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Cadmium-induced ototoxicity in rat cochlear organotypic cultures

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Abstract

Cadmium (Cd), a widely used industrial metal, is extremely nephrotoxic and neurotoxic; however, its effects on the peripheral auditory system are poorly understood. To evaluate the ototoxicity of Cd, we treated cochlear organotypic cultures from postnatal day 3 rats with Cd concentrations from 10 to 500 μM for 24 h or 48 h. Afterwards, we evaluated the degree of damage to hair cells, auditory nerve fibers and spiral ganglion neurons. Damage to the hair cells, auditory nerve fibers and spiral ganglion neurons systematically increased in a dose and time-dependent manner. Exposure to Cd concentrations of 10 μM for 24 and 48 h resulted in minor inner and outer hair cell loss in the basal third of the cochlea. As Cd concentrations increased, toxicity spread towards the apex, also in a time dependent manner. Treatment with 100 μM Cd for 48 h resulted in substantial (>30%) hair cell loss over the entire cochlea. Cd was also toxic to auditory nerve fibers and spiral ganglion neurons; 100 μM of Cd for 24 h or 10 μM of Cd for 48 h resulted in considerable damage to auditory nerve fibers and spiral ganglion neurons. These findings are the first to demonstrate that Cd can cause significant lesions to peripheral auditory nerve fibers, spiral ganglion neurons, and sensory hair cells in organotypic cultures from postnatal cochleae.

Keywords

hair cells; spiral ganglion neurons; nerve fibers; cadmium; nerve fibers

1. Introduction

Cadmium (Cd), a heavy metal widely used in the production of batteries, solar panels, pigments, and plastic stabilizers (Basinger et al., 1987, Bertin and Averbeck, 2006, Herber, 1992, Huff et al., 2007) has been linked to a wide range of adverse medical conditions (Cartana et al., 1992, Goering and Klaassen, 1983, Nemery, 1990, Reeves and Rossow, 1996, Sullivan et al., 1984, Swiergosz-Kowalewska, 2001) including cancer, nephrotoxicity,

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bone disease and hepatotoxicity (Nordberg et al., 2012, Prozialeck et al., 2007). Cd typically enters the body from contaminated food and water as well as inhalation of polluted air and cigarette smoke (Friberg, 1983, Massadeh et al., 2005). This was exemplified by residents of the Jinzu river basin in Japan who developed nephrotoxicity and itai-itai, a painful joint, skeletal and bone disease as a result of Cd water pollution (Inaba et al., 2005, Kobayashi et al., 2009, Nogawa et al., 1983, Tsuchiya, 1976). Humans and laboratory animals can also develop severe and irreversible neurological disorder due to excess Cd intake (Fern et al., 1996, Hart et al., 1989, Klaassen and Wong, 1982, Kumar et al., 1996, Lopez et al., 2003, O'Callaghan and Miller, 1986, Okuda et al., 1997, Webster and Valois, 1981, Wong and Klaassen, 1982). Cd-induced neurotoxicity is thought to arises from increased production of reactive oxygen species, depletion of antioxidant enzymes and inhibition of cellular energy production resulting in apoptosis (1988, Almar et al., 1987, Bianucci et al., 2011, Cookson and Pentreath, 1996, El-Naggar and El-Sheekh, 1998, Helbig et al., 2008, Kang, 1992, MacKinnon and Kapron, 2010, Muller, 1986, Ochi et al., 1988, Rai et al., 1990, Shimizu and Morita, 1990, Singhal et al., 1987, Sura et al., 2006, Tynecka et al., 1998, Yang et al., 2007).

The half-life of Cd is in excess of 15 years accounting for its harmful accumulation in a variety of tissues. The highest concentrations of Cd are observed in the kidney, which is considered to be the major organ for the Cd related toxicity (Nogawa1981;Hamada et al. 1991). Several studies have suggested that the kidney is very similar to the cochlear portion of the inner ear given that many drugs that are nephrotoxic are also ototoxic (Humes, 1999, Walker et al., 1990). Although research on the ototoxic effects of Cd is sparse, there is, in fact, evidence suggesting that Cd is toxic to the inner ear and can lead to hearing loss. For example, rats treated with $CdCl_2$ (15 ppm) for 30 days in drinking water were found to have not only significant nephrotoxicity but hearing loss as well (Ozcaglar et al., 2001). Cd treatment caused a reduction in otoacoustic emissions, suggesting that damage occurred to outer hair cells (OHC) and/or the stria vascularis (Ozcaglar et al., 2001). The latency of wave I of the auditory brainstem response (ABR), which originates from the auditory nerve, was also greatly delayed, suggesting possible damage to the spiral ganglion neurons (SGN). Surprisingly, the latencies of waves III and V, which emanate from the auditory brainstem, did not change significantly, suggesting that the cochlea is more vulnerable to the toxic effects of Cd than neurons in the auditory brainstem. Unfortunately, this study failed to perform any experimental verification of the suggested cochlear histopathologies responsible for these hearing defects. In contrast to chronic treatment, acute dosing of rats with Cd (5 mg/kg, i.p.) failed to produce any permanent hearing loss 7 day post-treatment (Whitworth et al., 1999), however, hearing and hair cell loss was observed when Cd was co-administered with furosemide, a diuretic that disrupts the blood-labyrinth, which by itself did not cause hearing impairment.

Thus, the existing literature, though limited, suggests that Cd has the potential to cause permanent hearing loss. It is unclear, however, as to which cells in the cochlea are preferentially damaged by Cd and whether cell death occurs predominantly by necrosis or apoptosis. To address these issues, we treated postnatal day 3 cochlear ogranotypic cultures with increasing concentrations of Cd to determine which concentraions were toxic to the cochlear hair cells, support cells and SGN and to investigat some of the mechanisms by which Cd exerts its ototoxic effects.

2. Materials and Methods

2.1. Cochlear organotypic cultures

Procedures for preparing rat cochlear organotypic cultures have been described in previous publications (Ding et al., 2011a, Ding et al., 2002, Ding et al., 2009b, Qi et al., 2008, Wei et al., 2010). In brief, postnatal day 3 rat pups (SASCO Sprague-Dawley, Charles River Laboratory Inc.) were decapitated and their cochleae carefully removed. The whole cochlear basilar membrane containing the organ of Corti and SGN were dissected out in Hank's Balanced Salt Solution and placed in a culture dish coated with rat tail collagen Serum-free medium was added to the culture dish and the cochlear explants were subsequently incubated at 37 °C in 5% CO_2 overnight. To determine the effect of Cd on cochlear damage, explants were cultured in fresh medium containing various concentration of the metal for 24 or 48 h.

2.2. Histology

At the end of the incubation period, cochlear explants were fixed with 4% formalin in phosphate buffered saline (PBS) for 1 h. Specimens were rinsed in 0.01 M PBS, incubated overnight (4 °C) in a solution containing of mouse anti-neurofilament 200 kD antibody, Triton X-100 (10%), normal goat serum, and 0.01 M PBS. Specimens were subsequently rinsed with 0.01 M PBS, and then immersed for 1 h in a solution containing secondary antibody conjugated with Alexa Fluor 555 dissolved in 5% normal goat serum, 1% Triton X-100 in 0.01 M PBS. To label the stereocilia and the cuticular plate of cochlear hair cells, specimens were labeled with Alexa Fluor 488-phalloidin in PBS for 40 min. Afterwards, specimens were mounted on glass slides in glycerin, coverslipped and examined using a confocal microscope.

2.3. TUNEL Staining

Cochlear explants were cultured for 24 h in the presence or absence of 500 μM Cd. After fixation, specimens were fixed with 10% formalin and processed for terminal deoxynucleotidyl transferase dUTP end-labeling (TUNEL) staining using a TUNEL Assay Kit (Invitrogen) according to manufacturer's protocol. Cochlear cultures were incubated in DNA-labeling solution at 37 °C for 4-8 h. After washing with rinsing buffer, specimens were incubated with antibody (5.0 μl of Alexa Fluor 488 dye–labeled anti-BrdU antibody and 95 μl rinsing buffer) at room temperature for 1 h as previously described (Qi et al., 2008). Afterwards, specimens were stained with an antibody against neurofilament 200 kD and secondary antibody (see above) to label SGN (Ding et al., 2007, Ding et al., 2011b, Wei et al., 2010). The nuclei in the cochlear cultures were also labeled with To-Pro-3 solution (1 mM To-Pro-3 in 0.75 μl DMSO dissolved in 1 ml H_2O) at room temperature for about 30 min, then rinsed with 0.01 M PBS and mounted on glass slides in glycerin.

2.4. Cochleograms

To quantify hair cell loss induced by Cd, cochlear cultures were examined under a fluorescent microscope (Zeiss Axioskop 400X) equipped with appropriate filters for detection of Alexa Fluor 488 fluorescence (absorption 488 nm, emission 520 nm). The

numbers of missing IHC and OHC were counted in consecutive 0.24 mm segments along the entire length of the cochlea. Hair cells were counted as missing if the stereocilia and the cuticular plate were absent. Using lab norms of hair cell counts from normal SASCO Sprague-Dawley rats, a cochleogram was constructed showing the percent IHC and OHC loss as a function of the percent distance from the apex of the cochlea. Hair cell losses from individual cochleograms were averaged to generate a mean cochleogram for each condition using custom software (Ding et al., 2011a, Ding et al., 2009a, Qi et al., 2008, Wei et al., 2010).

2.5. Confocal microscopy

Specimens were examined under a confocal microscope (Zeiss LSM-510 meta, step size 0.5 μm per slice, approximately 10-20 slices) with appropriate filters for detection of Alexa Fluor 555 (absorption 555 nm, emission 565 nm), Alexa Fluor 488 (excitation 488nm, emission 520 nm) or To-Pro-3 (absorption 642 nm, emission 661 nm). Specimens were processed with a Zeiss LSM Image Examiner (Zeiss LSM-510, step size 0.5 μm per slice, 20 to 50 slices). Images from approximately 10-20 slices were typically merged into a single plane using the Zeiss LSM Image Examiner (version: 4,0,0,91). Adobe Photoshop (version 5.5) was used for additional processing of photomicrographs as described previously (Ding et al., 2011a, Ding et al., 2010, Ding et al., 2007, Qi et al., 2008, Wei et al., 2010, Yu et al., 2011).

To quantify shrinkage of SGN soma induced by Cd treatment, the sizes of 150 SGN soma (6 cultures per condition; 25 SGN per culture) were measured from samples taken from the upper middle turn of the cochlea 24 h after being cultured without (control) or with 10, 100 or 500 μM Cd. Soma area was measured using Confocal LSM Image Examiner software as described previously (Wei et al., 2010). Briefly, multiple layers of SGN images were selected and merged into a single layer in which the SGNs with largest cross sectional area were measured. To avoid repeated measurement of the same SGN, an interval of 20 μm was interposed between each of the merged layers. A polygon was drawn around the perimeter of the cell body of each well-defined SGN and the enclosed area was calculated automatically by the Zeiss LSM Image Examiner software (version: 4,0,0,91). Statistical analyses were performed with GraphPad Prism program (version 5.01).

3. Results

3.1. Hair Cell Loss

Initial studies were performed to examine the effects of Cd on hair cell viability. For these studies, cochlear cultures were labeled with Alexa Fluor 488-phalloidin in order to visualize the stereocilia bundle and the cuticular plate of the hair cells in both control and Cd-treated samples. Figure 1 shows a series of representative photomicrographs taken from the upper middle turn (~20% of the distance from the apex) of the cochlea. Three orderly rows of OHC and one row of IHC were present in normal cochleas cultured for 24 h (Figure 1A) or 48 h (Figure 1E). Cochlear cultures treated with 10 μM Cd for 24 h (Figure 1B) were similar to those of untreated controls. Cultures treated for 24 h with 100 μM Cd (Figure 1C) showed little evidence of actual hair cells loss; however, the rows of OHC were slightly distorted

due to inward and outward radial shifts of OHC (Figure 1C, arrows). Treatment with 10 μM Cd for 48 h also resulted in severe distortion of the cochlear architecture, but displayed little hair cell loss (Figure 1F). In some areas, the OHC shifted outward and away from the IHC while in other regions the OHC shifted inward toward the IHC; these radial shifts resulted in rows of hair cells with a scalloped profile (Figure 1F). When the dose of Cd was increased to 100 μM and treatment duration was 48 h, some hair cell rows disappeared and the rows became more distorted (Figure 1G). A dose of 500 μM for 48 h destroyed nearly all the hair cells near the upper middle turn of the cochlea (Figure 1H). Interestingly, when the treatment duration was decreased to 24 h, the 500 μM dose of Cd caused minimal hair cell loss, although considerable distortion of the hair cell rows was evident.

To quantify the toxic effects of Cd on sensory hair cells, we counted the numbers of missing IHC and OHC along the entire length of the sensory epithelium and computed a mean cochleogram showing the percentage of missing hair cells as a function of percent distance from the apex for each experimental condition. Little or no hair cell loss was seen in normal control cochleas cultured for 24 h except for small IHC and OHC losses that increased from less than 10% to approximately 30% in the region 75% to 100% of the distance from the apex. Control cochleas cultured for 48 h showed slightly greater loss of IHC and OHC 80-100% distance from the apex plus less than 10% OHC loss 0-10% distance from the apex. These lesions are most likely the result of mechanical damage that occurred during the dissection and preparation of the cultures as noted previously (Wu et al., 2011b). Cultures treated for 24 h with 10 μM Cd had greater IHC and OHC loss in the basal third of the cochlea (Figure 2B) than controls cultured for 24 h (Figure 2A). As the Cd concentration increased from 10 to 100 μM, IHC and OHC losses increased and expanded over the basal half (50-100%) of the cochlea (Figure 2C). The 500 μM dose for 24 h resulted in extensive IHC and OHC over the entire cochlea; the losses decreased from 100% near the base of the cochlea to approximately 40% IHC and 60% OHC loss in the apical third of the cochlea (Figure 2D). Treatment for 48 h with 10 μM Cd resulted in approximately 70% IHC and OHC loss in the extreme base of the cochlea to roughly 10% IHC and OHC loss in the middle of the cochlea (Figure 2F); these losses were considerable greater than those seen in the 48 h controls (Figure 1E). With 100 μM Cd, the IHC and OHC lesion expanded all the way to the apex; nearly all of the IHC and OHC were missing in the extreme base of the cochlea whereas roughly 30% of the IHC and 40% of the OHC were missing between 20% to 80% of the distance from the apex of the cochlea (Figure 2G). Treatment with 500 μM Cd for 48 h resulted in 100% loss of IHC and OHC over the basal half of the cochlea; OHC and IHC losses then declined to roughly 50% and 40% respectively near the apex of the cochlea (Figure 2H). The Cd-induced hair cell lesions generally progressed from base to apex as the dose increased; although the OHC lesions tended to be slightly greater than IHC, the differences were relatively small.

3.2. Auditory Nerve Fibers and Spiral Ganglion Damage

Studies were also performed to determine the effects of Cd on both the auditory nerve fibers and spiral ganglion. For these experiments, specimens were labeled with Alexa-Fluor 488-phalloidin to identify hair cells and neurofilament-Alexa-Fluor 555 antibody to visualize SGN and auditory nerve fibers (NF). Representative confocal images from the upper middle

turn of control and Cd-treated cochleas cultured for 24 h are shown in Figure 3A-D. In control samples, thick fascicles of NF can be seen emanating from the SGN and projecting out radially to the three rows of OHC and one row of IHC (Figure 3A). The NF and SGN in cultures treated with 10 μM Cd (Figure 3B) appeared similar to controls (Figure 3B). As the dose of Cd increased from 100 to 500 μM Cd (Figure 2C-D), the NF fascicles began to thin out and fragment whereas the soma of the SGN began to shrink and disappear. The dose-dependent damage to the NF increased at approximately the same rate as the damage to hair cells. The NF and SGN in untreated controls cultured for 48 h (Figure 3E) appeared normal and similar to those cultured 24 h. However, the NF fascicles started to thin out (Figure 3F) after 48 h treatment with 10 μM Cd. NF shrinkage and fragmentation and SGN shrinkage increased rapidly as the dose of Cd increased from 100 to 500 μM (Figure 3F-H). Nearly all the hair cells, NF and SGN were destroyed after 48 h treatment with 500 μM Cd.

The higher magnification confocal images displayed in Figure 4 further illustrate the changes in the size and shape of the soma and nucleus of SGN when cultured for 24 h with increasing concentrations of Cd. Soma and nuclear shrinkage was apparent in some SGN at the lowest concentration, 10 μM, whereas at the highest concentration, 500 μM, most of the soma and nuclei were severely shrunken and condensed, anatomical hallmarks of apoptosis. In addition, many SGN were missing. To quantify the shrinkage of SGN soma, we measured the cross sectional area of 25 SGN soma located in the upper middle turn of each sample (n = 6 samples per condition) after 24 h in culture. Measurements were obtained from untreated controls and cultures treated with 10, 100 or 500 μM Cd. The mean size of the SGN in normal controls was 220.2 μm^2 (SEM 2.24 μm2; Figure 5). Soma cross sectional area decreased in a dose-dependent manner from 207 μm^2 (SEM 1.96 μm^2) at 10 μM, 157 μm^2 at 100 μM (SEM 1.99 μm^2) and 90.8 μm^2 at 500 μM (SEM 2.52 μm^2). A one-way analysis of variance revealed a main effect of Cd dose ($F = 720.5, 3, 596$ df, $p < 0.0056$) and a Tukey post-hoc analysis showed a significant difference ($p < 0.05$) between all treatment groups as indicated by the arrows in Figure 5.

3.3. Cd-induced apoptosis

To determine if Cd-induced cochlear degeneration was mediated by apoptosis, cochlear explants treated with or without 500 μM Cd for 48 h were evaluated with TUNEL staining to identify DNA fragmentation. Results of these experiments obtained with untreated control cochlear explants revealed no TUNEL positive labeling in IHC, OHC or SGN (Figure 6A, C). In contrast, many TUNEL-positive cells were seen in the hair cell region (Figure 6B) and in the SGN region (Figure 6D) 48 h after 500 μM Cd treatment.

4. Discussion

Cd is an environmental contaminant most commonly produced from industrial pollution during smelting and refining of metals and manufacturing of batteries, alloys, and pigments used in production of inks, dyes, paints, enamels, and plastics. Significant exposure to Cd can also result from its high content in cigarette smoke. Cd is on the EPA National Emission Standards for Hazardous Air Pollutants (NESHAP) list of 189 hazardous air pollutants and is listed as one of 33 hazardous air pollutants that present the greatest threat to public health

in urban areas. Chronic exposure causes Cd to accumulate in the body as the biological half-life is estimated to be at least 15 years (Dickel et al., 2002, Viaene et al., 2000, Viaene et al., 1999). Since Cd absorption in the body is relatively low, when testing the toxic effects of Cd in experimental settings, it is difficult to fully imitate the cumulative process that occurs in animals with real exposure in nature. Although, not without its limitations, it is possible to shorten the long period of exposure to heavy metals in vitro by increasing their operative concentrations in the culture dish (Ding et al., 2011a, Kim et al., 2010, Leoni et al., 2002, Prins et al., 2010, Schmid et al., 1983, Waters et al., 1975, Wu et al., 2011a). In the current study, cochlear explants were treated with Cd at concentrations ranging from 10 μM to 500 μM. These were equivalent to 1.83 μg per milliliter in 10 μM Cd and 92 μg per milliliter in 500 μM Cd in culture medium, respectively. These were approximately 100 to 5,000 times higher than the safety standards (15 μg / L) for workers according to German law (Godt et al., 2006).

Numerous studies have demonstrated that Cd can produces severe neurotoxic manifestations especially in the central and peripheral nervous system in neonatal animals, which is believed to be caused by amplified sensitivity in the developing brain (Antonio et al., 2003, Rigon et al., 2008, Viaene et al., 1999). Although the ototoxic effects of Cd have previously been described in the literature (Agirdir et al., 2002, Kim et al., 2008, Kim et al., 2009), detailed information as to the mechanism of the actions of Cd on the different cells within the cochlea has not previously been described. In the current study, we provide the first evidence that Cd-induced ototoxicity involves structural degeneration of HCs, peripheral auditory nerve fibers and SGN.

As indicated by the results presented in this paper, Cd-induced damage to hair cells, auditory nerve fibers and SGN systematically increased in a dose and time-dependent manner. Exposure to Cd concentrations of 10 μM for 24 h and 48 h resulted in minor IHC and OHC loss in the basal third of the cochlea. As Cd concentrations were increased toxicity spread towards the apex, also in a time dependent manner. Treatment with 100 μM Cd for 48 h resulted in substantial hair cell loss over the entire cochlea as well as toxicity to auditory nerve fibers and SGN. Higher concentrations of Cd led to further degeneration of hair cells as well as breakdown of the peripheral auditory nerve fibers and the soma of SGN.

Loss of IHC and OHC followed a consistent pattern of damage that begins at the base of the cochlea and progresses toward the apex. This is similar to most ototoxic drugs, such as aminoglycoside antibiotics, antitumor platinum drugs, and other toxic reagents (Ding et al., 2011b, Ding and Salvi, 2004, 2007, Li et al., 2011, Qi et al., 2008). These findings are consistent with previous studies showing that Cd causes hearing loss and cochlear HC loss in experimental animal models in vivo (Agirdir et al., 2002, Ozcaglar et al., 2001, Prasher, 2009, Whitworth et al., 1999).

Previous studies indicated that Cd can accumulate in the inner ear following Cd treatment causing selective prolongation of latency in ABR wave I, but failing to change the latencies of either wave III or V (Ozcaglar et al., 2001). These findings suggest that cochlear HCs as well as peripheral auditory neurons are more vulnerable to Cd than cells within the central auditory system. Our results demonstrated a concentration and duration-dependent damage

effect on peripheral auditory nerve fibers and SGNs in rat cochlear organotypic cultures exposed to Cd. Since the ABR wave I is derived from the auditory nerve and SGN that contact cochlear IHC, the current findings are consistent with prior results demonstrating Cd-induced dysfunction in the auditory periphery in vivo (Ozcaglar et al., 2001).

Although Cd toxicity is implicated in a diverse array of pathological conditions, the precise mechanism by which it produces neurotoxicity and ototoxicity is poorly understood. The general consensus is that cell death is typically mediated by apoptosis, although necrosis has also been proposed to be contributing factor (Prozialeck et al., 2009). Necrosis, can induce an inflammatory response in vivo (Hughes and Gobe, 2007), and this would likely exacerbate any oxidative stress caused by Cd alone. Cd may also induce autophagy (Wang et al., 2008) which allows for the digestion of dysfunctional organelles within lysosomal autophagic vacuoles. Autophagy results in the removal of dysfunctional mitochondria which potentially can reduce excessive formation of ROS, but, may, also cause digestion of a sufficient number of mitochondria such that cell viability is compromised (Sansanwal et al., 2010). In the experiments reported here, TUNEL staining and cellular histopathologies demonstrated that Cd induced damage to IHC, OHC and SGN that resulted in nuclear condensations and/or fragmentations, morphologic features of apoptosis. These findings are consistent with numerous other studies demonstrating that Cd cell death is mediated by apoptosis (Chabicovsky et al., 2004, Chao and Yang, 2001, Chatterjee et al., 2009, Chuang et al., 2000, Coutant et al., 2006, Dong et al., 2001, Fernandez et al., 2003, Galan et al., 2000, Joseph, 2009, Jung et al., 2008, Kim et al., 2000, Kondoh et al., 2002, Lasfer et al., 2008, Li et al., 2003, Mao et al., 2007, Pathak and Khandelwal, 2006, Poliandri et al., 2003, Shih et al., 2004, Xu et al., 1996).

In conclusion, our results demonstrate for the first time both a time and concentration-dependent increase in the toxic effects of Cd on IHC, OHC, auditory nerve fibers and SGN in organotypic cultures from postnatal day 3 cochleae. Cell death most likely was mediated by apoptosis as indicted by TUNEL staining, indicative of DNA strand breaks, in IHC, OHC and SGN; these changes were associated with soma shrinkage and nuclear condensation and fragmentation.

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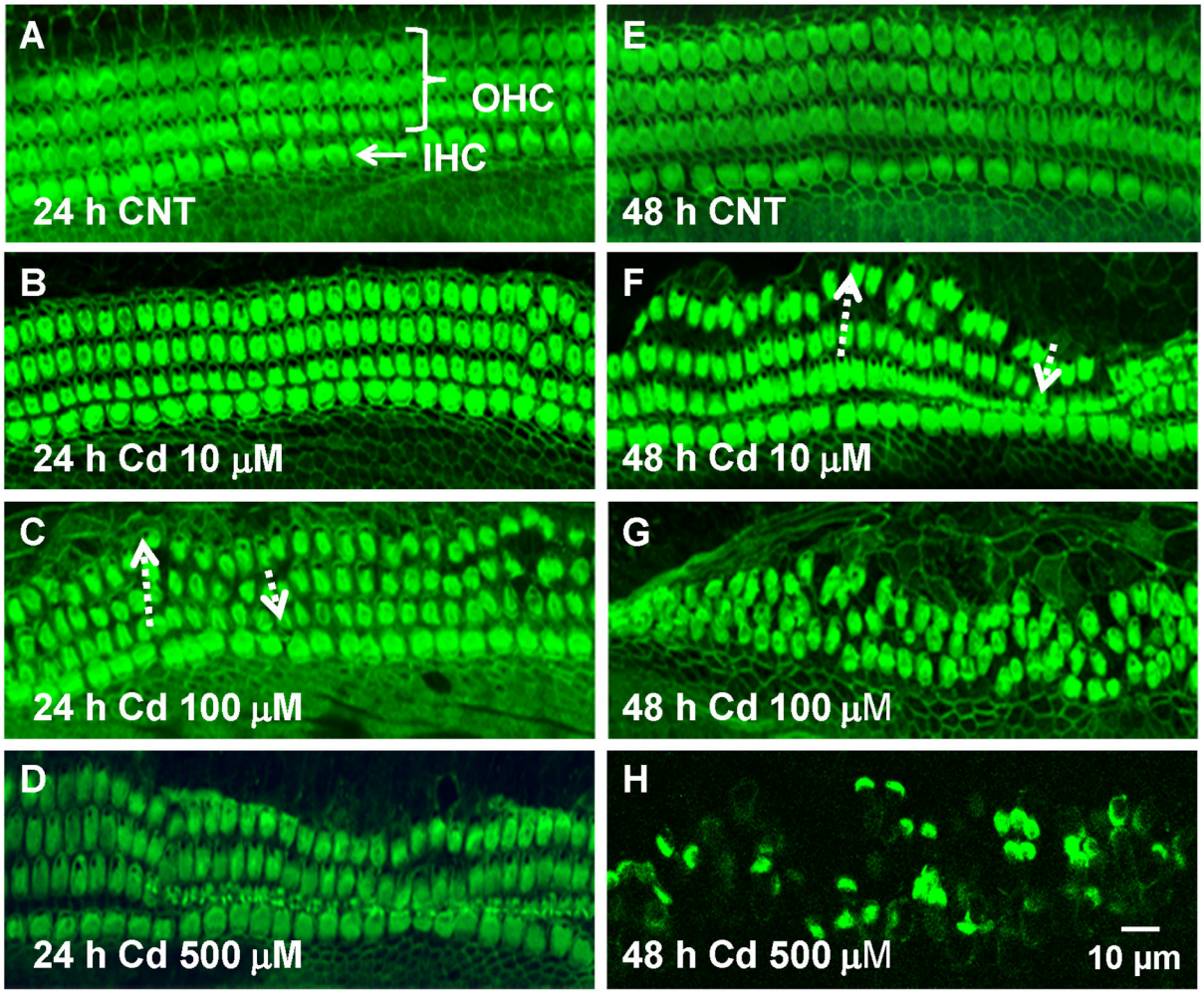

Figure 1:

Representative photomicrographs of cochlear surface preparations labeled with Alexa Fluor 488-phalloidin taken from the upper middle turn of the cochlea approximately 20% of the distance from the apex. Photomicrographs shown for normal controls (CNT) cultured for 24 h (A) or 48 h (E) and from cochleas treated for 24 h with 10 to 500 mM CdCl2 (B-D) or 48 h with 10 to 500 mM CdCl2 (F-H). Bracket in panel A shows 3 orderly rows of OHC; arrow shows the location of a single row of IHC. Note wavy row of OHC (Dashed arrows) after 24 h treatment with 100 mM CD and highly disorganized rows of OHC after 48 h treatment with 10 mM Cd. Dashed arrows show inward and outward radial shift of OHC (F). Scale bar shown in lower right of panel H.

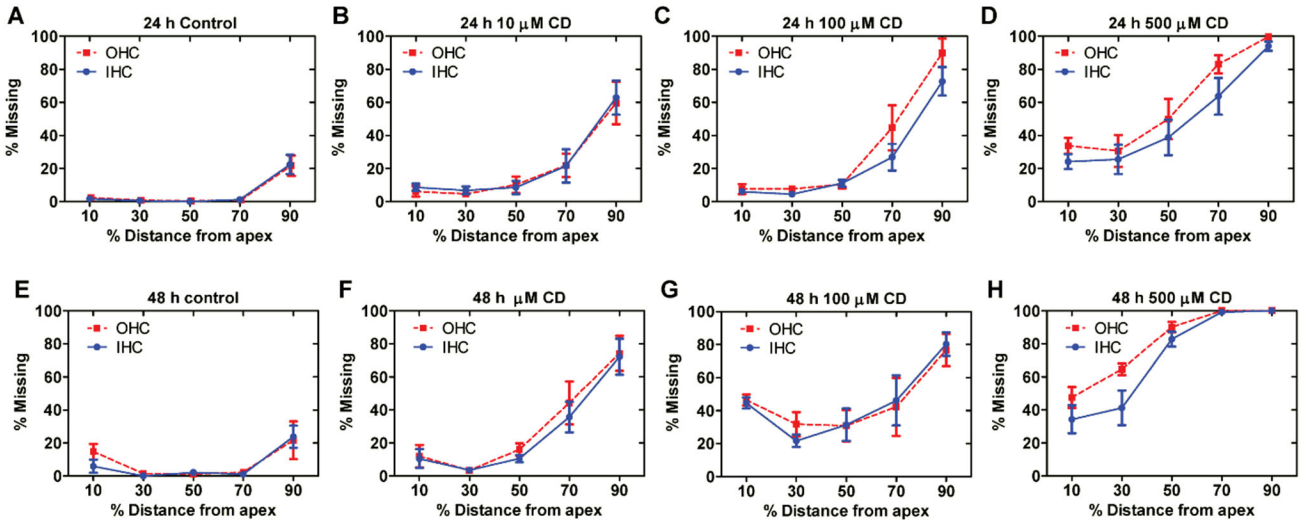

Figure 2:

Mean cochleograms (n = 6/condition) showing the percentage of missing IHC and OHC as function of percent distance from the apex. Data shown for normal controls maintained in culture for 24 h (A) or 48 h (B). Panels B, C and D show the mean cochleograms obtained from cultures treated for 24 h with 10, 100 or 500 μM of $CdCl_2$. Panels F, G and H show the mean cochleograms obtained from cultures treated for 48 h with 10, 100 or 500 μM $CdCl_2$. Approximately 30 - 40% of IHC and OHC were missing at the extreme base in normal control cultured for 24 or 48 h presumably due to mechanical damage during the sample preparations.

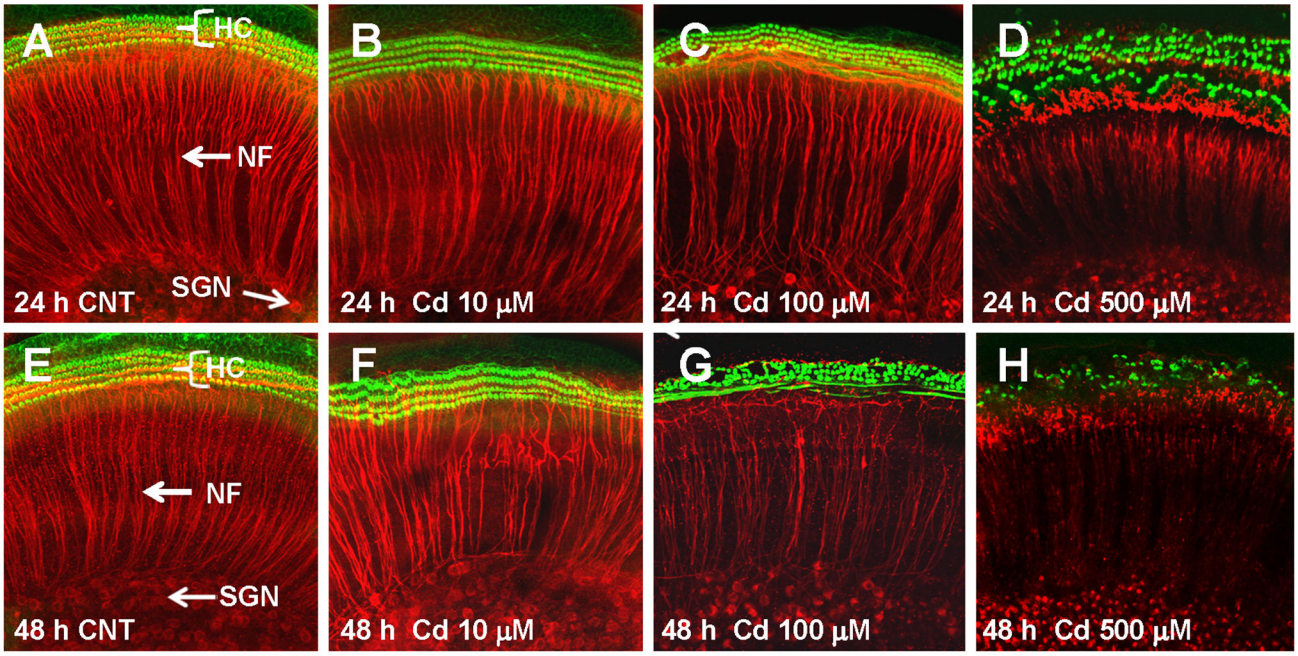

Figure 3:

Confocal images of control and cadmium-treated cochlear explants cultured for 24 h (A-D) or 48 h (E-H). Hair cells (HC) labeled with Alexa-Fluor 488 phalloidin (green, yellow label). Auditory nerve fibers (NF) and spiral ganglion neurons (SGN) labeled with neurofilament 200 kD-Alexa-Fluor 555 (red). In controls (CNT) cultured for 24 h (A) or 48 h (E), thick fascicles of NF project out radially from SGN soma towards the HC. Cochlear explants cultured with increasing concentrations of cadmium for 24 h (B-D) or 48 h (F-H). Note dose dependent loss of NF and SGN beginning with 100 µM after 24 h treatment and beginning with 10 µM after 48 h treatment.

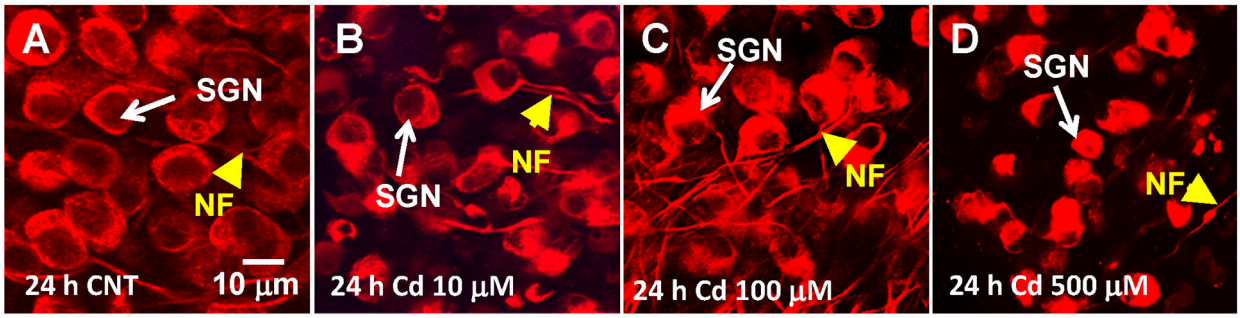

Figure 4:

Representative confocal images of SGN cultured for 24 h without and with Cd. SGN labeled with neurofilament 200 kD-Alexa-Fluor 555 (red). (A) SGN in controls (CNT) have a large soma (white arrow) and round nucleolus. Some thin nerve fibers (NF, yellow arrowhead) pass by the SGN. (B-D) Soma and nuclei of SGN became progressively smaller and more condensed as the dose of Cd increases from 10 to 500 μM. Note loss of SGN and NF in cultures treated with 500 μM Cd. Scale bar shown in panel A.

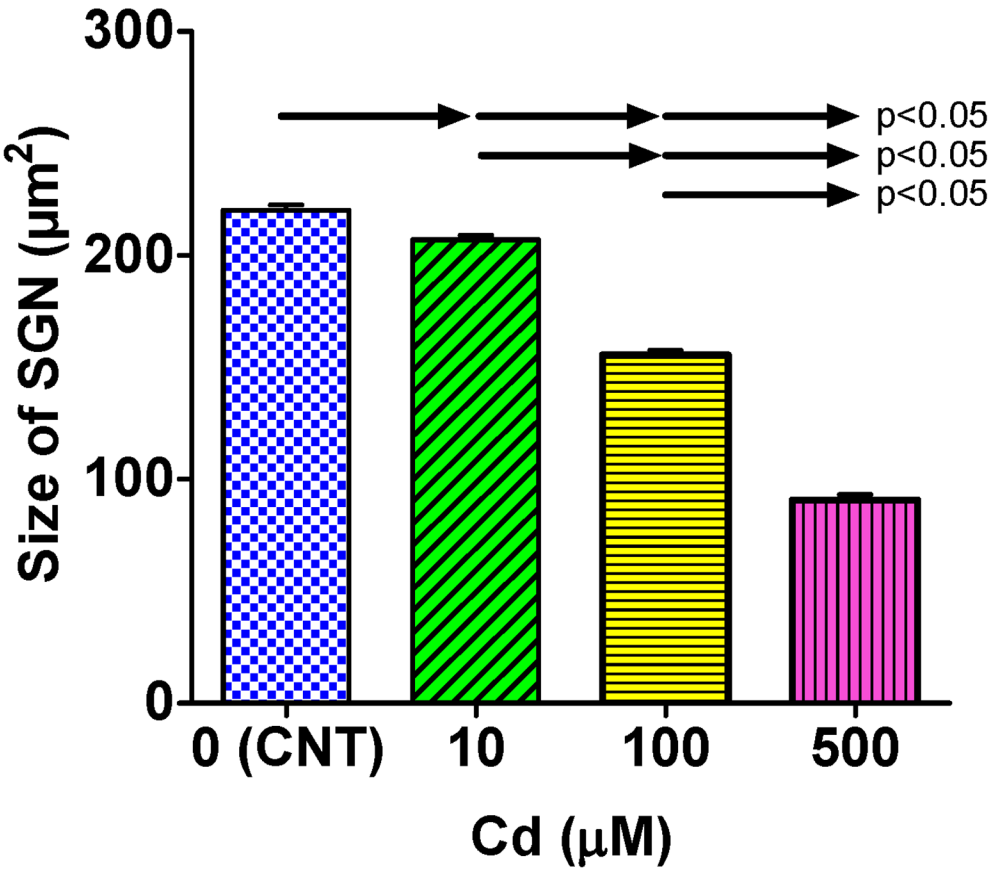

Figure 5:

Mean (n = 6, ± SEM) area of SGN soma in control and Cd (10 - 500 μM) treated cochleas cultured for 24 h. Arrows indicate conditions that were significantly different from one another ($p < 0.05$, Tukey post-hoc analysis).

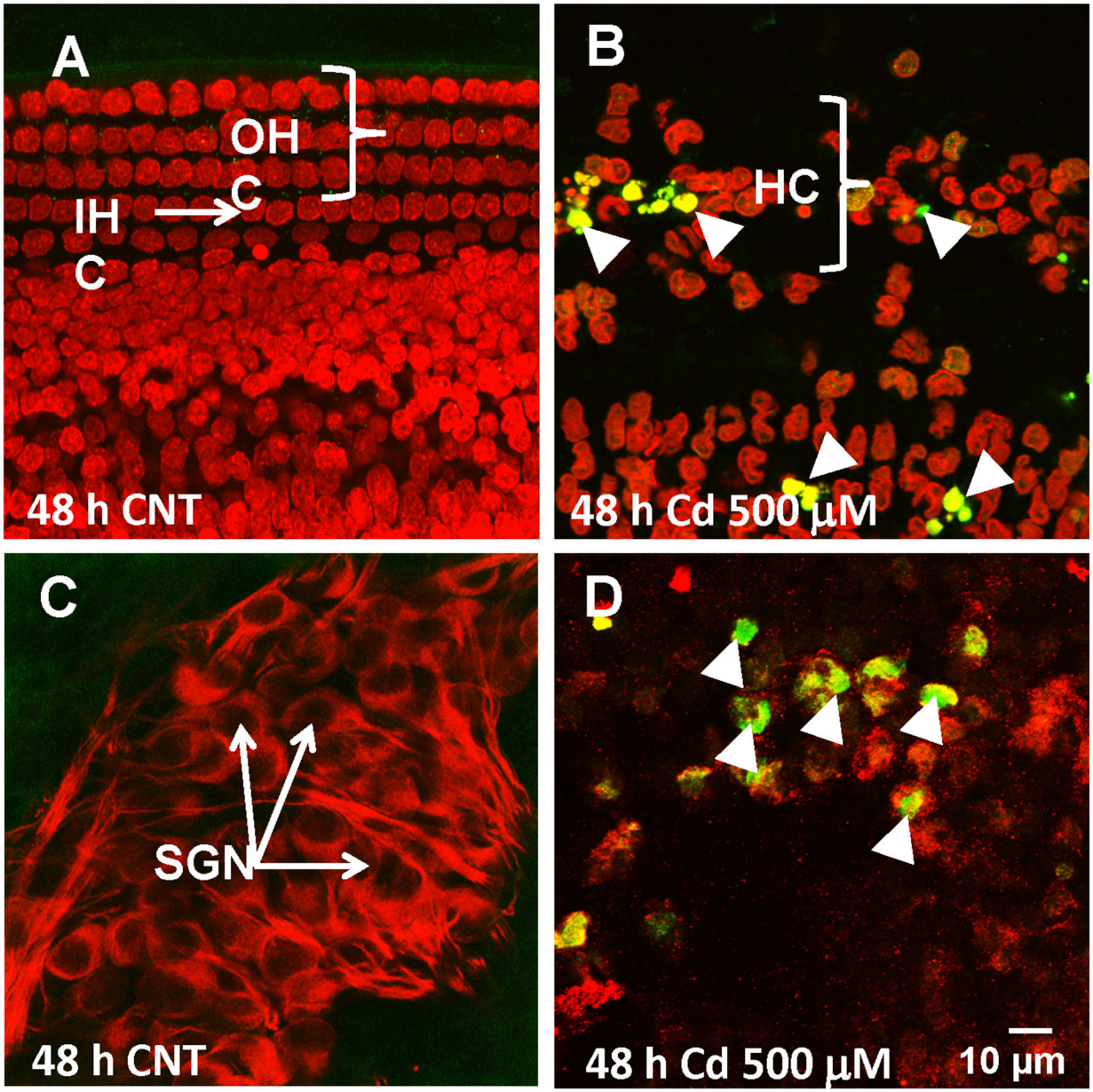

Figure 6:

(A-B) Representative confocal images of cochlear cultures stained with To-Pro-3 (red) and TUNEL Assay Kit (green). (A) Control cochlea cultured for 48 h; note robust To-Pro-3 in nuclei of IHC and OHC and absence of TUNEL labeling. (B) Cochlea cultured for 48 h with 500 µM Cd; note TUNEL labeling (green or yellow, white arrowheads) in region normally occupied by hair cells (HC). (C-D) Representative confocal images of SGN; specimens labeled with neurofilament 200 kD-Alexa-Fluor 555 (red) and TUNEL Assay Kit (green/yellow). (C) SGN in normal controls cultured for 48 h have a large round soma and large nucleolus (arrows); note absence of TUNEL labeling in controls. (D) SGN treated with 500

μM Cd for 48 h; note TUNEL labeling (white arrowhead) in SGN with shrunken soma (white arrowheads) and condensed nuclei.