg. In this study, 30 embryos received 10–50 μg of amikacin for 2 days, so the average daily exposure of each embryo is 0.17–0.83 μg of amikacin if the absorption rate is 100%. The body weight of each zebrafish embryo is around 1.2 mg. Thus, the daily exposure of zebrafish embryos was around 136–691 mg/kg (0.17–0.83 μg/1.2 mg). This is an extremely high dose in comparison with the recommended daily dose of amikacin in adult people (15 mg/kg). However, the teratogenic dosages of amikacin in zebrafish embryo should be acceptable in comparison with those used in rodents for toxicological studies (500–1000 mg/kg; Daudet et al., 1998; Parietti et al., 1998; Murillo-Cuesta et al., 2010). Thus, we suggest that 2–10 ppm of amikacin, exposure for 48 h is effective in this current model.

In conclusion, amikacin-induced malformation of fins could be easily observed *in vitro* during early embryogenesis in the present model. This could provide novel insights into the subtle changes induced by amikacin, which are worthy of further tests in higher vertebrates.

Declaration of interest

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