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Styrene Induced Alterations in Biomarkers of Exposure and Effects in the Cochlea: Mechanisms of Hearing Loss

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It is known that styrene is ototoxic and causes cochlear damage starting from the middle turn. However, the cellular mechanism underlying styrene ototoxicity is still unclear. In this study, rats were exposed to styrene by gavage at different doses once a day for varying periods. Styrene levels in the cochlear tissues, styrene-induced permanent hearing loss, cochlear disruptions, and cell death pathways were determined. Styrene concentration in the cochlea varied along with the basilar membrane with the lowest level in the basal turn being consistent with the lowest styrene-induced threshold shift and hair cell loss in this region. After 3 weeks of exposure (5 days per week), a dose-dependent permanent hearing loss and a hair cell loss, especially in the midfrequency region, were observed. The styrene exposure at a dose of 200 mg/kg, which induced a blood level of 6.0 ± 1.0 μg/g, caused an average of 4.4 ± 0.5% OHC (outer hair cell) loss and 2–5 dB threshold shift in the cochlear region of 20–70% from the apex. A significant OHC loss was not observed until 7 days of exposure at a dose of 800 mg/kg. Deiters cells appeared to be the most vulnerable target of styrene. When condensed nuclei were observed in Deiters cells after a few days of styrene exposure (800 mg/kg), other cells were still intact. Apoptotic cell death appeared to be the main cell death pathway in the cochlea after styrene exposure. In the styrene-induced apoptotic OHCs, histochemical staining detected activated caspases-9 and 8, indicating that both mitochondrial-dependent pathway and death receptor–dependent pathway were involved in the styrene-induced cell death.

Key Words: **styrene ototoxicity; cochlear injury; hair cell death; apoptosis; caspase.**

Styrene, an aromatic hydrocarbon, is used extensively in the production of plastics, synthetic rubbers, resins, insulators, and protective surface coatings (Johnson and Nylén, 1995; Rybak, 1992). It is among the top 10 synthetic organic chemicals produced in the United States. Human exposure occurs at levels of milligrams during production and industrial use (Diodovich *et al.*, 2004). In a previous report, styrene levels in whole blood in workers in a fiber-reinforced plastics industry ranged up to 2 mg/l, depending on styrene exposure level (Mizunuma *et al.*, 1993). Styrene exposure levels ranged from 0 to 194 ppm among individuals with a geometric mean level of 16.6 ppm (Mizunuma *et al.*, 1993). In their investigation, styrene in the air was sampled by attaching a box-type diffusive sampler with carbon cloth KF 1500 as absorbent on the chest of each worker. In a recent measurement in a boatyard and a plastics factory, styrene concentrations ranged from 0.05 ppm up to a maximum of 47 ppm (Sliwinska-Kowalska *et al.*, 2003).

Styrene occupational exposure-related hearing loss was observed in several investigations (Morata *et al.*, 2002; Morioka *et al.*, 1999; Sliwinska-Kowalska *et al.*, 2003) but not in others (Calabrese *et al.*, 1996; Möller *et al.*, 1990; Muijser *et al.*, 1988; Sass-Kortsak *et al.*, 1995). Animal experiments have also showed that exposure to styrene caused hearing loss (Pryor *et al.*, 1987). The lowest styrene dosage that caused hearing loss in rats was about 600–700 ppm for 4 weeks of exposure (Loquet *et al.*, 1999; Makitie *et al.*, 2002; Pouyatos *et al.*, 2002). Among industrial solvents, styrene is one of the most ototoxic solvents (Gagnaire and Langlais, 2005). Like other ototoxic solvents, styrene disrupts cochlear cells starting from the middle turn, leading to hearing loss in the midfrequency region (Crofton *et al.*, 1994; Yano *et al.*, 1992). Since most ototoxic drugs initially attack the base of the cochlea, it raises the question: why are cochlear cells in the middle turn more vulnerable to exposure of ototoxic solvents? Outer hair cells (OHCs) are known to be a vulnerable target of styrene with the third row of OHCs being attacked first, then the second and first rows (Campo *et al.*, 2001; Loquet *et al.*, 1999; Yano *et al.*, 1992). Inner hair cells (IHCs) are relatively insensitive to styrene exposure. While many OHCs are destroyed by styrene, only a few IHCs are affected (e.g., Campo *et al.*, 2001). A previous report further indicated that supporting cells were the most sensitive target of styrene in the cochlea (Campo *et al.*, 2001). However, it is unclear which supporting cells are the first targets.

Styrene exposure has been reported to induce necrosis in blood cells (Diodovich *et al.*, 2004). However, styrene 7,8-oxide (SO), the main metabolite of styrene, has been reported to induce apoptosis through activation of caspase in neurons (Boccellino *et al.*, 2003; Daré *et al.*, 2002, 2004). What

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is the cell death pathway in the cochlea after styrene exposure? Both necrosis and apoptosis have been observed in the cochlea (Hu *et al.*, 2006). Apoptotic cell death has been observed in the cochlea due to aging (Nevado *et al.*, 2006), disease (Labbe *et al.*, 2005), or traumatic exposures (Hu, 2007; Hu *et al.*, 2006; Nakagawa *et al.*, 1998; Xu and Huang, 2003).

This study was designed to determine: (1) distribution of styrene in the cochlea; (2) hearing loss and death or disruption of cochlear cells in different regions as a function of styrene dosage; and (3) relative vulnerability of cochlear cells and cell death pathways after styrene exposure.

MATERIALS AND METHODS

Subjects

Long Evans pigmented rats (male, 330 ± 32 g) were acquired from Harlan Sprague Dawley and housed in the University at Buffalo animal facility after delivery. All animal facilities are registered with the U.S. Department of Agriculture and are inspected semiannually by the members of the Institutional Animal Care and Use Committee (IACUC) serving the Research Foundation of State University of New York. Background noise level in the colony room was 45 dB (A-weighting). Temperature was maintained at 71°F. Lights were on from 6:00 A.M.–6:00 P.M. All procedures regarding the use and handling of animals and styrene exposures were reviewed and approved by the IACUC.

Styrene Exposure

To determine kinetics of styrene in the blood and effect of repeated exposures. Rats were exposed to styrene (≥ 99%, S4972, Sigma, St Louis, MO) once a day for 6 days by oral gavage at an exposure dose of 800 mg/kg body weight, which was mixed in olive oil (0.4 ml/kg body weight). Blood samples were collected on day 1 at the following time points (before, 0, 0.5, 1, 2, 3, 4, 5, and 6 h after the gavage treatment). Blood samples were collected again on day 6 at the following time points (before, 0, 0.5, 1, 2, 3, 4, 5, 6, 12, and 24 h after the treatment). The tail tip of the rats was cut, and 10 μl of blood was quickly collected using a heparinized capillary tube and transferred to a preweighed 10 ml SPME (solid phase microextraction) sample vial (Supelco, Bellefonte, PA), capped tight, and weighed again. Each sample collection took about 10 s. Duplicate samples were collected for each time point.

To determine styrene dose relationship to blood concentration. Rats were exposed to styrene by oral gavage at doses of 100, 200, 400, 600, and 800 mg/kg body weight. Three hours after the treatment, blood samples were collected as described above.

To determine styrene levels in the cochlear perilymph and in the cochlear tissues. Rats were exposed to styrene by oral gavage at an exposure dose of 800 mg/kg body weight. Blood, cochlear perilymph, and cochlear tissue in each turn were sampled separately for styrene level measurement 3 h after the gavage treatment. The blood samples were collected as described above. For cochlear perilymph sampling, rats were anesthetized with ketamine (50 mg/kg, im) and xylazine (6 mg/kg, im). The cochleae were removed, and the perilymph samples were collected using a pipette with its sharp tip inserted into the round window. About 2 μl of fluid was collected from each cochlea. The sample was immediately transferred to a preweighed 10 ml SPME sample vial, capped tight, and weighed again. After cochlear perilymph sampling, the cochlea was dissected. The cochlear tissues, including the basilar membrane with the organ of Corti, the spiral ligament with the stria vascularis, and a small part of nerve endings, were sampled from each turn and immediately transferred to a preweighed 10 ml SPME sample vial, capped tight, and weighed again. To avoid variability due to the order of dissection, the cochlear tissues in different turns were sampled randomly. Furthermore, to confirm the results obtained with the small individual samples, cochlear tissues in each turn in three additional rats (six cochleae) were pooled into a sample vial and then analyzed.

To determine permanent hearing loss and hair cell loss as a function of styrene exposure level. Rats were grouped and exposed to styrene by oral gavage once a day for 5 days per week for 3 weeks at doses of 0 (oil only), 200, 300, 400, and 800 mg/kg body weight. Auditory thresholds were determined 12 h and 3 weeks after the last day of styrene exposure, and then the cochleae were removed for hair cell examination.

To determine dynamics of styrene-induced hair cell loss. Rats were exposed to styrene by oral gavage at a dose of 800 mg/kg body weight for 3, 5, 7, 9 days, and 3 weeks (5 days/week). Cochleae were removed 12 h after the last exposure.

To determine cell death pathway in the cochlea after styrene exposure. Rats were exposed to styrene by oral gavage at a dose of 800 mg/kg body weight up to 7 days. Nuclei of cochlear cells were examined and active caspases-8 and 9 were detected.

Styrene Measurement

Headspace solid phase microextraction together with gas chromatography (HS-SPME-GC) has been used to measure styrene level in the blood and in the cochlear samples in a previous report (Campo *et al.*, 1999). The HS-SPME-GC used in this study was developed and validated. The fiber (carboxen/polydimethylsiloxane, 85-μm thick, Supelco) was exposed into the sample collection vial headspace for 4 min at ambient temperature. After sampling, the fiber was then inserted into the gas chromatography (GC) injection port, which was set at 250°C, for 30 s allowing styrene to be desorbed from the fiber into the GC column for analysis. A fused silica capillary column (SPB-624, 30 m × 0.25 mm, 1.4 μm film thickness, Supelco) was used on an Agilent (HP6890) GC equipped with a flame ionization detector. The oven temperature program started from 35°C, held for 1 min, and was increased to 150°C at a rate of 5°C/min. The amount of styrene was determined from a calibration curve established using whole blood as the sample matrix, expressed on a weight/weight basis (μg/g). The method was validated for linearity over a range of 0.5–500 ng per sample with a minimal level of detection of 0.43 ng per sample. Intraday and interday precision and accuracy were less than 5%.

Audiometric Evaluation

Rats were anesthetized by inhalation of isoflurane using a Surgivet (Smiths Medical Vet Division, Isotec 4) during auditory brainstem response (ABR) recording. The ABR response was recorded by the subdermal needle electrodes (one on the scalp midline; one posterior to the stimulated ear; and one on the shoulder), band-pass filtered (100-3000 Hz), and amplified with a bioamplifier (TDT HS4) (McFadden *et al.*, 1999). The ABR signals were averaged 250 times with a rate of 21/s using the BioSig 32 stimulate/record system (TDT system 2). Tone pips (1-ms duration) were generated using the SigGen32 (version 3.11) software with gating type of Blackman and gating time of 0.5 ms. The stimulation frequencies were 2.5, 5, 10, 20, and 40 kHz. The stimulation intensity varied from 100 to 0 dB SPL (peak level) in 5-dB steps. The stimulation intensity evoking the smallest recognized response was used as the threshold. ABR threshold shift was the threshold difference between those obtained before and after the exposure.

Histological Examination

Detection of active caspases. The methodology is based on carboxyfluorescein-labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and noncytotoxic. Once inside the cell, the inhibitor binds covalently to the active caspase (Amstad *et al.*, 2000; Bedner *et al.*, 2000; Nicotera *et al.*, 2003; Smolewski *et al.*, 2001). FAM-LEHD-FMK (Cat#: FAM400-1, APO LOGIX) is a caspase-9 inhibitor and FAM-LETD-FMK (Cat#: FAM300-1, APO LOGIX) is a caspase-8 inhibitor. The inhibitors were dissolved in dimethyl sulfoxide to make a 150× stock solution. The stock solution was diluted in artificial perilymph (APL, Chen and Zhao, 2006) to

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make a 3× working solution before use. Rats were anesthetized with ketamine (50 mg/kg, im) and xylazine (6 mg/kg, im). The cochlea was surgically exposed, and two small holes were made on the basal turn and the apex. Approximately 50 µl of 3× working solution was pumped into the scala tympani in the basal turn through the hole within a 15-min period. The 3× solution was used in consideration of dilution in the cochlea. After 1-h incubation (from the beginning of the perfusion), the cochlea was removed and fixed in 10% formalin. After fixation for about 3 h, the cochlea was dissected. After the nucleus staining with propidium iodide (PI) (see below), the basilar membrane with the organ of Corti was mounted on a slide with ProLong Gold antifade reagent (Invitrogen Molecular Probes, Carlsbad, CA, P36934) for microscopy examination under a Zeiss (Oberkochen, Germany) Axiophot fluorescence microscope and/or a 510 Zeiss Confocal microscopes.

F-actin staining (for hair cell counting). Rats were deeply anesthetized with ketamine (50 mg/kg, im) and xylazine (6 mg/kg, im). The cochleae were removed, fixed in 10% formalin, and dissected after the fixation. The basilar membrane with the organ of Corti was stained with fluorescein isothiocyanate–labeled phalloidin (5 µg in 1 ml phosphate-buffered saline [PBS] containing 0.25% Triton X-100 and 1% bovine serum albumin) (Sigma) for 30 min at room temperature (Hu *et al.*, 2002). After the nucleus staining with PI (see below), the specimen was mounted on a slide with ProLong Gold antifade reagent (Molecular probes, P36934) for microscopy examination as above.

Nucleus staining (for apoptosis examination). PI is a DNA intercalating fluorescent probe and is used to trace the morphological changes in nuclei. After caspase staining or F-actin staining, the tissue was colabeled with PI. The specimen was incubated in PI solution (5 µg/ml in PBS, Sigma-Aldrich, cat# P4170) for 10 min and then mounted on a slide for microscopy examination as above (Hu *et al.*, 2002).

Statistical Analysis

For analysis of styrene distributions in the cochlea, one-way ANOVA followed by Bonferroni multiple comparison test was run. For analysis of cochlear impairments (hearing loss and hair cell loss) at different frequencies or locations to the exposure of styrene at different exposure intensities (dosage or duration), two-way ANOVA was used. For analysis of relationship between styrene exposure level and styrene concentration in whole blood, regression analysis was run. A p value ≤ 0.05 was considered to be statistically significant.

RESULTS

To determine kinetics of styrene in the blood, rats ($n = 3$) were exposed to styrene once a day for 6 days by oral gavage at a dose of 800 mg/kg body weight. The kinetics of styrene in the blood was measured on day 1 and day 6 following the styrene treatment and is presented in Figure 1A. On day 1, the styrene concentration in the blood increased rapidly during the first 30 min after the treatment and was maintained at a relatively steady level for the 6-h observation period (see open circles). On day 6, the kinetics of styrene in the blood (see filled circles) was similar to that observed on day 1. The results indicate that after an oral gavage treatment, the elevated styrene level remained relatively steady for about 6 h and then declined. The two additional samples collected at 12th and 24th hour after the exposure on day 6 indicated a complete elimination of styrene every day. The repeated treatments did not alter styrene concentration achieved in the blood.

To determine dose response in the blood after styrene exposure, 23 rats were divided into five groups (n = 3, 6, 6,

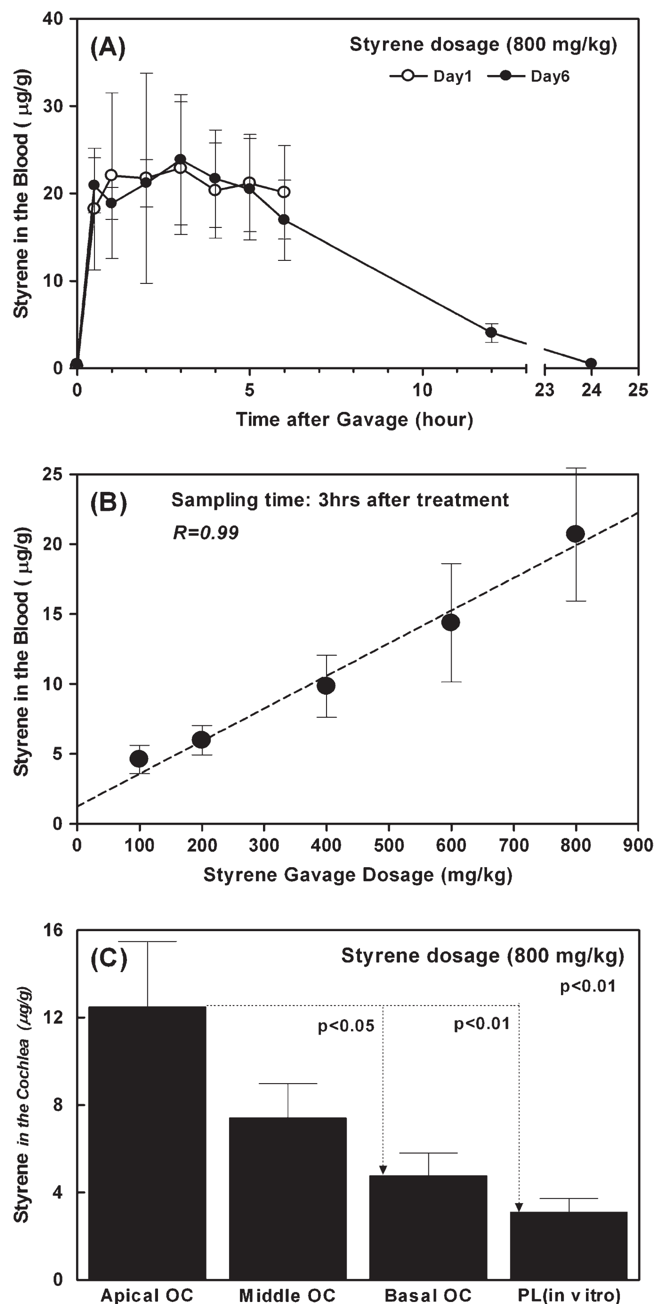

FIG. 1. Styrene levels in the body after an oral gavage. (A) Styrene concentrations (µg/g) in the blood as a function of time following an oral gavage at a dose of 800 mg/kg body weight. The rats ($n = 3$) were exposed to styrene once a day for 6 days. The blood samples (10 µl) were collected from the tail on day 1 (opened circles) and day 6 (filled circles). (B) Styrene concentrations (µg/g) in the blood as a function of styrene dosage. The blood samples were collected from the tail 3 h after the exposure. n = 3, 6, 6, 3, and 5 rats for the dosage groups of 100, 200, 400, 600, and 800 mg/kg, respectively. (C) Styrene concentrations (µg/g) in the cochlea. The cochlear perilymph was sampled from the isolated cochleae ($n = 14$) 3 h after an oral gavage at a dose of 800 mg/kg, and the cochlear tissues in different turns were then sampled by dissection. The difference between cochlear tissues from different turns and cochlear perilymph was significant ($p < 0.01$ by one-way ANOVA). *Post hoc* test (Bonferroni) showed a difference between apical turn and basal turn ($p < 0.05$) and between apical turn and perilymph ($p < 0.01$). Vertical bars are SEs.

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3, and 5) and exposed to styrene by an oral gavage at the following doses: 100, 200, 400, 600, and 800 mg/kg body weight. Figure 1B presents styrene concentrations in the blood (measured 3 h after the treatment) as a function of styrene dosage. Styrene concentration in the blood increased as the exposure level increased. A statistical significant linear regression ($p < 0.001$) indicated a linear relationship between styrene level in whole blood and styrene dosage and can be expressed as:

$$\mathrm{SLB} = 0.0234 \times D + 1.3$$

Here, SLB stands for styrene level in the blood (μg/g) and D is gavage dose (mg/kg).

To determine styrene levels in the cochlear perilymph and in the cochlear tissues, seven rats were exposed to styrene by an oral gavage at a dose of 800 mg/kg. The cochlear perilymph and the cochlear tissues in different turns were sampled 3 h after the treatment and are presented in Figure 1C. Styrene in the cochlear tissues was 12.5 ± 3.0 μg/g in the apical turn, 7.4 ± 1.6 μg/g in the middle turn, and 4.8 ± 1.0 μg/g in the basal turn. The styrene level in the cochlear perilymph was only 3.1 ± 0.6 μg/g. Since the perilymph samples might be contaminated with blood by inner ear bleeding from the cochlear aqueduct, perilymph was also sampled *in vivo* from the round window in three additional rats. The styrene concentration in these samples was 0.9 ± 0.1 μg/g. The difference of styrene distributions in the cochlea was significant ($p < 0.01$ by ANOVA). The differences between apical turn and basal turn ($p < 0.05$) and between apical turns and perilymph ($p < 0.01$) reached significance (*post hoc* test of Bonferroni). The styrene distribution in the cochlear tissues was confirmed in three additional rats in which the samples from each turn were pooled and then analyzed. Styrene levels in the pooled samples showed a similar pattern: apical (6.8 μg/g) > middle (3.6 μg/g) > basal turn (1.4 μg/g) as the individual samples. The lower styrene levels in the pooled samples may be due to the longer exposure time to the air due to repeated opening of the sample vial during pooled sample collection by cochlear dissection.

Figure 2A presents styrene-induced threshold shifts of ABR at different frequencies as a function of styrene exposure levels (measured 12 h after the last styrene treatment). The threshold shifts were styrene dose and frequency dependent with the largest threshold shift occurring at the midfrequencies (10–20 kHz). Two-way ANOVA showed significant difference between dosages ($p < 0.0001$) and between frequencies ($p < 0.01$). At frequencies of 10 and 20 kHz, each 100-mg/kg increase in styrene dose caused an extra 5-dB threshold shift (see dotted line). The threshold shifts, induced with the 3-week styrene exposure (1 per day, 5 days per week), were permanent. Figures 2B and 2C present threshold shifts measured 3 weeks after the last day of the exposure (stars), which are similar to those measured 12 h after the last treatment (opened circles).

The styrene-induced OHC loss was dose and location dependent, with OHC loss starting from the middle turn of

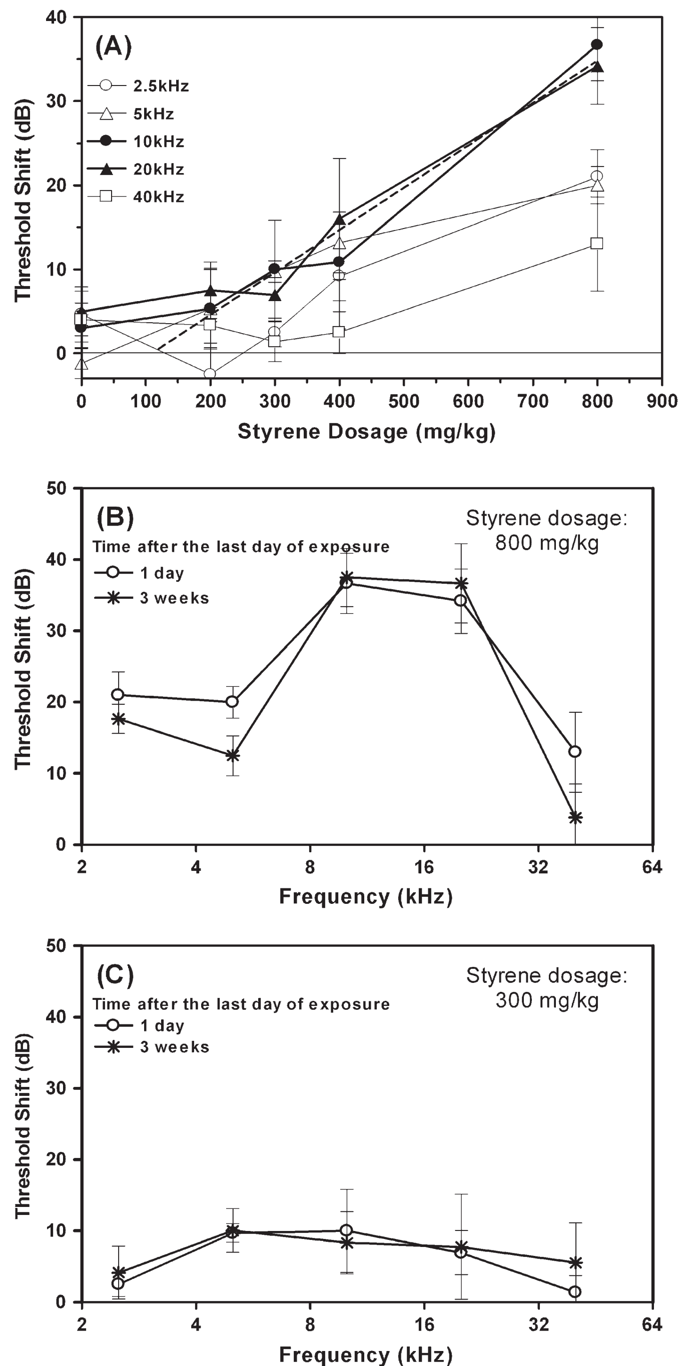

FIG. 2. Styrene-induced hearing loss. (A) Threshold shifts at different frequencies as a function of styrene dosage. The dashed line is a linear regression line of the threshold shifts at 10 and 20 kHz. (B) Threshold shifts determined 12 h (opened circles) and 3 weeks (stars) after the last day of the styrene exposure at a dose of 800 mg/kg as a function of frequency. (C) Threshold shifts 12 h (opened circles) and 3 weeks (stars) after the styrene exposure at a dose of 300 mg/kg as a function of frequency. The threshold shifts were determined by ABR recording. The rats ($n = 6$ in each group) were exposed to styrene by gavage at different dose once a day for 5 days per week for 3 weeks. Vertical bars are SEs.

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the cochlea (Fig. 3A). Two-way ANOVA showed significant difference between dosages ($p < 0.0001$) and significant difference along the basilar membrane ($p < 0.0001$). Figure 3B presents mean OHC losses in different regions as a function of styrene dosage.

To better understand the dynamics of styrene-induced hair cell loss, rats were exposed to styrene at a dose of 800 mg/kg once a day for varying periods, and hair cells were examined 12 h after the last treatment. Figure 4 presents OHC losses as a function of cochlear location in different groups receiving different periods of treatment. The styrene exposure for a period shorter than 3 days did not cause OHC loss (see stars). The 5-day exposure induced an OHC loss of about 1% (opened circles). Then, with the increase of exposure period, number of missing OHCs increased and the damaged area extended along the basilar membrane, especially toward the apical turn. Two-way ANOVA showed significant difference between treatment periods ($p < 0.0001$) and significant difference along the basilar membrane ($p < 0.0001$).

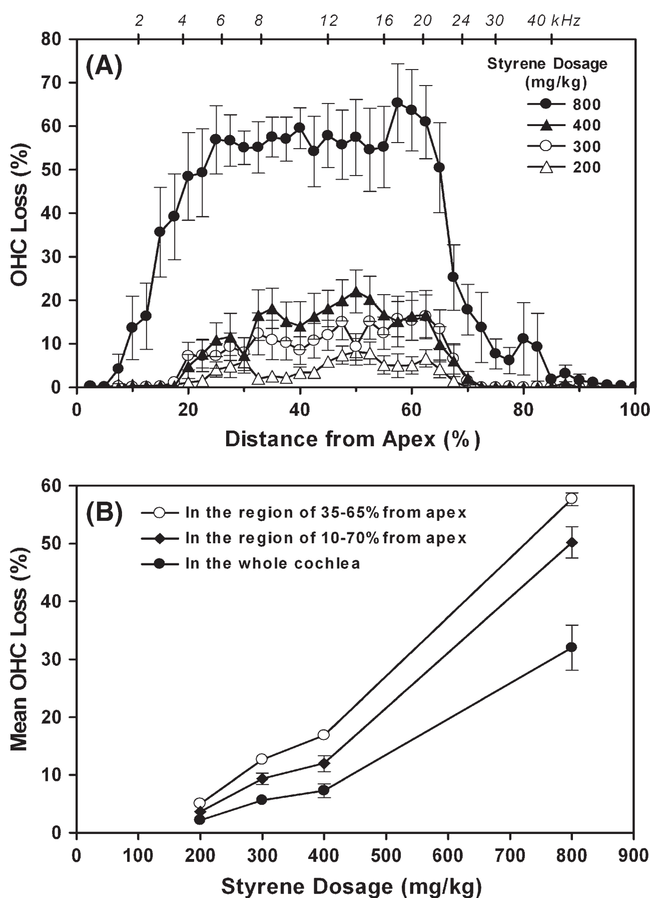

FIG. 3. (A) OHC losses after styrene exposure at different doses as a function of the basilar membrane. The rats were exposed to styrene by gavage once a day for 5 days per week for 3 weeks ($n = 6$, 9, 10, and 12 in groups exposed at doses of 200, 300, 400, and 800 mg/kg, respectively; (B) mean OHC losses in different area as a function of styrene dosage. Vertical bars are SEs.

As shown in Figure 4, significant OHC loss was observed only when the styrene exposure (800 mg/kg by gavage, 1 per day) exceeded 3–5 days. However, traumatic changes were already observed in Deiters cells after 3 days of the exposure. Figure 5 shows images from different cochlear regions (A: 45%; B: 56%; and C: 67% from the apex) in a rat receiving the treatment (800 mg/kg) for 3 days. Nuclei of all OHCs and IHCs look normal. However, nuclei of some Deiters cells in the third row were condensed (marked with "<"), indicating apoptotic cell death. Consistent with OHC loss, apoptotic Deiters cell death was cochlear location dependent. There were many nucleus-condensed Deiters cells in the cochlear region of about 45% from the apex (A) in this rat but only a few in its surrounding area (B) and none in the apical and basal turns (C). Figure 6 presents nucleus-condensed Deiters cells as a function of cochlear location in four rats exposed to styrene by gavage at a dose of 800 mg/kg (once per day) for 7 days. The results were consistent with the OHC losses shown above.

Figures 7A and 7B present images from a cochlear region of 48% from the apex in a rat being exposed to styrene by gavage at a dose of 800 mg/kg, once a day for 7 days. Figure 7A1 is a sectional picture showing relative positions of OHCs, Deiters cells, and Hensen cells at a cochlear location marked by the green line in A2. Figure 7A2 is a confocal image at a depth marked by the blue line in A1, showing normal nuclei of OHCs and Hensen cells. Figure 7B1 is the same image as A1. Figure 7B2 is a confocal image at a depth marked by the blue line in 7B1, showing condensed nuclei of Deiters cells (marked by "<"). The data indicate that while Deiters cells are injured by styrene exposure, OHCs and Hensen cells can be intact. Figures 7C and 7D are images from an intact area in the basal turn (78% from the apex) in the same cochlea as a control of normal Deiters cells (D2). Explanation is similar as that for A and B.

Death of OHCs was often through apoptotic pathway (condensed nucleus). To further determine mechanisms of the

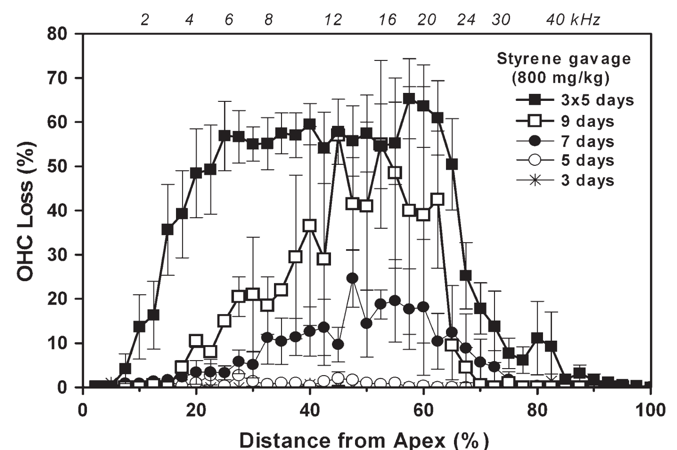

FIG. 4. OHC losses after styrene exposure at a dose of 800 mg/kg (by gavage) for varying periods as a function of the basilar membrane. Rats ($n = 4$, 3, 6, 3, and 6) were used in each group (3, 5, 7, 9 days, and 3 weeks). Vertical bars are SEs.

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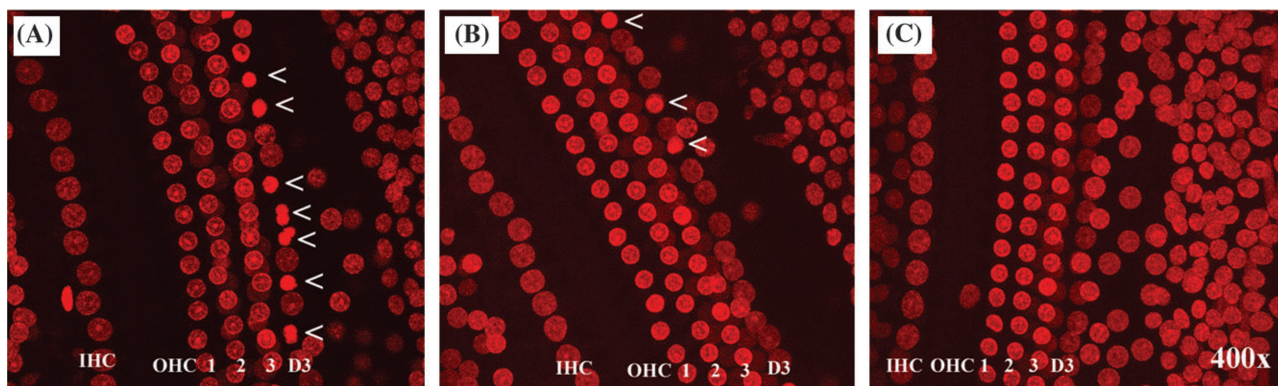

FIG. 5. Representative images showing nuclei of the cochlear cells in the early stage of styrene exposure. The rat was exposed to styrene by an oral gavage at a dose of 800 mg/kg once a day for 3 days. (A) 45% from the apex. Seven condensed nuclei of Deiters cells are marked with "<". (B) 56% from the apex. Three condensed nuclei of Deiters cells are marked with "<". (C) 67% from the apex. No condensed nucleus is seen. D3 Deiters cells of the third row.

apoptotic cell death pathway in the OHCs, active caspases-9 and 8 were examined by using carboxyfluorescein-labeled FMK-peptide inhibitors of caspases by *in vivo* incubation. Both active caspases-9 and 8 were observed in the OHCs with condensed nuclei. Figure 8A1 is a representative image showing an OHC (pointed with an arrow) with a condensed nucleus and activated caspase-9 staining (green). Other OHCs in the image had normal nuclei without detective caspase. Figure 8A2 presents the positive -stained OHCs as a function of cochlear location. Figure 8B1 is a representative image showing an OHC (pointed with an arrow) with a condensed nucleus and activated caspase-8 staining (green). Similar as A1, other OHCs in the image had normal nuclei without detective caspase. Figure 8B2 presents the positive-stained OHCs as a function of cochlear location. The data indicate that both the mitochondrial-dependent pathway (caspase-9 as the initiator caspase) and the death receptor–dependent pathway (caspase-8 as the initiator caspase) were involved in the styrene-induced OHC apoptosis.

DISCUSSION

Styrene Exposure

In many studies of styrene ototoxicity, subjects were exposed to styrene by inhalation because workers are usually exposed by this way. This experiment was designed to study mechanisms underlying styrene ototoxicity and the oral gavage approach was used (Gagnaire and Langlais, 2005). Although the subjects were exposed to styrene by gavage only once per day, a single oral gavage treatment induced a relatively steady styrene level in the blood for about 6 h (see Fig. 1A).

Styrene Blood Levels and Induced Toxic Effects in the Cochlea by Gavage and by Inhalation Exposures

Styrene levels in the blood in this study by gavage treatment were directly proportional to the exposure levels (see Fig. 1B). The styrene concentrations in the blood were about 2–3% of the oral gavage dose on a weight basis. A blood styrene level of 20.7 μg/g was obtained after an oral gavage at a dose of 800 mg/kg. This is similar to the blood level of 22.8 μg/g obtained in rats via inhalation exposure at 1000 ppm (Lataye *et al.*, 2003). A similar relationship of blood styrene level versus inhalation exposure level was also found at a much higher exposure level in rats (Campo *et al.*, 1999). Inhalation exposure of styrene at 1750 ppm produced a blood level of 37.5 μg/g (Campo *et al.*, 1999). Although the gavage exposure at a dose of 800 mg/kg in this study produced a similar blood styrene level (20.7 μg/g) as the inhalation exposure at 1000 ppm (22.8 μg/g, Lataye *et al.*, 2003), the gavage styrene exposure induced a much higher threshold shift (35 dB at 10 and 20 kHz) than the threshold shift of 16 dB in a similar frequency region induced by an inhalation exposure at 850 ppm (Loquet *et al.*, 1999). In contrast to the linear blood level/exposure relationship observed in this study, Ramsey and Young (1978) and subsequent analyzes (Ramsey and Andersen, 1984) showed

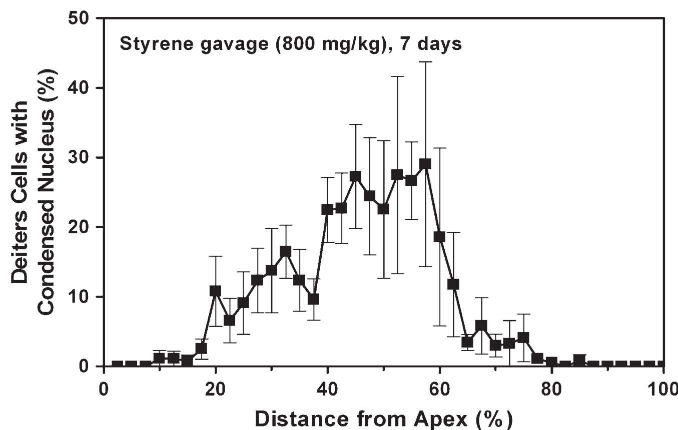

FIG. 6. Deiters cells with condensed nucleus after styrene exposure by an oral gavage at a dose of 800 mg/kg once per day for 7 days. $n = 4$ rats. Vertical bars are SEs.

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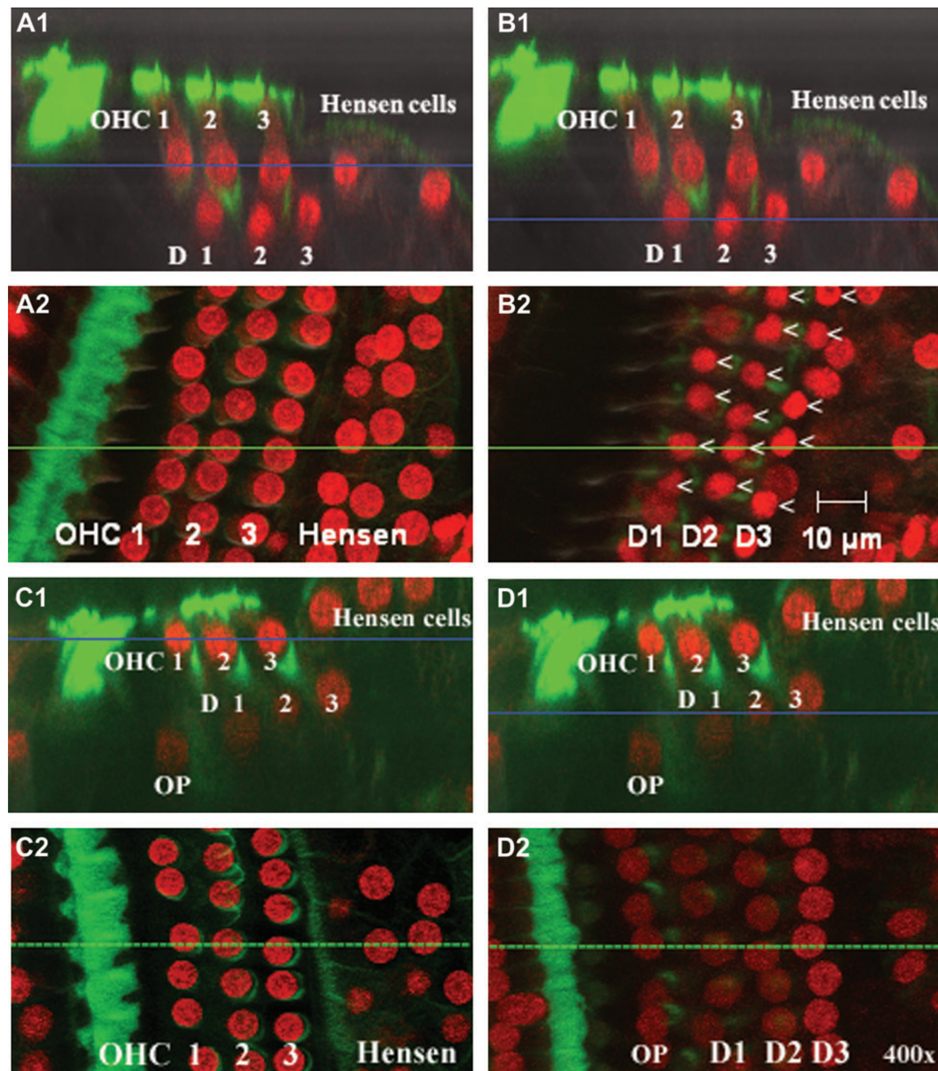

FIG. 7. Representative images showing condensed nuclei of Deiters cells, but with normal OHCs and Hensen cells, after styrene exposure by gavage at a dose of 800 mg/kg once a day for 7 days. A1 and B1 show positions of OHCs, Deiters cells, and Hensen cells. The blue lines indicate depth of images in A2 and B2. The green lines in A2 and B2 indicate location of the section in A1 and B1. Condensed nuclei are marked with "<". Images of A and B are from the middle turn (48% from the apex). Images of C (C1 and C2) and D (D1 and D2) are from the basal turn (78% from the apex) in the same cochlea as a control of Deiters cells. Explanations of C and D are similar as in A and B. OHC1, 2, 3: OHCs of row 1, row 2, and row 3; D1, D2, and D3: Deiters cells of row 1, row 2, and row 3; OP: outer pillar cells.

a nonlinear relationship between blood level and external exposure concentration. Styrene metabolism appeared to be saturated at inhalation levels above 200 ppm. As exposure levels exceeded 200 ppm, blood concentrations of styrene were higher than those estimated. For example, a blood level of 25 μg/ml was observed after exposure to styrene at 600 ppm, but a blood level of 64 μg/ml was measured after exposure at 1200 ppm. The saturation of styrene metabolism may reflect the smaller capacity of relevant enzymes in the lung than in the liver. In the other aspect, given a similar blood level of styrene by gavage and inhalation, a difference in blood levels of styrene metabolites may exist. It would be useful to measure the kinetics of styrene metabolites produced in future studies in order to assess the role that the metabolites play in ototoxicity.

Styrene Blood Levels in Workers in Fiber-Reinforced Plastics Industry

Styrene levels in the blood of workers in fiber-reinforced plastics industry ranged up to about 2 μg/g (Mizunuma *et al.*, 1993). The lowest styrene dosage (200 mg/kg) used in this study to induce hearing loss produced a blood level of 6.0 ± 1.0 μg/g (see Fig. 1B), which destroyed OHCs of about 5% in the cochlear region of 20–70% from the apex (see Fig. 3). Although styrene levels in the workers in fiber-reinforced

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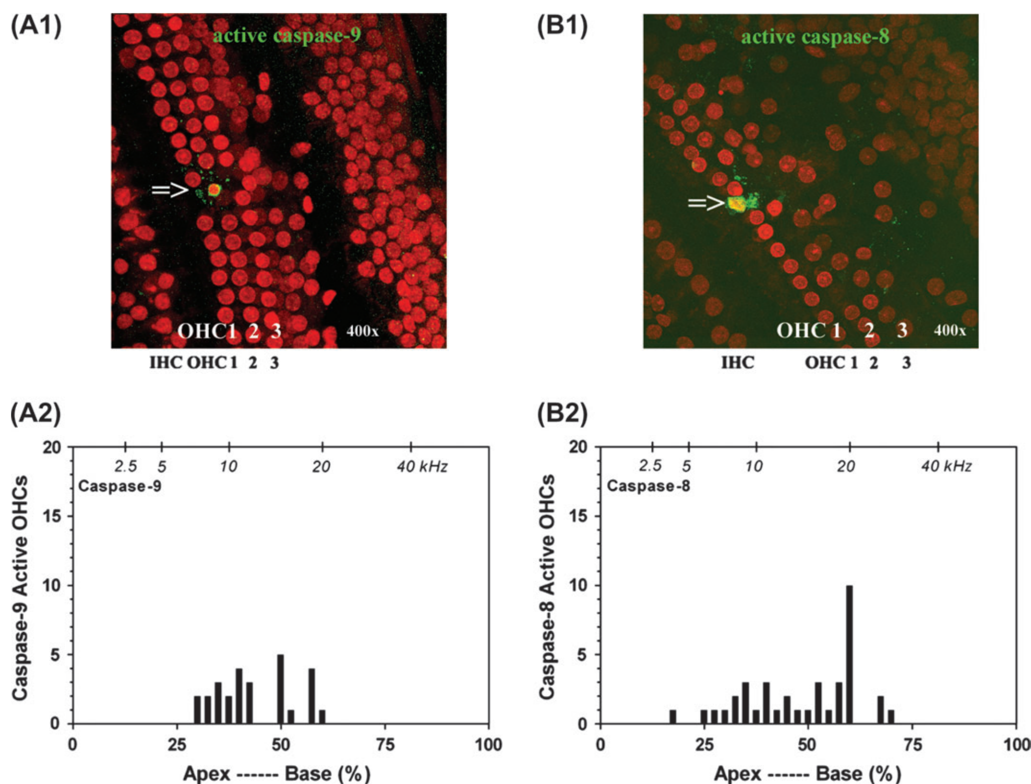

FIG. 8. (A1) A representative image showing condensed nucleus of an OHC (pointed with the arrow) with detected active caspase-9 (green staining). (A2) Distribution of the caspase-9 active OHCs in the cochlea. (B1) A representative image showing condensed nucleus of an OHC (pointed with the arrow) with detected active caspase-8 (green staining). (B2) Distribution of the caspase-8 active OHCs in the cochlea. The rats ($n = 6$) were exposed to styrene by gavage at a dose of 800 mg/kg once a day for up to 7 days.

plastics industry are much lower than 6.0 μg/g, it is unclear whether a risk may exist for lifelong exposures.

The Mechanism Underlying Selective Styrene Ototoxicity

Styrene exposure caused hearing loss starting in the midfrequency region and correspondingly hair cell loss starting in the middle turn. This study revealed that styrene concentration in the cochlear tissue in the basal turn was lower than that in the middle turn, which is consistent with the physiological and histological observations. Thus, the low susceptibility of OHCs in the basal turn to styrene exposure is because of the low-level styrene distribution in the basal turn tissue.

Styrene is proposed to enter the cochlear tissue through the vascular system (Campo *et al.*, 1999; Lataye *et al.*, 2001). Styrene concentration in the cochlear perilymph (3.1 ± 0.6 μg/g) was lower than that in the cochlear tissue (see Fig. 1C). The cochlear perilymph was collected from the isolated cochleae (for a pure cochlear fluid without cerebrospinal fluid (CSF)). However, the samples might be contaminated with blood since inner ear bleeding from the cochlear aqueduct was observed in some isolated cochleae. The real styrene level in the cochlear perilymph might be lower. Indeed, when the cochlear perilymph was sampled *in vivo* from the round window, only 0.9 ± 0.1 μg/g styrene concentration was measured. We believe that the auditory sensory epithelium (the organ of Corti) is contaminated with styrene from the blood (Campo *et al.*, 1999) and the styrene in the cochlear tissue is equilibrated with cochlear perilymph (largely driven by the lipid contention in each compartment). The styrene washout efficiency in the basal turn may be relatively higher than that in the apical and middle turns, because of the relatively larger space of scala vestibuli and scala tympani, which is directly connected to the CSF by the cochlear aqueduct in the basal turn. Although styrene concentration in the apical turn was higher than that in the middle turn, less OHC loss was observed in the apical turn. It is known that auditory cells in the apical turn are generally less susceptible to ototoxic agents such as chemical asphyxiants and some cancer chemotherapeutic agents (e.g., McWilliams *et al.*, 2000; Tawackoli *et al.*, 2001). It was reported that the antioxidant glutathione in OHCs in the apical turn was higher than that in the basal turn (Sha *et al.*, 2001). The lower susceptibility to styrene in the apical turn could result from the high level of antioxidants. It has to be mentioned that with the increase of styrene exposure level, the cochlear-damaged area extends more toward the apical turn. The interaction between the styrene gradient in the cochlea (high in the apex and low in the base) and the hair cell vulnerability gradient (high in the

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base and low in the apex) results in less damage in both the basal and apical turns.

It was reported that styrene-induced hearing loss was by tissue intoxication, and the outer sulcus was used as an intoxication route to reach the organ of Corti (Campo *et al.*, 1999). The histological findings demonstrated that supporting cells are the first targets of the solvent. Then, the OHCs of the third row (OHC3) were disrupted, followed successively by OHC2 and OHC1 (Campo *et al.*, 2001). Further observation of the study indicated that disruption of Hensen cells occurred earlier than Deiters cells. This study does not support this order of cell death. Deiters cells seemed more susceptible to styrene than Hensen cells (see Fig. 7). In fact, in our recent experiment Hensen cells were observed to be intact after all OHCs and Deiters cells were destroyed.

Hair Cell Death Pathway After Styrene Exposure

Ototoxicity of aromatic solvents was related to their structures (Gagnaire and Langlais, 2005). Generally, to be ototoxic, there must be a single side chain without branch. Styrene is an aromatic solvent with an unsaturated single side chain, which has a similar ototoxicity as ethylbenzene, which has a saturated single side chain. However, mechanisms underlying ototoxicity of the aromatic solvents may be much more complicated, since their metabolites in the body may also be involved in toxic activity. For example, active dihydroxylated metabolites of ethylbenzene (ethylhydroquinone and 4-ethylcatechol) was reported to be involved in the mechanism of carcinogenesis by ethylbenzene (Midorikawa *et al.*, 2004).

Styrene in the body is mainly metabolized, by cytochrome P450, to SO, which is considered to be the principal reactive and genotoxic intermediate of styrene (Green *et al.*, 2001; Hynes *et al.*, 1999; Johanson *et al.*, 2000; Kim *et al.*, 1997). SO forms covalent adducts to DNA, RNA, and proteins (Koskinen *et al.*, 2001a,b; Liu *et al.*, 2001; Vodicka *et al.*, 2001) and is further proposed to have a direct oxidative stress effect on cells (Marczynski *et al.*, 2000), possibly through the depletion of the major cellular antioxidant glutathione as well as other non-protein sulfhydrils (Coccini *et al.*, 1996; Dypbukt *et al.*, 1992; Katoh *et al.*, 1989; Trenga *et al.*, 1991; Vettori *et al.*, 2005). SO was reported to induce apoptosis in neurons through mitochondrial-dependent pathway (Boccellino *et al.*, 2003; Daré *et al.*, 2002, 2004).

It is known that there are two fundamentally different forms of cell death, apoptosis and necrosis (Vermeulen *et al.*, 2005). Apoptotic cell death has been observed in the cochlea due to aging, disease, and traumatic exposures (e.g., Hu, 2007; Hu *et al.*, 2006; Labbe *et al.*, 2005; Nakagawa *et al.*, 1998; Nevado *et al.*, 2006; Xu and Huang, 2003). In this study, the majority of dying cochlear cells after styrene exposure were characterized with condensed nucleus (recognized as apoptosis), but only a few dying cells were characterized with swollen nucleus (recognized as necrosis). Thus apoptotic cell death appeared to be the main cell death pathway in the cochlea after styrene exposure. Apoptosis is a programmed cell death process, which is further divided into caspase-dependent apoptosis and caspase-independent apoptosis (Vermeulen *et al.*, 2005). Pathways to caspase activation during apoptosis can be generally categorized as the mitochondria-mediated pathway using caspase-9 as the initiator caspase and the death receptor-mediated pathway using caspase-8 as the initiator caspase (Vermeulen *et al.*, 2005; Wang *et al.*, 2005). In the early stage of styrene exposure (< 3 days), condensed nuclei were observed in Deiters cells, indicating apoptosis. When the styrene treatment (800 mg/kg/day by gavage) exceeded 3 days, condensed nuclei were observed in the OHCs. Both caspases-9 and 8 were detected, indicating that both the mitochondria-mediated pathway and the death receptor–mediated pathway were involved in the styrene ototoxic effect.

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