Acute copper exposure induces oxidative stress and cell death in lateral line hair cells of zebrafish larvae

Francisco A Olivari, Pedro P Hernández, Miguel L Allende*

Center for Genomics of the Cell, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

ABSTRACT

Numerous physical and chemical agents can destroy mechanosensory hair cells in the inner ear of vertebrates, a process that is irreversible in mammals. Few experimental systems allow the observation of hair cell death mechanisms in vivo, in the intact animal, one of these being the lateral line system in the zebrafish. In this work we characterize the behavior of dying lateral line hair cells in fish exposed to low doses of copper in the water. The concentration of copper used in our study kills hair cells in a few hours, but removal of the metal is followed by robust regeneration of new hair cells. We use a combination of membrane and nuclear live stains, ultrastructural analysis and measurement of reactive oxygen species to characterize the events leading to the death of hair cells under these conditions. Our results show that a combination of necrotic cell death, accompanied by apoptotic features such as rapid DNA fragmentation, lead to the loss of these cells. We also show that hair cells exposed to copper undergo oxidative stress and that antioxidants can protect these cells from the effects of the metal. The study of this process in the zebrafish lateral line allows rapid morphological analysis of hair cell death and may be used as an efficient end point for molecule screens aimed at preventing these effects.

Keywords: Lateral line, Neuromast, Mechanosensory cell, Metal toxicity, Necrosis, Apoptosis

1. Introduction

The death of hair cells induced by a variety of environmental factors has been extensively documented. The effects of acoustic trauma, aminoglycoside antibiotics and, more recently the anti-cancer drugs cisplatin and other platinum derivatives, have been used to study hair cell death and there is an accumulating body of literature on the mechanisms and clinical outcomes of damage induced by these agents (Cheng et al., 2005; Henderson et al., 2006; Rizzi and Hirose, 2007; Selimoglu, 2007). Hair cells in the mammalian ear are highly sensitive to oxidative stress induced by aminoglycosides and cisplatin, but loading them with antioxidant scavengers prior to exposure can effectively protect the cells (Priuska and Schacht, 1995; Clerici et al., 1996; Hirose et al., 1997; Ton and Parng, 2005). Both necrotic and apoptotic pathways of cell death have been documented in the mammalian inner ear after toxic or mechanical damage (Yang et al., 2004; Cheng et al., 2005; Rybak and Witworth, 2005; Eshraghi and Van de Water, 2006; Bohne et al., 2007).

Recently, dissolved copper as CuSO₄ in low micromolar concentrations has been reported to be toxic to hair cells, a finding first analyzed in lateral line neuromasts of zebrafish larvae (Hernández et al., 2006; Linbo et al., 2006). This sensory system is extremely sensitive to copper concentrations that do not affect the survival of the fish. Copper generates a dose-dependent toxicity in neuromasts, producing death of hair cells followed by proliferation and regeneration of these cells,
or permanent damage if concentrations are higher than 50 μM (Hernández et al., 2006).

The lateral line system in zebrafish offers a unique experimental setting to study cellular and molecular events during hair cell death and regeneration, mainly because of the accessibility of neuromasts for examination and direct application of different compounds. Its utility has also been proven in chemical screens for assessing the effect of different ototoxic and otoprotective compounds (Ton and Parng, 2005; Owens et al., 2008). In zebrafish larvae, the lateral line is composed of neuromasts located at stereotypical positions on the head and the body. Neuromasts contain discrete hair cell clusters and two described populations of accessory cells: mantle cells which cover the apical surface of hair cells forming a single cell-wide sheet, and supporting cells forming a basal cell layer projecting apical processes that interdigitate with hair cells (Balak et al., 1990; Williams and Holder, 2000).

The transparency of larvae and accessibility of neuromasts allows for live hair cell labeling with fluorescent markers of mechanotransduction such as FM1-43 (Gale et al., 2001; Meyers et al., 2003; Corey et al., 2004) and other membrane and nuclear stains (Murakami et al., 2003; Santos et al., 2006).

Copper is an essential micronutrient but misregulation of intracellular levels can become toxic to many cell types. In fishes this metal can accumulate when excess is found in the water though dietary sources are tightly regulated (Clearwater et al., 2002). One of the effects of copper toxicity in zebrafish is oxidative damage in gills and the liver as well as changes in the expression of genes involved in cellular inflation (Craig et al., 2007). In lateral line neuromasts of zebrafish larvae, copper concentrations beginning from 0.15 μM, can significantly diminish hair cell number in a matter of minutes (Limbo et al., 2006). Short treatments (2 h) with as low as 1 μM CuSO₄ can completely eliminate all hair cells in neuromasts and higher concentrations differently affect accessory cells and the regeneration capacity of this sensory system (Hernández et al., 2006, 2007).

Several methods have been used to characterize hair cell and accessory cell damage, including staining of hair cells with vital dyes (Daspe, Di-Asp and FM1-43), transgenic lines that label neuromasts with GFP, Acridine Orange, immunofluorescence, light microscopy and scanning electron microscopy. These methods were useful to demonstrate that toxicity produced by these copper concentrations is restricted to neuromasts, hair cells being the most sensitive cell type, and to characterize the temporal and concentration-dependent aspects of hair cell disappearance. However, the occurrence of specific cell death pathways and the molecular events associated with copper exposure in hair cells remain unclear.

In the present work we sought to provide insights on processes occurring during copper-induced toxicity in hair cells using the zebrafish model. As a first approximation we developed a simple method to identify dying hair cells in lateral line neuromasts in vivo, using confocal microscopy and fluorescent dyes to monitor hair cell morphology during exposure. Hair cells were stained with two dyes, one for membrane labeling (Bodipy TR C5-ceramide) and a second with DNA binding properties (SytoGreen 24). In this way we could visualize HC shape and the fate of nuclear material during copper exposure. We analyzed the effects of 1 and 10 μM CuSO₄, copper concentrations previously used to study hair cell regeneration (Hernández et al., 2007), and we compared the outcomes that these two conditions produce in terms of the death pathways followed by HCs. To characterize further events occurring in hair cells exposed to copper we used transmission electron microscopy to observe dead cells in neuromasts. DNA fragmentation and ROS production was analyzed using methods such as TUNEL, antioxidant otoprotection and dichlorodihydrofluorescein labeling.

Our results show that copper exposure elicits necrotic hair cell death in neuromasts, an effect that is partially prevented by antioxidants. In addition, we detect significant DNA damage and an increase in ROS levels in copper-exposed hair cells.

2. Results

2.1. Hair cell death in neuromasts recognized by confocal microscopy and fluorescent vital dye staining

Hair cell (HC) labeling was performed in 3-day-old larvae (72 hpf), by incubating them in a mix of Bodipy TR C5-ceramide and SytoGreen 24 in fish medium, at concentrations that result in only HC staining (see Experimental procedures). Labeled neuromasts in untreated fish were monitored over time and no morphological changes were observed 15 h after staining, indicating that these dyes produce no toxic effects on HCs. Specific labeling of HCs was confirmed using the SqET4 transgenic line, which expresses GFP in neuromasts exclusively in HCs (Hernández et al., 2007). We observed co-localization of Bodipy TR with GFP expressing cells in transgenic larvae (Fig. 1A). Subsequent staining with SytoGreen 24 showed co-localization with Bodipy TR-labeled HCs as well.

Photographs of HCs sharing a focal plane (2.4 μm) were taken (Figs. 1A-E). For 10 μM CuSO₄ a temporal sequence of dying HCs was obtained, and for 1 μM, single representative images of the process. We also analyzed untreated fish as controls immediately after staining with the dyes and we found occasional dying HCs due to normal neuromast cell turnover (Fig. 1B, arrows). This turnover process has been described to be dependent on Caspase activity (Williams and Holder, 2000). Thus, HCs dying in this context can be considered as apoptotic.

The results show that 10 μM CuSO₄ produces a rapid death process in HCs, beginning with visible morphological changes only 5 min after copper exposure (Fig. 1E). HCs become round-shaped and swollen, as shown by membrane staining, suggesting necrotic death. The photographic sequence shows that the entire population of HCs follows this death pathway with this copper concentration, reaching necrotic features in only 35 min. Nuclear membranes conserve relatively the same size, however DNA appears condensed and in some cases fragmented. A similar result was observed in hair cells exposed to 50 μM CuSO₄ (not shown). Thus, morphological criteria support the notion that HCs are dying due to necrosis when exposed to 10 μM CuSO₄.

With 1 μM CuSO₄ HCs begin to degenerate approximately in 15 min. Necrosis was observed in many cells, as they become
round-shaped and swollen (Fig. 1C, arrowheads), comparable to 10 μM treated neuromasts. However, other HCs acquire a different morphology. Membranes look shrunken and nuclei become pyknotic (Fig. 1C, arrows), suggesting that other death pathways may be occurring as well. Remarkably, some cells detach from the centrally located rosette of HCs and become shrunken and fragmented (Fig. 1D, arrow). This morphology is similar to that found in HCs dying during normal turnover (Fig. 1A, arrows).

At least three death pathways are recognized in hair cells using morphology (Bohne et al., 2007). The double-staining method allowed us to easily recognize necrotic HCs and differentiate them from cells entering other death pathways. Non-necrotic HCs found in treatments using 1 μM copper morphologically resemble apoptotic cells dying during turnover. However, using only this criterion, we cannot unequivocally establish the kind of death program followed by HCs exposed to 1 μM copper.
2.2. Effect of copper on supporting cells and TEM analysis

To evaluate copper-induced damage in supporting cells we used an antibody against ClaudinB as a structural marker. This protein is strongly expressed in the membrane of all accessory cells in neuromasts (supporting cells and mantle cells) (López-Schier et al., 2004), and is useful to evaluate the state of these cells by immunofluorescence staining. Confocal microscopy allows a clear identification of the cell layer that corresponds to supporting cells.

We exposed 72 hpf larvae to 1, 10 or 50 μM CuSO4 for 2 h and compared them with untreated larvae (Fig. 2). Fixation was performed immediately after treatments and specimens were processed for immunofluorescence with anti-ClaudinB. 1 μM copper produced no difference in supporting cell structure compared with untreated larvae (Figs. 2A and B). 10 μM produces partial damage to these cells, with some cells appearing swollen and/or misshapen (Fig. 2C). 50 μM copper-treated neuromasts show widespread damage to the supporting cell layer; extremely swollen cells are observed as well as cell debris (Fig. 2D). This result shows that damage to the supporting cell layer is highly dependent on copper concentration. Moreover, the 2-hour treatments with different copper concentrations elicit specific effects that range from hair cell ablation only (1 μM), to partial and complete supporting cell damage (10 and 50 μM). Table 1 summarizes the data obtained on the morphological observations and damage to the different cell types in neuromasts at the three copper concentrations tested.

<table>
<thead>
<tr>
<th>Copper concentration (μM)</th>
<th>Hair cell degeneration time (minutes)</th>
<th>HC death pathway (morphology)</th>
<th>Cell type affected in neuromasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>120</td>
<td>Necrosis and apoptosis</td>
<td>Hair cells</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>Necrosis</td>
<td>Hair cells and partial loss of accessory cells</td>
</tr>
<tr>
<td>50</td>
<td>30</td>
<td>Necrosis</td>
<td>Hair cells and all accessory cells</td>
</tr>
</tbody>
</table>

Table 1 - Summary of observations in copper-exposed neuromasts

To obtain a more accurate view of events taking place in neuromasts during copper exposure, transmission electron micrographs (TEMs) were obtained to observe ultrastructural changes in 1 μM copper-treated fish compared with untreated larvae. As shown above, this concentration does not affect the supporting cell layer in a 2-hour treatment, and is thus useful for analysis of HC ultrastructure after the treatment. Dying cells can be observed in a copper-treated neuromast as opposed to a control neuromast (Fig. 3). Affected cells (Fig. 3B, arrows) manifest a pale cytoplasm, multiple vacuolar formations and membrane accumulation (Fig. 3B, curved arrow). Also some apoptotic debris can be observed (arrowheads in Fig. 3B). Supporting cells look normal and comparable to those in untreated larvae.

2.3. DNA damage in copper-exposed neuromasts

To investigate the extent of DNA damage in copper-exposed neuromasts we used the TUNEL (Td-mediated d-UDP-rhodamine Nick-End-Labeling) technique to evaluate DNA degradation. This method allows the identification of fragmented DNA strands in cells, an event that has been considered a characteristic feature of apoptosis though it is well known that other forms of cell death also present DNA fragmentation detectable by TUNEL (Kressel and Groscurth, 1994; Ben-Sasson et al., 1995; Nishizaki et al., 1999). In this study, we used transgenic zebrafish lines to detect co-localization of TUNEL label with different GFP-expressing cell-types in neuromasts (Fig. 4).

Larvae from the SqET4 line were used to monitor TUNEL labeling specifically in hair cells. In untreated larvae, TUNEL-positive hair cells were occasionally found in a few neuromasts (Fig. 4A). We treated 72 hpf larvae with 1 μM CuSO4, a concentration that does not affect accessory cells. Treatments were performed for 40 and 90 min to analyze DNA fragmentation at two stages in the process of copper toxicity in hair cells. Co-localization of TUNEL signal and GFP was found in hair cells for both cases (Figs. 4B, C).

In a second experiment we used a cross between SqET4 and SqET20 transgenic lines to obtain larvae expressing GFP in both hair cells and mantle cells (Parinov et al., 2004; Hernández et al., 2007). 72 hpf larvae were left untreated or were treated with 1 or 10 μM CuSO4 for 60 and
At these times, half of the total number of GFP-expressing hair cells are lost in neuromasts, as analyzed in the SqET4 line for each concentration (not shown). After processing for TUNEL staining, we observed and counted labeled nuclei in hair cells and mantle cells (Fig. 4G). Results show that 10 μM CuSO₄ treatments produce a significant increase in TUNEL-positive hair cells compared to the 1 μM treatment (One way ANOVA, p<0.01). Almost no mantle cells present TUNEL label with 1 μM CuSO₄, while 10 μM treatments significantly increase the number of labeled mantle cells (One way ANOVA, p<0.003) (Figs. 4, D–G). We did not include TUNEL-positive hair cells found in untreated larvae in our analysis because their appearance in neuromasts is too low compared to numbers found in copper treated larvae (two cells in 25 analyzed neuromasts).

These experiments show that DNA fragmentation is a common event during the death process in copper-exposed zebrafish neuromasts. The increase in TUNEL-positive hair cell number generated with 10 μM CuSO₄, a concentration that produces mainly necrotic hair cell death, suggests that DNA degradation is likely to be caused by processes different from apoptosis.

2.4. Protection with Amiloride, antioxidants, and copper-induced ROS production in hair cells

To investigate possible mechanisms involved in copper toxicity in lateral line hair cells we analyzed the protective effect of two kinds of compounds, the mechanotransduction channel inhibitor Amiloride (Meyer et al., 1998; Seiler and Nicolson, 1999; Chan and Hudspeth, 2005) and antioxidants. We used SqET4 larvae, taking advantage of GFP-expression in hair cells as viability indicator.

To assess Amiloride protection, 72 hpf SqET4 larvae were previously stained with the styryl dye FM1-43, which labels only mechanotransductively active hair cells, allowing the differentiation of two populations of hair cells in neuromasts, GFP labeled cells and GFP+FM1-43 double labeled cells (Hernández et al., 2007). Groups of 20 larvae were incubated in Amiloride-supplemented E3 medium for 5 min or in E3 medium only, and then incubated in 1 and 10 μM CuSO₄ for 60 min in the presence of the channel inhibitor or left untreated as control. Larvae were then washed in fresh media, anesthetized and mounted for observation in a fluorescence microscope. Hair cells were counted and numbers were statistically compared with those obtained from larvae treated with copper by itself (Fig. 5). No protection of hair cells by Amiloride was observed when fish were treated with 10 μM copper (results not shown). However, protection from the effects of 1 μM copper was evident in mechanotransductively active hair cells of Amiloride-treated larvae, as the number of surviving hair cells in this case was significantly different from copper-treated fish, while it was indistinguishable from the control group (One-way ANOVA, p<0.001. Multiple comparisons; Holm–Sidak method). No differences between groups were observed for non-active hair cells. Representative images of neuromasts from each case are presented in Figs. 5A–C and quantification of the results are shown in Fig. 5D.

To assess antioxidant protection, 72 hpf SqET4 larvae were treated with three different compounds: Reduced glutathione (GSH), N-acetyl L-cysteine (l-NAC) and Dimethylthiourea (DMTU). These compounds have been proven to promote hair cell and neuronal survival in diverse contexts by attenuating the effect of ROS produced by aminoglycoside and cisplatin toxicity (Tabuchi et al., 2002; Ton and Parng, 2005).

Groups of 20 larvae were incubated in E3 medium supplemented with antioxidants and 1% DMSO or E3 medium and DMSO alone for 4 h. Then larvae were washed three times with fresh medium and immediately incubated in 1 μM CuSO₄ for 60 min, leaving one group untreated as a control. In this
way we hoped to load the cells with the antioxidants prior to copper exposure thus avoiding possible interactions with copper ions in the extracellular environment. After the copper treatment, larvae were washed with fresh medium, anesthetized and mounted for observation in a fluorescence microscope. Hair cells expressing GFP were counted for all groups in the O and P1 neuromasts of each larvae and statistical comparison of obtained data was performed (Fig. 6A). Again, no protection was observed when fish were incubated with 10 μM copper. However, with all three antioxidants, significantly more hair cells remained viable in neuromasts of 1 μM copper treated fish compared to 1 μM copper-treated larvae that were not previously incubated with antioxidants (one way ANOVA, \( p < 0.01 \); Multiple comparisons Holm–Sidak method). GSH produces the most robust protective effect, as it is only with this reagent that the number of viable hair cells is not different from the control group. This result suggests an intracellular effect of antioxidants that protects hair cells from moderate copper toxicity and possible increases in ROS.

To determine if ROS levels increase in hair cells during copper exposure, we used H$_2$DCFDA as an indicator for production of these species (Keller et al., 2004; Bopp et al., 2008). This compound acquires green fluorescence in the presence of ROS and is designed to act as an intracellular indicator because of modifications performed by nonspecific intracellular esterases. Hair cells in neuromasts of 72 hpf wild-type larvae were incubated in H$_2$DCFDA-supplemented E3 medium and hair cell nuclei were stained with DAPI as described in Experimental procedures. Larvae were then incubated with 1 or 10 μM CuSO$_4$ for 40 or 10 min, respectively, or 400 μM neomycin for 20 min as a positive control (data not shown for 10 μM CuSO$_4$). It is well known that aminoglycosides increase ROS levels in hair cells (Cheng et al., 2005). A group of larvae were treated with GSH (as described above for antioxidant otoprotection) prior to H$_2$DCFDA application and then treated with 1 μM CuSO$_4$ for 40 min. These three treated groups of larvae were compared to untreated control larvae. After the treatments, larvae were anesthetized and mounted for observation under a fluorescence microscope. In both copper (Figs. 6B, F) and neomycin (Figs. 6C, G) treatments, green fluorescence appeared in hair cells which also show shrinkage of the nuclei (Figs. 6B, C, F, G) indicating ROS production and cell damage, while in GSH-treated and in untreated larvae hair cells present unaltered nuclei together with a lack of green fluorescence (Figs. 6D, E, H, I).

3. Discussion

Lateral line neuromasts in zebrafish larvae have emerged as a very useful model to study hair cell ototoxicity and regeneration. Recent studies have validated this model showing that lateral line hair cells are sensitive to aminoglycosides and cisplatin (Song et al., 1995; Williams and Holder, 2000; Murakami et al., 2003; Harris et al., 2003; Ton and Parng, 2005; Santos et al., 2006; Owens et al., 2007; Ou et al., 2007) demonstrating a functional parallel with hair cells found in the mammalian inner ear. A remarkable difference between these systems is that lateral line hair cells can robustly regenerate after damage, a phenomenon particular to amniotes (Balak et al., 1990; Jones and Corwin, 1993, 1996; Williams and Holder, 2000; Harris et al., 2003; Hernández et al.,

![Fig. 4](image_url) – DNA fragmentation in neuromasts detected by TUNEL. (A–C) Neuromast images from 72 hpf SqET4 larvae untreated (A) or treated for 40 (B) or 90 (C) minutes with 1 μM CuSO$_4$. Co-localization of TUNEL label and GFP expression in dying hair cells was observed at both time points. In untreated larvae, TUNEL labeled hair cells are only occasionally found. (D–F) 72 hpf larvae from a cross between SqET4 and SqET20 lines were untreated (D) or exposed to 1 μM CuSO$_4$ for 60 min (E), or to 10 μM CuSO$_4$ for 15 min (F). With 1 μM copper, TUNEL label is observed only in hair cells (centrally located GFP labeled cells), while 10 μM treatment extends the TUNEL label to mantle cells (peripherally located GFP labeled cells). (G) TUNEL-labeled hair cells (HC, black bars) and mantle cells (MC, gray bars) were counted and plotted as mean ± SD for copper treated larvae. 10 μM copper significantly produces more TUNEL-labeled hair cells (\( p < 0.01 \)), and mantle cells (\( p < 0.003 \)) (asterisk) than 1 μM copper. (n=25 larvae).
Moreover, zebrafish larvae have been used to study hair cell regeneration and the presence in neuromasts of cell populations that express the neural stem cell marker Sox2, linked to hair cell precursor maintenance, has been demonstrated (Pevny et al., 2005; Kiernan et al., 2005; Hernández et al., 2007; Ma et al., 2008). The present work establishes that, in zebrafish lateral line neuromasts, short copper treatments produce hair cell death that displays necrotic features, and different death pathways appear to occur in response to a change in concentration from 1 to 10 μM CuSO$_4$. We were able to recognize necrotic hair cells by using a membrane bound vital fluorescent dye, Bodipy TR C5-ceramide. This represents a new tool that can be used for monitoring cell death mechanisms in real time. Necrosis was apparent in hair cells of copper treated fish as they appear swollen and round-shaped as has been described in studies on noise damage in outer hair cells of the chinchilla organ of Corti (Bohne et al., 2007). Using a nuclear stain, Syto24, we also observed that nuclei become pyknotic, as stained DNA appears condensed and fragmented, a commonly described feature of apoptotic cells. Previous studies had shown similar effects in dying hair cells of zebrafish larvae exposed to neomycin and cisplatin using other fluorescent nuclear stains such as YO-PRO1 (Santos et al., 2006; Owens et al., 2007; Ou et al., 2007). Thus, it is likely that multiple cell death mechanisms are operating under our experimental conditions. TEM analysis shows that in 1 μM copper-treated neuromasts the cytoplasm is pale and the hair cells are swollen, features characteristic of necrotic cells, in contrast to the dark and compact debris found in neomycin-treated neuromasts, typical of apoptosis (Murakami et al., 2003). Small amounts of apoptotic debris were observed in copper-treated neuromasts, which is consistent with our observations of the coexistence of different cell death pathways occurring in hair cells with this treatment.

This work complements our previous observations concerning the effects of copper on hair cell survival and subsequent regeneration. We show here that functional hair cells are extremely sensitive to low micromolar concentrations of copper in the water, and that death of these cells is likely due to oxidative stress. Manifestations of the effect of copper include rapid DNA damage and an increase in ROS levels in hair cells. These features are commonly described in copper induced toxicity in cultured cells as a consequence of copper participation in Fenton chemistry (Lloyd et al., 1997; Gaetke et al., 2003; Valko et al., 2005). Moreover, we were able to

---

**Fig. 5** - Amiloride otoprotection in neuromasts. (A–C) 72 hpf SqET4 larvae showing GFP labeled hair cells (GFP-HC, green) were stained with FM1-43 which labels mechanotransductively active hair cells (yellow). Larvae were then incubated in Amiloride and treated with 1 μM CuSO$_4$ for 60 min in presence of this mechanotransduction channel inhibitor (B) or left untreated (A). A group of larvae not incubated with Amiloride was treated with copper only for comparison (C). (D) Hair cells (GFP and FM1-43 labeled) were counted in the O and P1 neuromasts and the total numbers plotted as mean±SD. Statistical comparison shows a significant difference in mechanotransductively active hair cell numbers between Amiloride-treated larvae incubated in copper and those incubated only in copper ($p<0.001$, asterisk). Cells labeled only with GFP (immature hair cells) do not show significant differences among treatments. ($n=20$ neuromasts).
to significantly protect hair cells against the effects of low concentrations of waterborne copper by pre-loading the cells with antioxidant compounds. It is also known that ROS generation plays an important role in aminoglycoside, cisplatin and noise induced hair cell death (Yamashita et al., 2004; Cheng et al., 2005; Henderson et al., 2006).

Lipid peroxidation and DNA damage are common consequences of oxygen radical production, generating both apoptotic and necrotic cell death (Van Campen et al., 2002; Ohinata et al., 2003; Valko et al., 2005). Copper can cause DNA strand breaks via ROS formation (Van Campen et al., 2002; Brezova et al., 2003) and dose-dependent changes in DNA conformation in oligonucleotides (Sorenson, 2002). Membrane damage facilitates the release of cell contents and chromatin degradation can be produced by various factors in cell death processes, as it is a consequence of Caspase-
mediated cascades and mitochondrion-specific nuclease release (Han et al., 2006; Boujrad et al., 2007).

10 μM copper exacerbates the observed effects in hair cells as nuclear material becomes rapidly condensed and fragmented, possibly caused by a combination of cell death activation and ROS increase. It is possible that the regular occurrence of hair cell turnover in zebrafish neuromasts predisposes these cells to activate cell death mechanisms quickly, under any kind of stress. Copper treatments cause the hair cells to fragment in a matter of minutes and all cellular material disappears after only 2 h. Therefore, it is unlikely that de novo activation of cell death programs requiring complex signaling events are operating under these conditions. We note that under the conditions tested (1 and 10 μM copper), underlying supporting cells are not affected and do not show strong signs of cellular stress. Either they are intrinsically insensitive to these conditions, perhaps lacking a mechanism for copper entry, or their position within the neuromast protects them from the effects of environmental factors. However, 50 μM copper strongly affects supporting cells and there is no regeneration of hair cells in neuromasts treated with these conditions (Hernández et al., 2006).

Interestingly, blocking mechanotransduction channel activity with Amiloride partially protected mature hair cells against copper toxicity at low doses. It has been described that hair cells at early stages of the differentiation (when mechanotransduction is not yet active), are less sensitive to copper (Hernández et al., 2006) and aminoglycosides (Murakami et al., 2003; Santos et al., 2006) suggesting an important role of this property in toxicity. The identity of the mechanotransduction channel remains unclear, but it appears that these cells allow entry of several substances by this means. In the case of copper, other mechanisms of access to the cells may be in play, such as other channels, or the copper specific transporter Ctr1. Ctr1 has been related to the entry of cisplatin into cells, a drug that is extremely toxic to hair cells in mammals and in fish (Holzer et al., 2006; Ou et al., 2007). The elucidation of the mechanisms of copper entry into hair cells awaits further work but we suggest that the zebrafish lateral line hair cells offer a convenient experimental system in which to study the properties that make these cells susceptible and for finding ways in which to confer otoprotection.

4. Experimental procedures

4.1 Maintenance of zebrafish, copper application and image acquisition

Our own breeding colony of the Tübingen wild-type strain of zebrafish (Danio rerio) was maintained at 28.5 °C on a 14-h light/10-h dark cycle (Westerfield, 1994). The transgenic strains SqET4 and SqET20 (previously known as ET4 and ET20) were obtained from Vladimir Korzh (Parinov et al., 2004). All embryos were collected by natural spawning, staged 1996), unless otherwise noted. We express the larval ages in hours post-fertilization (hpf) or days post-fertilization (dpf). We chose to study copper treatments in larvae of 72 hpf since, at this stage, the primary lateral line is completely developed and functional (Rable and Kruse, 1995). Copper was added as CuSO₄ (Merck, Darmstadt, Germany) dissolved in E3 medium. All protocols involving animals have been reviewed by the Animal Welfare and Ethics Committees of the University of Chile. For visualization of fluorescent dyes and antibody stains, we used a Model III Zeiss photomicroscope or a confocal microscope (LSM META510, Carl Zeiss) and a Plan-Apochromat 63/1.4 oil immersion lens. Digital images were handled with Photoshop CS3 for Macintosh. For all statistical analysis, we used SigmaStat 3.1 software.

4.2 Hair cell death detection with vital fluorescent dyes

Hair cells in lateral line neuromasts were labeled in 72 hpf larvae by incubating with 500 μM Bodipy TR C₁₀-ceramide (Molecular probes, B34400) and 3 μM Sytogreen 24 (Molecular probes, 57559). Both dyes were diluted from original stocks in fish medium and larvae were incubated for 1 h at 28 °C. Labeled larvae were then washed several times with fresh medium and incubated in petri dishes with 1 μM CuSO₄ for 10 min and 10 or 50 μM for 5 min, then anesthetized with MS222 (3-aminobenzoic acid ethyl ester, methanesulphonate salt, Sigma) and placed on cover-slips. They were also immobilized with low-melting point agarose prepared in fish medium containing the desired copper concentration and anesthetic. During the observation, a constant temperature of 28 °C was maintained. Observations were performed in a LSM510 Carl Zeiss confocal microscope.

4.3 Immunohistochemistry

Larvae were fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Embryos were rinsed three times for 5 min in PBS and then incubated in methanol at −20 °C overnight, rinsed two times for 5 min in PBS, and washed one time for 1 h in distilled water. The embryos were then incubated in acetone for 7 min, washed one time in distilled water, two times in PBS plus 0.1% Tween 20 (polyoxyethylene–sorbitan monolaurate; Sigma, USA) for 5 min, and transferred to blocking solution for 30 min. Embryos were then incubated overnight at 4 °C with the rabbit anti-ClaudinB antibody (López-Schier et al., 2004) diluted at 1:500 in blocking solution. Larvae were then washed four times for 20 min in PBS-Tween 20, incubated for 30 min in blocking solution, and incubated overnight at 4 °C with the secondary antibody Alexa Fluor 594 goat anti-mouse IgG highly cross-adsorbed (Molecular Probes, A11032) diluted in blocking solution at 1:200. Fish were then rinsed four times for 20 min in PBS plus 1% Tween 20 before mounting for imaging.

4.4 Transmission electron microscopy

Neuromasts of control and copper-exposed (1 μM copper) 72 hpf zebrafish larvae were visualized using transmission electron microscopy to observe ultrastructural changes after copper treatment. Larvae were fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4)+0.001% CaCl₂ for 1 h. Fish were then placed in fresh fixative and held overnight at 4 °C.
Larvae were washed in 0.1 M cacodylate buffer and post-fixed in 1% OsO4 in 0.1 M cacodylate + 0.001% CaCl2 for 1 h on ice. Fish were again washed in cacodylate and then dehydrated through graded ethanols, infiltrated and embedded in Spurr’s epoxy resin via propylene oxide. Cross-sections were obtained in a caudal to rostral manner. Semi-thin sections (2 μm) were stained with 1% toluidine blue to locate neuromasts. Ultra-thin sections (90 nm) were cut on a Leica Ultracut S microtome and mounted on 200-mesh Athene thin-bar grids and contrasted with uranyl acetate and lead citrate. Grids were examined using a Zeiss EM-109 transmission electron microscope. Low magnification (3000×) survey micrographs were made to record the entire neuromast. Higher magnification (4000× and 12000×) micrographs were made to document ultrastructural details of neuromast.

4.5. TUNEL

To obtain TUNEL (Terminal dUTP Nick-End-Labeling) label in neuromasts of copper-treated or control larvae, fixation was performed in 4% PFA in phosphate-buffered saline (PBS). They were then washed three times with PBS and permeabilized by incubating in proteinase K (10 μg/ml; Gibco, Carlsbad, CA) for 40 min at RT and re-fixed with 4% PFA for 20 min at RT. The in situ cell death detection kit TMR red (Roche, 12.156.792.910) was used. Rhodamine labeling of fragmented DNA was obtained according to the manufacturer’s instructions. As a positive control, a group of permeabilized larvae was incubated for 5 min with DNAse I (10 mg/ml; Fermentas, EN0521) in DNAse buffer and then processed with the kit. This treatment produces widespread TUNEL labeling in nuclei throughout the larvae bodies (not shown).

72 hpf SqET4 larvae were used to observe co-localization of TUNEL label and GFP in hair cells. They were left untreated or treated with 1 μM CuSO4 for 40 or 90 min. Larvae from a cross of SqET4 and SqET20 transgenic lines were used to observe TUNEL label in hair cells and mantle cells, respectively. Twenty-five larvae were treated with 1 or 10 μM CuSO4 for 60 or 15 min respectively or left untreated as control, and then processed for TUNEL labeling. The total number of TUNEL-positive hair cells and mantle cells were counted in the O, OC, P1, P2 and P3 neuromasts of each larvae and conditions statistically compared (see neuromast nomenclature in Harris et al., 2003).

4.6. Amiloride and antioxidant application

Mechanotransductively active neuromast hair cells were labeled by incubating 72 hpf SqET4 transgenic zebrafish larvae in 3 μM [N-(3-triethylammoniumpropyl]-4-(4-dibuty-l-amino] pyridinium] dibromide] (FM1-43, Molecular Probes T-35356) in E3 medium for 30 s. Stained larvae were incubated with 1 mM Amiloride in E3 medium for 5 min. They were then treated for 60 min with 1 μM CuSO4 supplemented with 1 mM Amiloride or left untreated. A group of larvae not incubated with Amiloride was treated with 1 μM CuSO4 for comparison. After treatments larvae were washed, anesthetized and mounted for observation.

Antioxidants were dissolved in E3 medium plus 1% dimethylsulfoxide (DMSO) at the following concentrations: Reduced glutathione (GSH) 200 μM, N-acetyl-l-cysteine (l-NAC) 200 μM and Dimethylthiourea (DMTU) 400 μM. 72 hpf SqET4 transgenic larvae were incubated in antioxidant containing solutions or E3 medium plus DMSO only for 4 h at 28 °C, then washed with fresh E3 medium and treated with 1 μM CuSO4 for 60 min. Larvae were finally washed, anesthetized and mounted for observation. Hair cell numbers were counted in O and P1 neuromasts and conditions statistically compared. Compounds used in these experiments were purchased from Sigma Chemical Corporation (St. Louis, MO).

4.7. ROS detection with H2DCFDA

Generation of reactive oxygen species was determined using the membrane-permeable fluorescent dye 2’7’-dichlorodihydrofluorescein diacetate (H2DCFDA) (Molecular probes, D-399). H2DCFDA is hydrolyzed inside cells to the nonfluorescent compound 2’7’-dichlorodihydrofluorescein, which emits fluorescence when oxidized to 2’7’-dichlorofluorescein (DCF). Thus the fluorescence emitted by DCF directly reflects the overall oxidative status of a cell. Larvae were incubated for 1 h with 10 μM H2DCFDA (prepared in E3 medium from a 10 mM stock solution) and then washed in fresh E3 medium. Hair cell nuclei in neuromasts were labeled by incubating with 0.1 μg/ml DAPI (Sigma D-9542) dissolved in E3 medium for 5 min at 28 °C. Larvae were then left untreated or treated with 1 μM CuSO4 for 40 min, 10 μM for 10 min or 400 μM neomycin sulphate (Sigma, N-1876) for 20 min. One group of larvae was incubated in 200 μM GSH (as described above) before H2DCFDA application and they were then treated with 1 μM CuSO4 for 40 min. After these treatments larvae were anesthetized and mounted for observation under a fluorescence microscope.

Acknowledgments

We are especially indebted to Juilieta González and Nancy Olea for discussions and help with electron microscopy. We thank Vladimir Korzh and Hernán López-Schier for transgenic fish lines and valuable reagents. Catalina Lafourcade and Florencio Espinoza provided technical help. This work was supported by the Millennium Science Initiative, Grant number P06-039F and by FONDECYT, Grant number 1070867.

REFERENCES


Owens, K.N., Santos, F., Roberts, B., Linbo, T., Coffin, A.B., et al., 2008. Identification of genetic and chemical modulators of...