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Functional and structural changes in the chinchilla cochlea and vestibular system following round window application of carboplatin

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Abstract

Objective—In chinchillas, moderate doses of carboplatin administered systemically selectively destroy inner hair cells and type I vestibular hair cells; however, it is unclear whether this unique damage pattern persists if carboplatin is applied directly to the cochlea, how quickly the damage develops and what cell death pathways are involved.

Study design—To address these questions, carboplatin (5 mg/ml, 50 µl) was applied to the round window.

Results—Carboplatin caused a rapid decline in distortion product otoacoustic emissions, significantly increased compound action potential thresholds and caused massive inner hair cell loss and less severe outer hair cell loss. Hair cell loss was initially more severe in the base than the apex of the cochlea, but by 28 days post-treatment most cochlear hair cells were missing and hair cell density in the utricle, saccule and lateral crista was greatly reduced. At one day post-treatment, many hair cell nuclei were condensed or fragmented indicative of apoptosis, and expressed initiator caspase-8 and executioner caspase-3, but not initiator caspase-9. Carboplatin-treated animals circled towards the treated ear and during the swim test rolled towards the treated ear.

Conclusion—These results indicate that local application of carboplatin causes loss of hair cells that begins near the base of the cochlea and spreads towards the apex with increasing survival time. Hair cell loss is initiated by caspase-8 followed by executioner caspase-3.

Keywords

hair cells; cochlea; crista ampullaris; utricle; apoptosis; distortion product otoacoustic emissions; compound action potential; caspase

Introduction

Carboplatin (cis-diamine-1,1-cyclobutane dicarboxylate platinum(II), CBDCA) is a second generation anti-cancer agent that is significantly less ototoxic and nephrotoxic than cisplatin

(1,2). Nevertheless, at high doses carboplatin can cause hearing loss and destruction of sensory hair cells in both the auditory and the vestibular sensory epithelium (1,3–6).

The ototoxic effects of carboplatin have been studied in many species. Carboplatin as well as other platinum analogs such as cisplatin typically damage the outer hair cells (OHC). In the guinea pig, carboplatin preferentially destroyed OHC and the severity of hair cell loss decreased along a base-to-apex gradient (3,6,7). These findings are consistent with the pattern of high-frequency hearing loss seen in patients treated with carboplatin (8). In contrast, the pattern of hair cell loss seen in carboplatin-treated chinchilla is remarkably different from that observed in other species. At low to moderate doses, carboplatin selectively damages inner hair cells (IHC) relatively uniformly along the length of the cochlea leaving the OHC functionally and structurally intact. However, at high doses, carboplatin also destroys the OHC according to the classical base-to-apex gradient (4,6,9–12).

One clinical report suggests that carboplatin may also affect the peripheral vestibular system (13). Studies in the chinchilla show that carboplatin not only damages hair cells in the cochlea, but also those in the vestibular system (4,9). Lesions in the chinchilla vestibular system were observed five days or more following carboplatin treatment (7). Morphological damage varied in degree and location with the posterior crista showing the most consistent and severe damage. The earliest functional change observed after carboplatin treatment was an abnormal nystagmus response; this was associated with missing and fused stereocilia, and damage to centrally located type I hair cells and vestibular neurons (4,9,10).

Little is currently known about the effects of carboplatin when applied directly to the inner ear and knowledge of the vestibulotoxic effects from local drug delivery have yet to be evaluated (14). Knowledge of its local ototoxic effects could prove useful for future studies designed to examine the functional and structural changes associated with unilateral cochlear or vestibular damage that involve a particular subpopulation of hair cells or neurons (e.g. central plasticity, tinnitus, and vestibular compensation). The cell death pathways involved in carboplatin ototoxicity are also poorly understood, but would be important to understand given the unique lesion pattern. To address these issues, carboplatin was applied directly to the chinchilla round window membrane (5 mg/ml, 50 μ l) and its functional and structural changes were examined at regular time intervals to 28 days post-treatment. In addition, the contributions of intrinsic and extrinsic apoptotic cell death pathways were investigated by studying the pattern of caspase activation that occurred after local carboplatin administration.

Methods

Subjects

Adult chinchillas (480–600 g) were divided into five groups (1 day, 3 days, 7 days, 14 days and 28-days groups); the 1-day group contained eight animals and the other groups contained five animals. The right ear of each animal was treated with carboplatin and the left ear served as a normal control. The balance behaviour, nystagmus response, distortion product otoacoustic emission (DPOAE) and compound action potential (CAP) threshold were measured.

Afterwards, morphological studies were carried out on the cochlea and vestibular system. The cochleas of three chinchillas in the 1-day group were perfused with fluorescent probes for caspase-3, caspase-8, or caspase-9, 1 h before sacrifice. DPOAE measurements were obtained from animals in the 28-day group at 1, 3, 7, 14 and 28 days post-treatment.

Balance behaviour

Balance behaviour was assessed at different times after carboplatin treatment by two methods. Animals were placed on a table and observed for 2–3 min for signs of circling behaviour and

spontaneous nystagmus. Subsequently, animals were placed in a container filled with warm water for 20–40 s to determine if the animal would swim or roll over in the water.

Surgery

Chinchillas were anesthetized with isoflurane (2% induction, 1–1.5% maintenance) and placed on a temperature-controlled heating pad with a rectal probe to maintain core temperature at 37°C. Animals were positioned in a standard stereotaxic frame and the head was held in place with ear bars. Under sterile conditions, the right bulla was exposed via a dorsal lateral approach. A 50- μ l drop of carboplatin (Sigma, C2538, 5 mg/ml in lactated Ringer's solution) was placed on the round window membrane with the aid of a surgical microscope. Afterwards, the bulla was closed with dental cement and the wound closed with suture material.

DPOAE

Our procedures for measuring DPOAE have been described previously (12). Briefly, DPOAE were recorded using a Smart Distortion Product Otoacoustic Emission System (Intelligent Hearing System, version 4.53). Awake chinchillas were placed in a custom restraint and tested in a sound attenuating booth lined with sound absorbing foam. An earpiece containing a low noise microphone (Etymotics 10B+) and two earphones (Etymotics ER-2) was inserted into the ear canal for DPOAE testing. Two HIS-3738 high frequency transducers were used to deliver the primary tones, f1 and f2, to the ear canal via flexible tubes connected to the earpiece. The f2/f1 ratio was set at 1.2. The output of the microphone was fed to the input of the Smart DPOAE system, digitized and evaluated using the system. DPOAE input/output functions were measured at f2 values of 1, 2, 4, 8 and 16 kHz. The intensity of f1 was varied from 15 dB to 60 dB SPL in 5-dB steps and the intensity of f2 was 10 dB lower than that of f1. Animals were tested two times, at pre-treatment and prior to sacrifice.

CAP

Recordings were carried out in a double-walled sound booth (Tracoustics). Animals were anesthetized and the bulla opened as described above. A Teflon-coated silver-ball electrode was placed on the round window and held in place with dental cement. A silver wire reference electrode was placed in the neck muscles. The CAP was measured using Tucker Davis Technologies (TDT) software (BioSigRP) and hardware consisting of a stimulus generator (RP2.1), real-time processor (RA16BA), programmable attenuator (PA5), headphone amplifier (HB7) and loudspeaker (Fostex FE127E). The loudspeaker was located 10 cm directly in front of the entrance to the ear canal. The output of the speaker was calibrated with a sound level meter (Larson Davis 824) equipped with a half-inch free-field, condenser microphone (Larson Davis 2540). The CAP was elicited with tone bursts presented at 1, 2, 4, 8 and 16 kHz (1 ms Blackman gating, 2 ms duration, 9/s, 50 kHz sampling rate). Stimulus intensity was varied from approximately 90 to 0 dB SPL in +5 dB steps. Responses were amplified (5000x), filtered (100–3000 Hz), digitized (~25 kHz, 10 ms window), averaged (100x) and displayed over a range of intensities. Threshold was defined as the lowest intensity at which the N₁ peak of the CAP could be reliably detected (~5 μ V) from visual inspection of the waveforms.

Histology

Animals were sacrificed and the cochleae removed and prepared for analysis as described previously (15). Briefly, the anesthetized (sodium pentobarbital, 80–100 mg/kg i.p.) animals were perfused transcardially with saline (phosphate buffered, pH 7.4 at 37°C), followed by 10% phosphate buffered formalin (4% formaldehyde). Animals were decapitated, the bulla removed, and the inner ear processed as described previously (12). The stapes was removed,

the round window membrane opened and 10% phosphate buffered formalin perfused through the round window. The cochleae were immersed and stored overnight in fixative.

Specimens were stained with Harris hematoxylin solution or FITC labeled phalloidin (Sigma, P5282), which heavily labels the stereocilia bundles and circumferential ring surrounding the hair cell cuticular plate (16). For hematoxylin staining, Harris' hematoxylin solution was perfused through the round window and then each cochlea was immersed in staining solution for 5 min. Afterwards, the organ of Corti was dissected out as a flat surface preparation. If the staining was too dark, specimens were dipped into 0.3% hydrochloric acid to remove excess color. For FITC-phalloidin staining, the cochlear basilar membrane was dissected out and then immersed in FITC-phalloidin solution for 30 min. The sensory epithelium from the cochlea and vestibular systems was mounted as a flat surface preparation on glass slides in glycerin, and coverslipped.

Some cochleae were stained by succinate dehydrogenase (SDH) histochemistry (10,17). The cochlea was removed and SDH staining solution (1:1:2 parts 0.2 M sodium succinate, 0.2 M phosphate buffered saline (pH 7.6), 0.1% tetranitro-blue tetrazolium) was gently perfused through the round and oval windows. Subsequently, the cochlea was immersed in SDH solution for 45 min at 37°C. Specimens were later fixed with 10% formalin for 24 h. The sensory epithelium was removed from the cochlea and mounted in glycerin on glass slides as a surface preparation. Specimens were examined under a light microscope at 400x (Zeiss Axioskop) and photographed (SPOT Insight, Diagnostic Instruments Inc.). Images were transferred to a PC computer and processed with Adobe Photoshop (version 5.5).

For cochleograms, the cochlear surface preparation was examined with a microscope (Zeiss Standard 400x magnification) and the numbers of missing IHC and OHC were determined over 0.24-mm intervals along the entire basilar membrane. Hair cells were counted as present if the cell body and the cuticular plate were intact. Data were entered into a computer using custom cochleogram software to obtain a cochleogram showing the percent IHC and OHC loss as function of percent total distance from the apex; percent loss was based on laboratory norms of hair cell density for young normal chinchillas (16). Percent distance from the apex was related to frequency using a frequency place map (18). Mean cochleograms were computed over 10% intervals along the cochlea and the OHC and IHC loss for each group was plotted as an average cochleogram, as described previously (12).

The bone surrounding the vestibular sensory end organs was removed and the sensory epithelium from the saccule, utricle and lateral crista was carefully dissected out as a flat surface preparation for hematoxylin or FITC-phalloidin staining. Subsequently, the specimens were trimmed and mounted in glycerin on glass slides. The sensory epithelium was viewed at 400x under a light microscope (Zeiss Standard) for hematoxylin staining or observed with a fluorescent microscope (Nikon Eclipse E400) equipped with appropriate filters for FITC-phalloidin staining.

Our procedures for quantifying vestibular sensory hair cells have been described previously (19). The vestibular hair cells were counted as present if the cuticular plate and nucleus appeared intact. Hair cell density was assessed by counting the hair cells in a 0.1×0.1 mm square grid in each of the specified regions from utricle, saccule and lateral crista from each animal. Because of differences in hair cell density in the marginal and striolar regions of the maculae, hair cell counts were obtained from both marginal and striolar regions in each utricle and saccule. In the lateral crista ampullaris, hair cell density was assessed in the central region and border region. Mean hair cell counts were obtained from five specimens per group for each location noted above.

Fluorescent staining of the cuticular plate and nucleus

The entire basilar membrane containing the organ of Corti was carefully dissected out as a flat surface preparation as described previously (16). Specimens were stained with TRITC-conjugated phalloidin (Sigma, P1951) for 30 min. Subsequently, the samples were counter-stained with TO-PRO-3 (Invitrogen, T3605) for 10 min in order to visualize the nuclei. Basal, middle and apical turns were trimmed and mounted in glycerin on glass slides.

Caspase labeling

Caspase-8, caspase-9 and caspase-3 activity were assessed with fluorescently labeled probes that detect activated caspases in living hair cells using carboxyfluorescein (FAM) labeled peptide fluoromethyl ketone (FMK) caspase inhibitors (FAM-peptide-FMK) that selectively label caspase-9, caspase-8 or caspase-3 (Cell Technology, Inc.) as described previously (20). Twenty-four hours following carboplatin treatment, chinchillas were anesthetized as described above. The round window and oval windows were opened and 10 μ l of solution containing a specific caspase probe was gently perfused into the perilymph according to the manufacturer's instructions. After 1 h, the animals were sacrificed and the cochleae harvested and fixed by perfusing 10% formalin in PBS into the cochlea and then immersing the specimen in fixative for 3 h. The sensory epithelium containing the organ of Corti was carefully dissected out, stained with TRITC labeled phalloidin (Invitrogen) for 30 min and then labeled with TOPRO3 for 10 min. Basilar membranes were mounted in glycerin on glass slides and examined under a confocal laser scanning microscope (Zeiss LSM510) using appropriate filters to detect green carboxyfluorescein of activated caspases, TRITC-labeled phalloidin and TOPRO3. Images were captured and analyzed with a Zeiss LSM Image Examiner (version 4.0.0.91, Carl Zeiss GmbH) and processed with Adobe Photoshop (version 5.5).

All procedures regarding the use and care of animals in this study were approved by the Institutional Animal Care and Use Committee at the University at Buffalo.

Results

DPOAE

Figure 1 shows the average ($n = 5$) DPOAE amplitudes for the group of chinchillas that was treated with carboplatin and allowed to survive 28 days. In the control condition, prior to carboplatin treatment, DPOAE amplitudes increased with intensity reaching levels of approximately 35–40 dB when L2 was 70 dB SPL. One day after carboplatin treatment, DPOAE amplitudes were reduced at 2, 4, 8 and 16 kHz. The largest reductions occurred at 8 and 16 kHz. At 3 days post-treatment, DPOAE amplitudes were greatly reduced at low and moderate intensities at all frequencies. DPOAE amplitudes declined more slowly beyond 3 days and by 28 days post-treatment DPOAE were largely absent except at intensities greater than 60 dB SPL.

CAP

The CAP thresholds were measured in each animal in the 1-day, 3-day, 7-day, 14-day and 28-day groups prior to sacrifice. Mean CAP thresholds obtained from normal control ears (Figure 2, $\pm$ 1 SEM, $n = 5$), ranged from 14 to 28 dB SPL between 1 and 16 kHz with the lowest thresholds at 8 kHz. Mean CAP thresholds ($\pm$ SEM) increased to between 71 and 88 dB SPL across the frequency range 1 day post-carboplatin. CAP threshold increased more slowly from 3 days to 14 days post-treatment, and by 28 days post-treatment thresholds exceeded 90 dB SPL (maximum stimulus output) at all frequencies (upward arrows).

Cochleograms

Twenty-four hours after carboplatin treatment, the stereocilia bundles were fused, in disarray or missing on some OHC (Figure 3). The normal complement of IHC and three rows of OHC can be seen in normal ears stained with SDH (Figure 4). However, many IHC were missing 24 h post-treatment, but nearly all the OHC were still present (Figure 4B). At 3 days post-carboplatin, extensive IHC and OHC loss was observed (Figure 4C). To quantify these trends, cochleograms were prepared from each of the carboplatin treated ears in the 3-day, 7-day, 14-day and 28-day groups. Figure 5 shows the mean cochleograms for the 3-day, 7-day, 14-day and 28-day groups with the average IHC and OHC loss in 10% intervals along the length of the cochlea. Position in the cochlea is expressed as percent distance from the apex (lower abscissa) or as a function of frequency (upper abscissa) (18). At 3 days post-treatment IHC loss was substantially greater than OHC loss. Mean IHC loss declined from 100% in the basal half of the cochlea to approximately 20% in the extreme apex while OHC loss declined from approximately 90% in the base of the cochlea to less than 10% near the apical third of the cochlea. The mean cochleograms from the 7-day and 14-day groups were similar: IHC loss ranged from 90% to 100% over most of the cochlea except in the extreme apex where the loss declined to approximately 50%. The OHC loss declined from 100% in the base to roughly 15–20% near the apex of the cochlea. At 28 days post-treatment, IHC and OHC loss were nearly 100% in the basal 60%; IHC and OHC losses declined to roughly 80% and 60% at the apex, respectively.

Apoptosis

Fluorescent probes against caspase-8, or caspase-9, or caspase-3 were infused into the perilymph 24 h following carboplatin treatment and afterwards the cochleas were removed and labeled with TRITC phalloidin and TOPRO3. Figure 6 shows typical images from the basal turn of the chinchilla cochlea 1 day after carboplatin treatment. TRITC-phalloidin (pseudo-green) and TOPRO3 (pseudo-red) staining showed that the cuticular plate and hair cell nuclei were present in most hair cells at this time (Figure 6A,C,E). However, many of the TOPRO3 labeled hair cell nuclei were condensed and fragmented (arrows, Figure 6A,C,E), indicative of cells undergoing apoptosis. In a few cells (jagged arrows), TOPRO3 labeling appeared condensed, fragmented and unevenly dispersed near the edges of the nuclear envelope. The right panel of Figure 6 shows the same images labeled with caspase-8 (panel B), caspase-9 (panel D) or caspase-3 (panel F). Importantly, hair cells with condensed or fragmented nuclei (Figure 6A,C,E, arrows) also expressed caspase-8 (Figure 6B) or caspase-3 (Figure 6F), but not caspase-9 (Figure 6D). In contrast, the nuclei of normal chinchilla hair cells were large, round and evenly stained and did not express caspase-3, -8 or -9 (data not shown), as noted in earlier reports (20).

Balance function

One day after the carboplatin treatment, the animals exhibited spontaneous nystagmus. In addition, all the carboplatin-treated animals showed a pronounced head tilt and circling behaviour towards the damaged ear 1 day post-treatment. By 7 days post-treatment, the circling behaviour had greatly subsided in most animals. When the animals were placed in a tub of warm water for the swim test, all the carboplatin-treated animals rolled over in the direction of the damaged ear even 28 days after drug treatment.

Vestibular hair cell degeneration

One day after carboplatin treatment, there were early signs of stereocilia damage in the utricle, saccule and lateral crista. The cilia bundles on many hair cells were missing, in disarray or fused into a single, long thread (data not shown). These changes were mainly confined to the striolar region in the maculae of the utricle and saccule and to the central region of the ampulla

of the lateral crista. However, after 3 days, massive hair cell damage was seen throughout much of the vestibular sensory epithelia. Figure 7 shows typical surface views of the saccule from normal controls and experimental animals at 1 day, and 3 days post-carboplatin. In normal ears, the sensory epithelia are packed with hair cells decorated with long stereocilia bundles. Twenty-four hours after carboplatin treatment, the stereocilia were missing from sensory hair cells, and at 3 days post-carboplatin, the stereocilia bundles were completely missing on surviving hair cells and many hair cells were also missing.

To quantify the damage, vestibular hair cells were counted in a 0.01×0.01 mm grid in the central and peripheral regions of the macula of the utricle and saccule and the ampulla of the lateral crista. Hair cells were counted as missing if the stereocilia bundle and/ or the cuticular plate were missing. Mean hair cells numbers ($n = 5$, +SEM) for these six regions are shown in Figure 8. At 1 day post-carboplatin, approximately 50% of the hair cells in the striolar region of the utricle and saccule were missing, whereas hair cell losses near the margins of these two regions were 15% or less. By 14 days post-treatment, nearly all the hair cells in the utricle and saccule were missing. In the ampulla of the lateral crista, hair cell losses at 1 day post-treatment were approximately 65% and 45% in the central region and near the margin, respectively. By 14 days post-treatment nearly all the hair cells were missing from both regions of the ampulla.

Discussion

Relatively little is known about the cell death pathways involved in carboplatin ototoxicity in the chinchilla inner ear or the time-course and pattern of damage that occurs in the cochlear and vestibular sensory epithelia after round window drug application. In the present study, local application of carboplatin (5 mg/ml; 50 μ l) caused a remarkably rapid deterioration of cochlear and vestibular function and widespread destruction of most hair cells in the cochlea and vestibular epithelium.

Hair cell degeneration

Although there was a large reduction in DPOAE amplitudes and a large increase in CAP thresholds 1 day post-carboplatin, most cochlear hair cells were still present at this time; however, the stereocilia bundles on many hair cells appeared abnormal and in the early stages of degeneration (Figure 3B). Importantly, TOPRO3 labeling showed that many hair cell nuclei in the basal turn of the cochlea were abnormally condensed and/or fragmented, morphological features characteristic of cells in the early stages of programmed cell death. Tissues were labeled with caspase-3 and -8, but not caspase-9 (21). Programmed cell death is often initiated by caspase-8 or caspase-9, which reside upstream of executioner caspase-3. Caspase-8 is activated by pro-apoptotic signals such as TNF, FAS or TRAIL that contain a death domain (22). In contrast, caspase-9 mediated cell death is regulated by mitochondrial signaling involving members of the Bcl-2 family (23). Activation of pro-apoptotic Bcl-2 family members such as Bax and Bid can trigger a sequence of events leading to alterations in mitochondrial permeability transition, release of mitochondrial cytochrome c into the cytosol, and activation of pro-caspase-9 (24). One day following carboplatin treatment, caspase-8 was heavily expressed in the cytoplasm of hair cells with condensed or fragmented nuclei; however, caspase-9 was absent at this time. Downstream executioner caspase-3 was also expressed in hair cells with condensed or fragmented nuclei; however, caspase-3 labeling tended to be restricted to hair cells with the smallest and most condensed nuclei presumably in the later stages of degeneration. These results closely parallel previous studies from our laboratory showing that systemic administration of cisplatin plus ethacrynic acid induced heavy expression of caspase-8 in damaged hair cells, but little expression of caspase-9 (20). Our results differ from earlier *in vivo* studies showing that systemic administration of cisplatin up-regulated the expression of caspase-9, but not caspase-8 in guinea pig hair cells (25). The

reasons for these differences are unclear, but may be related to rate of onset of cochlear damage. Local carboplatin treatment or cisplatin plus ethacrynic acid causes massive and rapid onset of cell death in the chinchilla, whereas the onset of hearing loss and hair cell loss from systemic administration of cisplatin alone, occurs slowly over days or weeks. Alternatively, the cell death pathways induced by carboplatin may be different from those induced by cisplatin.

Degeneration gradient

The gradients of carboplatin induced hair cell death have been most thoroughly studied in the cochlea. Systemic administration of low to moderate doses of carboplatin causes a diffuse loss of IHC along the entire length of the cochlea; the broad loss of IHC along the length of the cochlea is highly unusual and is distinctly different from that seen with most ototoxic drugs in other species. On the other hand, high doses of carboplatin can destroy the OHC, but the gradient of cell loss progresses from base to apex as is typically seen with other ototoxic compounds (26). The pattern of IHC loss that occurs when carboplatin is delivered directly to the round window differs from that seen when the drug is given systemically. Instead of causing a relatively uniform loss of IHC along the length of the cochlea, the IHC lesion begins in the base and spreads towards the apex. The difference in the pattern of IHC seen with local versus systemic administration is most likely due to two factors: the diffusion of carboplatin from the round window towards the apex of the cochlea, combined with a weaker antioxidant enzyme system near the base versus apex of the cochlea (27). On the other hand, the pattern of OHC loss seen with local application of carboplatin is similar to that which occurs with systemic administration.

The time-course of hair cell loss occurs in two distinct phases – an early rapid phase during the first three days followed by a slow and progressive phase over the next 2–4 weeks (Figure 5). The rapid induction of the hair cell lesion and loss of function may be due its rapid penetration through the round window as opposed to slow and diffuse distribution likely to occur with systemic administration (28). The mechanisms that give rise to the slow, progressive loss of hair cells in the apical half of cochlea is more difficult to account for, but could be related to the fact that hair cell antioxidant defenses increase from base to apex resulting in slower onset of hair cell death in the apex.

DPOAE

DPOAE amplitudes were greatly reduced 1 day post-carboplatin; these effects were most severe at 8 and 16 kHz. Surprisingly, few hair cells were missing 1 day post-treatment; however, the stereocilia appeared abnormal and many hair cells were in the early stages of apoptosis, as reflected by caspase-3 and -8 labeling and nuclear condensation and fragmentation. DPOAE amplitudes showed a second large decline between 1 day and 3 days post-treatment at 2 and 4 kHz and to a lesser extent 8 and 16 kHz. Cochleograms measured 3 days post-treatment showed more than 50% loss of OHC from cochlear regions spanning 2–16 kHz (Figure 5). These results suggest a relationship between the degree of OHC damage at 3 days post-treatment and the DPOAE frequencies with the greatest reduction. On the other hand, the reduction in DPOAE amplitude at 1 day post-treatment occurred several days before we observed substantial OHC damage. This discrepancy is most likely due to subtle stereocilia pathology and early stages of apoptosis. Another factor that may contribute to the rapid decline in DPOAE amplitude 1 day post-treatment is drug-induced impairment of the endocochlear potential that provides the driving voltage for the OHC receptor potential and electromotile response. Several studies report a reduction in the endocochlear potential shortly after local or systemic administration of cisplatin; however, the magnitude of the effect may depend on the dose and mode of administration (29). On the other hand, long-term carboplatin treatment did not cause a permanent reduction in the endocochlear potential (3).

CAP

The CAP reflects the synchronized onset response from many type I neurons that synapse on IHC (30). Round window application of carboplatin caused a rapid increase (50–60 dB) in the CAP threshold over a wide frequency range at 1 day post-treatment and by 28 days the CAP response was undetectable. The rapid and widespread decline in CAP was expected given that carboplatin not only damages the IHC, but also type I neurons. Type I neurons begin to show signs of degeneration as early as 6 h following systemic administration of carboplatin roughly 1–2 days before the IHC begin to degenerate (31). The complete loss of the CAP response at 28 days post-carboplatin is consistent with the massive loss of IHC seen at this time.

Vestibular degeneration

The present study described for the first time the time-course and pattern of damage to the chinchilla vestibular system that occurs after round window application of carboplatin. Early studies in the albino guinea pig indicated that carboplatin had no obvious morphological effect on the vestibular system (32). However, clinical reports suggest that carboplatin affects the peripheral vestibular system (13). Previous studies in the chinchilla indicated that systemic carboplatin damaged the hair cells in the crista and the macula and caused vestibular dysfunction (4,9). In the present study, round window application of carboplatin resulted in severe head tilt and circling behaviour towards the damaged ear 1 day post-treatment. These results are consistent with previous reports showing functional deficits in the cold caloric nystagmus response as early as 6 h following systemic treatment (9). The functional deficits noted here were mainly associated with damage to the stereocilia bundles on vestibular hair cells located in the striolar region of the maculae and in the central region of the ampulla of the lateral crista. More than 50% of the hair cells in the striola and central region of the maculae and ampulla, respectively, were missing at 1 day post-treatment. In contrast, most of the hair cells near the margins of the utricle and saccule were present and only a third were missing near the margin of the ampulla. Vestibular hair cell damage progressed rapidly and by 14 days, virtually all the hair cells in these three sensory epithelia were missing. These results suggest that hair cell damage from local carboplatin treatment progresses rapidly in both cochlear and vestibular sensory epithelium. In addition, vestibular hair cells located near the center of the epithelium (type I) are more vulnerable than those near the margins (type II), consistent with earlier reports obtained with systemic administration (4,9).

Our results show that round window application of 50 μ l of carboplatin (5 mg/ml) causes rapid and severe onset of auditory and vestibular deficits. These functional deficits are associated with early structural deficits to the stereocilia bundles. Round window application of carboplatin triggers an apoptotic response in the hair cells that is initiated by the extrinsic cell death pathway involving caspase-8 followed by caspase-3 and does not involve the intrinsic cell death pathway initiated by caspase-9.

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Figure 1.

DPOAE input/output functions (mean $\pm$ SEM) at $f_2 = 2, 4, 8$ and 16 kHz measured in control ears ($n = 5$) and carboplatin treated ears 1, 3, 7, 14 and 28 days after carboplatin treatment.

Figure 2. CAP thresholds (mean $\pm$ SEM) in normal control ears ($n = 5$) and in carboplatin treated ears ($n = 5$) of the 28-day group. Thresholds that exceeded the maximum output of the system are indicated by arrows.

Figure 3.

Photomicrographs of the organ of Corti stained with FITC labeled phalloidin from a control animal (A) and a carboplatin treated animal 1 day post-treatment (B). Some IHC were missing 1 day post-carboplatin (arrows) and the stereocilia on many OHC were missing (jagged arrow) or blurry (arrowhead). Outer hair cells (OHC), inner hair cells (IHC) and pillar cells (PC).

Figure 4.

Surface preparations of organ of Corti stained with SDH. (A) Photomicrograph of cochlea from normal animal. Scale bar 10 μ M. (B) Photomicrograph of cochlea obtained 1 day post-carboplatin; most OHC present, most IHC missing. (C) Photomicrograph of cochlea obtained 3 days post-carboplatin; most IHC missing and most OHC missing or damaged.

Figure 5.

Mean cochleograms showing percent IHC and OHC cell loss as a function of percent distance from the apex of the cochlea in 3-day, 7-day, 14-day and 28-day groups. Cochlear frequency position maps for the chinchilla cochlea are shown on the upper abscissa.

Figure 6.

Typical surface preparation views in the middle turn of the chinchilla cochlea obtained 24 h following round window application of carboplatin. Panels A, C and E show labeling with TRITC-phalloidin (green) and TOPRO3 (red); some TOPRO3 labeled nuclei are condensed and fragmented (arrows) and in a few cells the labeling is condensed and unevenly dispersed (jagged arrow). The same three images as on left, but green labeling, showing caspase-8 (B), caspase-9 (D) and caspase-3 (F) plus TOPRO3 (red). Many hair cells have condensed and/or shrunken nuclei (arrows) and express caspase-8 and caspase-3 (panels B and F, respectively), but not caspase-9 (panel D). A few normal cells with large, round, evenly stained nuclei and absence of caspase labeling are present (arrowheads, panels C,D,F). Three rows of OHC (1, 2, 3) and row of pillar cells (P).

Figure 7.

Typical surface preparation images of the macula of saccule stained with phalloidin in normal chinchillas (A) or those treated with carboplatin and allowed to survive for 1 day (B) or 3 days (C). Stereocilia bundles present on all vestibular hair cells in normal chinchillas (A). In contrast, a few stereocilia are present in the vestibular sensory epithelium at 1 day post-treatment (B). At 3 days post-carboplatin, no stereocilia are visible on the macula of saccule (C).

Figure 8.

The number of hair cells per 0.01 mm² (mean $\pm$ SEM) in indicated region of control ($n = 5$) and in carboplatin-treated chinchillas at survival times of 1, 3, 7, 14 or 28 days.