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Low-Level Toluene Disrupts Auditory Function in Guinea Pigs

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Toluene appears to have adverse effects on the human auditory system, but it is difficult to estimate its potency since it is commonly present in the workplace in combination with noise exposure; workplace noise exposures are often highly variable. Studies designed to assess toluene ototoxicity specifically have been limited to high-dose studies in a single laboratory animal model, the rat. Here permanent hearing loss has been observed at concentrations of 1000 ppm toluene and greater after inhalation exposure for 5 days, 6 h/day. The OSHA threshold limit value for toluene is only 100 ppm. The current study focuses on the onset of toluene ototoxicity acutely in the guinea pig and in adducing a mechanism of effect. In this study, evidence is presented for the impairment of auditory function by toluene in the guinea pig, at a concentration substantially lower than that used for studying permanent impairment in the rat. The impaired function was correlated with reduced energy metabolism in outer hair cells. Assessment of auditory function was made using distortion product otoacoustic emissions (DPOAE) with subsequent measurement of succinate dehydrogenase (SDH) staining density in hair cells using surface preparations. Temporary disruption of auditory function in guinea pigs is seen in subjects exposed to 250, 500, and 1000 ppm toluene for 8 h/day, 5 day/week for 1 and 4 weeks. Concentrations as low as 250 ppm toluene were able to disrupt auditory function acutely in the guinea pig, and 500 and 1000 ppm toluene produced greater acute dysfunction. SDH staining suggests that reduced enzyme activity in the midfrequency region of the cochlea occurs acutely following toluene exposure. Although the auditory dysfunction progressed between 1 and 4 weeks of exposure, a permanent loss did not develop for these subjects and hair cell death was not seen. The current study identifies early evidence of auditory system impairment in the guinea pig at low toluene concentration and evidence for impairment of energy production in hair cells. While even a transient auditory impairment has implications for workplace safety, additional study on the transition from such acute effects to permanent impairment is essential. © 2000 Academic Press

Key Words: **Toluene; hearing loss; ototoxicity; cochlea; DPOAE; surface preparation; guinea pig; succinate dehydrogenase staining.**

During the last decade, workplace exposures to organic solvents, like toluene and simultaneous noise, have been shown to cause hearing loss. Morata *et al.* (1993), for example, reported that workers from printing and paint manufacturing industries exposed to approximately 100–365 ppm toluene and noise between 88 and 93 dBA were far more likely to suffer significant hearing loss than matched reference groups exposed to noise only or solvent only. The workers revealed an odds ratio for hearing impairments of approximately 4 for the noise exposed group, 5 for the mixed solvent group, and nearly 11 for subjects receiving combined exposure to noise and solvents compared to controls. Whereas the toluene concentration in Morata's study exceeded the U.S. PEL values, Abbate *et al.* (1993) determined that 12–14 years of 97 ppm toluene exposure resulted in significantly elevated latency in the brainstem auditory evoked potential among normal hearing workers. The initial component of the brainstem response, which is indicative of cochlear function, was especially affected, demonstrating subclinical changes in auditory function in a solvent-exposed population.

In addition to toluene, exposures to other organic solvents have resulted in measurable auditory dysfunction. Szulck-Kuberska *et al.* (1976) demonstrated loss in auditory sensitivity among workers studied who were exposed to trichloroethylene (TCE). Auditory impairments were more common among workers exposed to TCE for a longer duration. Additional evidence of solvent-induced hearing loss among workers has been reported for carbon disulfide and noise (Morata, 1989), styrene (Muijser *et al.*, 1988), and mixed solvent exposure (Jacobsen *et al.*, 1993; Bergstrom and Nystrom, 1986).

Along with workplace exposure, solvent ototoxicity has been reported when individuals were exposed to products that contain toluene. For example, actual hearing loss has been reported in glue sniffers who expose themselves to high toluene doses (Ehyai and Freemon, 1983). When toluene's ototoxicity was evaluated within animal models, Sullivan *et al.* (1988) found both a loss of auditory sensitivity and a loss of outer hair cells. Toluene has also demonstrated an ability to potentiate the effects of noise. Studies such as the one by Johnson and colleagues (1988) showed an additive effect for successive exposure to toluene and noise in the rat. Additionally, Layate and Campo (1997) have found that simultaneous exposure to toluene and noise, each of which alone resulted in hearing loss in the rat, generated an auditory deficit that ex-

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ceeded the summated losses for successive exposure to toluene and noise.

The characterization of toluene's ototoxicity, specifically in the absence of noise exposure, has entailed experimental studies performed in the rat. Most published studies suggest that toluene produces a loss in midfrequency auditory function, but exposures have been done at concentrations considerably higher than permissible exposure limits for humans. For example, Campo *et al.* (1997) found that 4 weeks of 1000 ppm toluene (6 h/day, 5 days/week) resulted in cochlear lesions. Layate and colleagues (1999) were able to generate significant hearing loss and outer hair cell loss, but with an exposure of 1750 ppm toluene (6 h/day, 5 days/week, 4 weeks). In two short-term studies, Johnson and Canlon found that 3 days of 1400 ppm toluene exposure caused no acute hair cell loss (1994b) but did result in lower acute distortion product otoacoustic emissions (DPOAE) amplitudes and elevated acute auditory brainstem response thresholds (1994a), which developed into permanent threshold shifts. Rebert *et al.* (1989) showed that concentrations as low as 500 ppm toluene could have effects on the amplitudes of the late portion of click-evoked brainstem auditory-evoked responses after 2 h.

Even though toluene ototoxicity has been reported, the absence of data at levels approaching permissible human workplace exposure levels raises some concern about the utility of such laboratory work for understanding mechanisms of human solvent ototoxicity. The determination that toluene exposures produces ototoxicity in a second species would increase confidence that such a relationship has some generality. The identification of a sensitive laboratory species and the identification of a mechanism of ototoxicity may be helpful in determining human susceptibility to toluene ototoxicity. This study monitors the onset and progression of toluene ototoxicity in the guinea pig, the most commonly used nonhuman mammal in hearing research. To undertake this investigation, DPOAEs (Hofstetter *et al.*, 1997; Trautwein *et al.*, 1996) and succinyl dehydrogenase (SDH) staining (Chen *et al.*, 1999) were used to determine if toluene acts on outer hair cells (OHCs) (Johnson and Canlon, 1994a; Campo *et al.*, 1997; Liu and Fechter, 1997) or other components of the auditory system.

METHODS

Subjects

Thirty-two pigmented guinea pigs (approximately 60 days old) were either obtained from Kuiper Rabbit Ranch or bred from animals obtained from Kuiper Rabbit Ranch at the University of Oklahoma Health Sciences Center (OUHSC) main animal facility. All animals were housed in an OUHSC animal facility, which is registered with the U.S. Department of Agriculture and inspected semiannually by the members of the Institutional Animal Care and Use Committee (IACUC). All procedures regarding the use and handling of animals were reviewed and approved by the IACUC serving the University of Oklahoma Health Sciences Center. Prior to use, subjects were housed under a 12:12-h light–dark cycle with free access to food and water with a room temperature controlled at 22°C. Weight gain was measured weekly as a marker of general toxicity.

General Experimental Protocol: Experimental Design and Group Identification

This study evaluated both the acute and persistent effects of toluene on the auditory system of the guinea pig after 1 and 4 weeks of exposure. Exposure conditions for the present study were selected to span a broad range up to a level that produces ototoxicity in the rat (Campo *et al.* 1997). In the present study, eight guinea pigs were randomly assigned to each of four treatment groups. These included a control group that was exposed to clean air within exposure chambers and experimental subjects that received 250, 500, and 1000 ppm toluene by inhalation for 8 h/day for 5 days. A within-subjects design was employed whereby DPOAE assessment was undertaken before inhalation exposure and again following exposure. At the conclusion of the toluene exposure and DPOAE assessment, the 250 ppm toluene subjects were discontinued. Six of the eight 1000 and 500 ppm subjects were allowed to recover for 3 days, at which time DPOAEs were recorded once again. Since auditory function returned to the preexposure level after this recovery period, toluene exposures were resumed for control ($n = 6$) and 500 ppm subjects ($n = 4$) for 8 h/day, 5 days/week for an additional 3 weeks. For each exposure condition, upon completion of DPOAE assessment, cochleae were obtained from a limited number of subjects for comparison of functional and histological data.

Toluene Inhalation Exposure Protocol

All inhalation exposures were performed in freely moving animals placed in a wire-mesh cage within a custom-built glass chamber (0.3 m diameter × 0.6 m long). Evaporating technical-grade toluene that was metered into a heated round-bottom flask generated toluene vapor. A peristaltic pump (Masterflex 7521–40, Cole-Parmer Instrument Co., Vernon Hills, IL) was used to control toluene delivery onto glass beads within the round-bottom flask. A thermostatically controlled heating mantle (Cole-Parmer 89000 electronic heater) controlled evaporation rate. Lab air passed through the round-bottom flask forcing toluene vapor into the chamber. A secondary lab air supply from an oil-less compressor was combined with the toluene vapor flow to generate the desired toluene concentration prior to entering the chamber. The toluene vapor and secondary air flow rate provided 16 air changes each hour. Toluene concentration was monitored continuously using an infrared vapor analyzer (Miran 203, Miran, Foxboro, MA). The sound level in the exposure chamber was 60 dB(A) and was below 60 dB_{lin} for octave bands centered above 2 kHz as measured with a Quest (Marlboro, MA) sound level meter with air supply flowing through the exposure chamber. No food or water was available during the exposure period.

Cubic Distortion-Product Otoacoustic Emission (CDP)

Measurements of the CDP-gram and input/output functions were made using two sound sources for generation of primary tones (F_1 and F_2) coupled to an Etymotic Research (Elk Grove Village, IL) ER 10B low noise microphone. The calibration of the ER 10B at high frequencies was achieved using a Larson Davis 2200C Preamp PC (Larson Davis, Provo, UT) with $\frac{1}{4}$-inch microphone fitted with a probe tube that also fits into the coupler. The primary-tone signals were produced by a dual-channel frequency synthesizer and attenuated under computer control by matched Tucker Davis Technologies (Gainesville, FL) programmable attenuators (PA4) and transduced by two speakers (Vifa D25AG-05–06, Speakerlab, Seattle, WA). The CDP was measured in all guinea pigs for six primary frequency pairs between 6 and 24 kHz. The size of the CDP was maximized by using an F_2:F_1 ratio of 1.28. For input/output function measurements, the intensity of the primaries was varied in 5-dB steps with the sound level of F_1 ($L_1 = 50$ to 80 dB SPL) and the level of F_2 ($L_2 = L_1$-10). Recordings were averaged for 1.97 s.

Preexposure Screening and Determination of Baseline.

Subjects were anesthetized by an im injection of ketamine (30 mg/kg) and xylazine (5 mg/kg) and CDP measurements were made in a double-walled sound attenuating room. The external ear canal was inspected otoscopically for

infection and any significant cerumen was carefully removed. The anesthetized animal was placed on a temperature-controlled surface to maintain normal body temperature. Using a micromanipulator, the coupler containing the sound source and measuring microphone probe was placed into the ear canal. The angle of the micromanipulator was not changed to ensure that the probe position would be repeatable between sessions with each subject.

Posttoluene Exposure Screening

For measurements of toluene ototoxicity acutely following exposure, subjects were anesthetized (as above) immediately following the final toluene inhalation exposure, the external ear canal was inspected otoscopically, and testing was performed within 5 min of exposure using the same protocol as in preexposure screening and determination of baseline (see above).

For measurements of CDP recovery, subjects were allowed 3 days in the animal care facility without solvent exposure and with free access to food and water. On the third day, CDP amplitude was measured using the same protocol as used for preexposure measurements (see above).

Histology

Histological sections of the cochlea were made to evaluate loss and damage to hair cells as determined by SDH staining density (Chen *et al.*, 1999) in two randomly chosen subjects from each exposure condition endpoint (e.g., 250, 500, and 1000 ppm 1 week; 500 and 1000 ppm 1 week plus 3-day recovery; 500 and 1000 ppm 4 weeks; and 500 and 1000 ppm 4 weeks plus 3-day recovery). The animals were decapitated while deeply anesthetized after CDP recording. Both the round and oval windows were opened and the apex of the cochlea was drilled open to facilitate perfusion. The cochleae were perfused with SDH incubative solution (0.05 M sodium succinate, 0.05 M phosphate buffer, and 0.05% TetraNitro Blue Tetrazolium) and immersed in the solution for 1 hour (37°C) (see Wang *et al.*, 1990; Zhai *et al.*, 1998). The cochleae were then fixed with 10% formalin for at least 2 days. After fixation, the cochleae were decalcified in 10% EDTA solution containing 5% glutaraldehyde for 3 days or longer as needed. Cochlea microdissection was accomplished under a light microscope with images stored using a frame grabber under 10× magnification. Surface preparations of the organ of Corti were evaluated to determine SDH staining density (via computer-generated density readings) in sensory cells in subjects taken from each exposure group. SDH staining density was plotted as a function of location along the basilar membrane to permit a comparison between functional impairment and SDH staining density. This was achieved using Scion Image software based upon established cochlear maps relating cochlear location and frequency (Wada *et al.*, 1998). Hair cell loss was quantified by counting of all visibly present cells within each section obtained with the Scion imaging software.

Statistical Analysis

CDP amplitude data are analyzed by a split-plot factorial ANOVA (NCSS 2000). Treatment (control vs toluene groups) is analyzed between subjects while tone frequency and time relative to toluene exposure serve as the repeated measures within subjects. Therefore, the most important source of variance from the perspective of the hypotheses addressed is the interaction term of treatment × test day with respect to toluene exposure. Post hoc analysis was performed by Scheffe's multiple-comparison test.

RESULTS

CDP-grams at High Sound Intensity

The CDP-gram provides a general assessment at the effect of toluene on CDP output across multiple frequencies at one stimulation level. Since suprathreshold tone intensities are used, normal outputs should be significantly above the just-detectable 3 dB CDP response. The CDP response data showed that 250, 500, and 1000 ppm toluene all significantly altered the CDP amplitude compared to the control.

Figure 1 compares the effect of five daily toluene inhalation exposures of 0, 250, 500, and 1000 ppm on CDP-gram amplitude for the stimulation level of $F_1 = 80$ dB and $F_2 = 70$ dB. The CDP-gram demonstrates the minimal effect of toluene on the function of the outer hair cells. At this level of effect, frequency specificity is not seen. Figure 1A demonstrates that CDP outputs for all groups were equivalent prior to toluene exposure. Further, CDP output was stable in control subjects over the 7-day period during which toluene exposures were performed in parallel groups (see Fig. 1B). CDP amplitudes averaged 23 dB above the noise floor for these control subjects across test frequencies.

Figure 1C represents the CDP-gram generated by the 250 ppm toluene subjects. A loss of CDP amplitude of approximately 5–10 dB occurred at all test frequencies. At 500 ppm (see Fig. 1D), a loss of approximately 15 dB occurred at all frequencies tested with no one frequency having a greater loss than any other. When the toluene concentration increased to 1000 ppm, the loss of CDP was equivalent to that found for 500 ppm, averaging 10–15 dB loss across all frequencies (see Fig. 1E). Therefore, 500 ppm may represent a saturation point for acute loss of CDP by toluene exposure. There were no obvious signs of general toxicity (e.g., loss of weight) observed in the toluene-treated subjects.

When the effects of toluene exposures on acute auditory threshold were compared statistically, each dosage was found significantly different from the controls but not from one another. Prior to toluene exposures, a repeated-measures ANOVA revealed no significant difference between groups ($F_{[3,28]} = 0.39$, $p = 0.763$) or for the group–frequency interaction ($F_{[15,140]} = 0.99$, $p = 0.465$). However, when CDP amplitudes were compared within subjects before and following toluene exposure, a significant treatment × day interaction ($F_{[3,28]} = 3.82$, $p = 0.021$) was found. Scheffe's post hoc analysis revealed significant difference for each toluene treatment from the preexposure CDP measurement to the posttest (250 ppm, $p = 0.02$; 500 ppm, $p = 0.002$; and 1000 ppm, $p = 0.02$), but no shift in the CDP-gram in the control subjects ($p = 0.99$).

Relationship between Dosage and Frequency as Measured with CDP Input/Output Functions

While the CDP-gram provides an index of the effects of toluene on maximal CDP amplitudes, input/output functions are helpful in determining stability of differences between treated and control subjects by tracking CDP output growth as stimulus intensity increases from the lowest level that provides a just-detectable CDP of 3 dB above the noise floor to sound levels producing a maximal CDP. This portion of the study shows that toluene exposure reduces CDP amplitude across

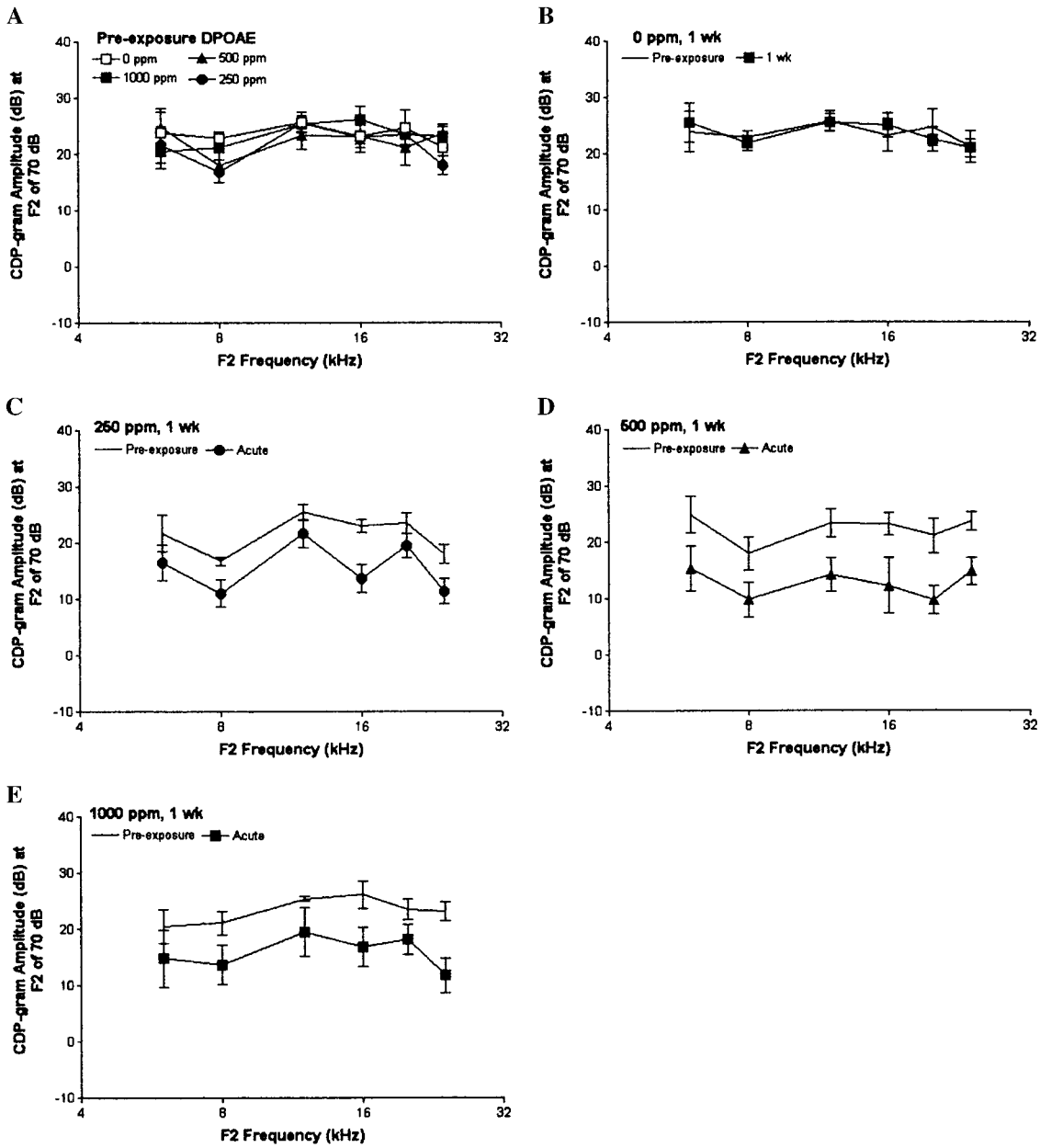

FIG. 1. DP grams of 1-week toluene inhalation exposures. Measurements made at $F_2 = 6$, 8, 12, 16, 20, and 24 kHz at $F_1 = 80$ dB and $F_2 = 70$ dB in guinea pigs (means ± SE). (A) Compares the preexposure CDP output of all groups; 0 ppm (□), 250 ppm (●), 500 ppm (▲), and 1000 ppm (■). (B) The change in CDP output in untreated animals over the parallel period of toluene exposure. Preexposure (no symbol) and postexposure (solid square) are shown. (C–E) Three CDP measurements are shown: treated animal preexposure (no symbol) and immediately after (solid symbol) toluene exposure (8 h/day for 5 days). Three dosages are shown; (C) 250 ppm (●), (D) 500 ppm (▲), and (E) 1000 ppm (■).

multiple sound intensities with the effect being more pronounced as the toluene concentration increased from 250 to 1000 ppm. With increasing toluene concentrations, sound levels required to generate the smallest detectable CDP increase and the peak CDP output decreases, especially for frequencies in the middle of the frequency range tested.

Figures 2–5 show the CDP input/output functions for subjects exposed to air and each toluene level for 5 days, 8 h/day and tested acutely on the final exposure day. Data that did not generate at least a 3-dB output was not considered to be an accurate measurement. For the control animals (see Fig. 2), the CDP output at all frequencies remains stable over the 1-week test period. The difference in CDP output observed in control subjects from pretest to posttest was ±2.0 dB over the 30-dB tone stimulation range represented in each input/output function.

For the animals exposed to 250 ppm toluene (see Fig. 3), the postexposure CDP amplitudes are reduced from the preexpo-

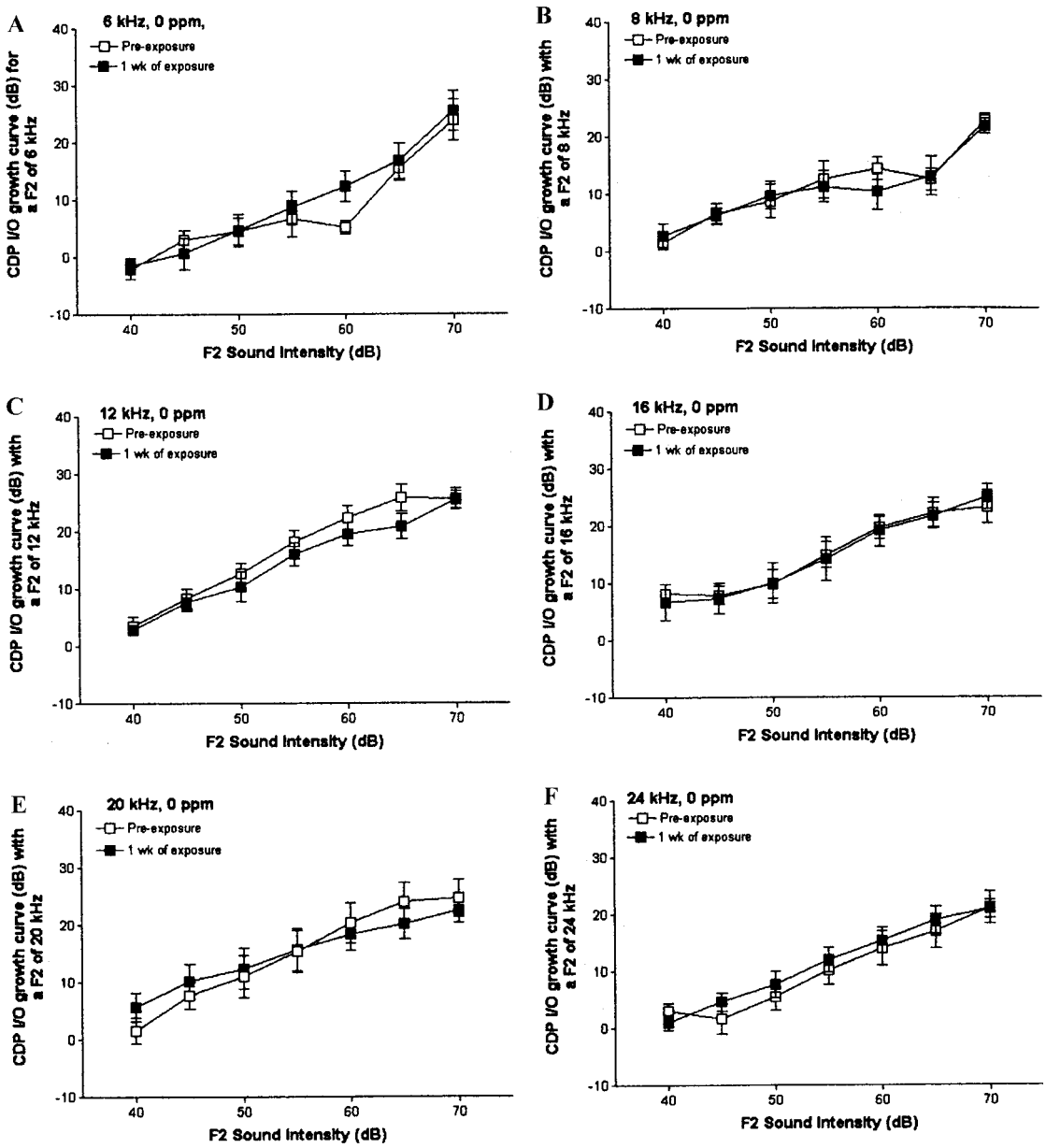

FIG. 2. CDP input/output (growth) functions for untreated animals with stimulation from F_2 = 40 to 70 dB and at six frequencies between 6 and 24 kHz. Measurements were obtained before (open symbol) and after (filled symbol) a 7-day period that paralleled toluene exposures (means ± SE). (A) 6 kHz, (B) 8 kHz, (C) 12 kHz, (D) 16 kHz, (E) 20 kHz, and (F) 24 kHz.

sure condition. A loss of function is most apparent for mid- to high frequencies. For example, at 12 kHz, an increase of 10 dB in the sound intensity was required to generate a CDP above the 3-dB criterion; CDP amplitude is depressed at all sound intensities that initially generated at least a 3-dB output, except the maximal level where the preexposure CDP output was achieved. At 16 kHz, toluene exposure resulted not only in an elevation of 5 dB in the sound level required to produce the criterion 3-dB CDP, but also depressed the maximal CDP at the highest test intensities by about 10 dB from preexposure levels. The later finding suggests a loss not only in the number of outer hair cells that could be recruited but may also suggest impairment in other parts of the auditory periphery.

At 500 ppm (see Fig. 4) some loss of CDP amplitude occurred at all frequencies, with the greatest effect observed above 8 kHz. When CDP output was measured at the most intense stimulus level (70 dB), a 10-dB loss in CDP output was observed between 12 and 24 kHz, suggesting a loss in the number of responding cells compared to the preexposure assessment. The lowest stimulus intensity needed to elicit a CDP was elevated by toluene exposure at 12 and 20 kHz by about 15 dB. Impairment of function at 16 kHz was sufficiently severe

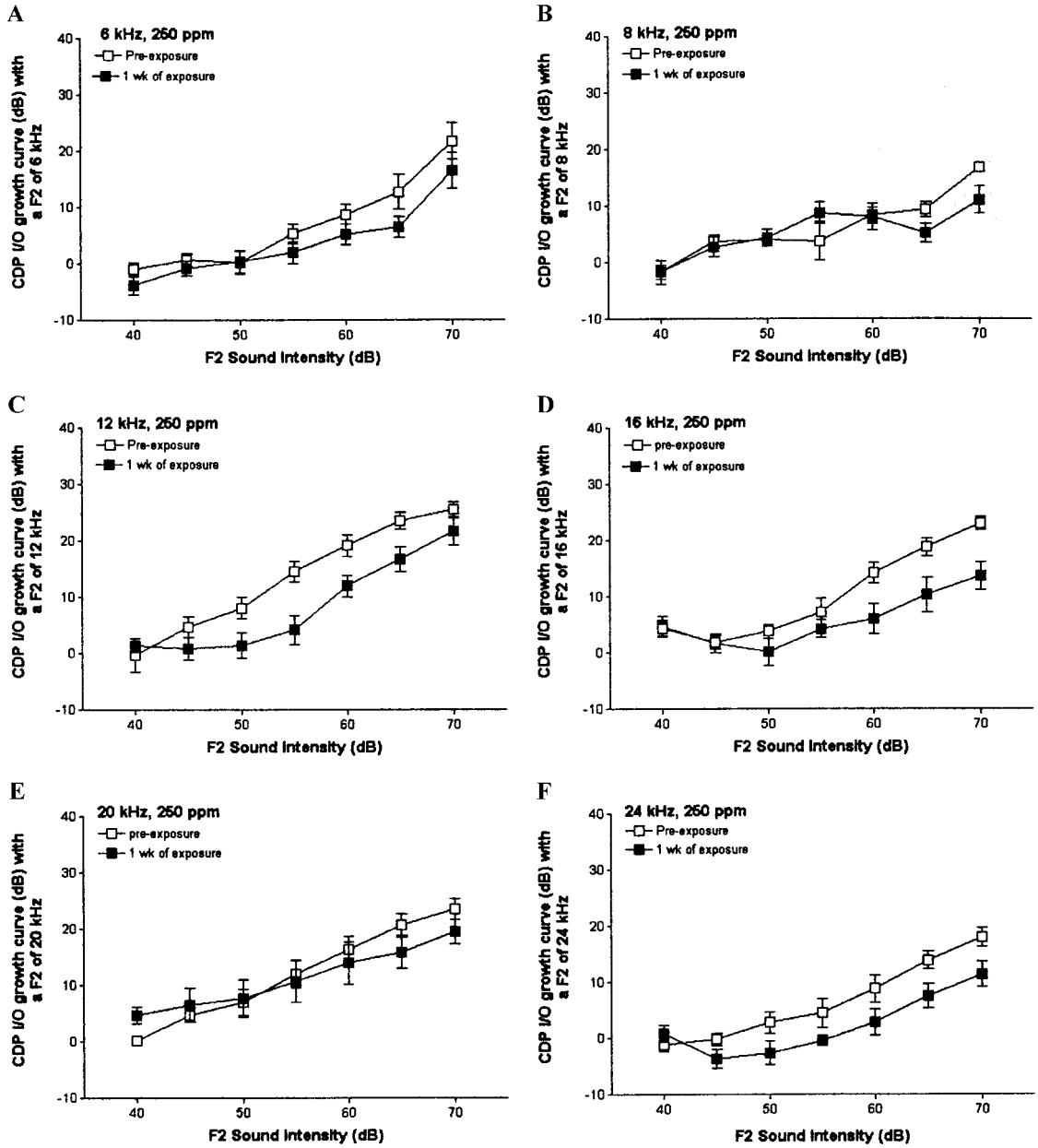

FIG. 3. CDP input/output (growth) functions for animals exposed to 250 ppm toluene with stimulation from $F_2 = 40$ to 70 dB and at six frequencies between 6 and 24 kHz. Measurements were obtained preexposure (open symbol) and immediately after (filled symbol) 8 h/day, 5-day toluene exposure (means ± SE). (A) 6 kHz, (B) 8 kHz, (C) 12 kHz, (D) 16 kHz, (E) 20 kHz, and (F) 24 kHz.

such that CDP output increased by only 6 dB across the 30-dB increase in stimulus intensity. The corresponding dynamic range of CDP amplitude was 17 dB prior to toluene exposure. For all the other affected frequencies, growth of CDP output of at least 10 dB was obtained following toluene, while prior to exposures these values were 15–20 dB.

At 1000 ppm, the CDP output is decreased for all frequencies above 8 kHz. For the 1000-ppm subjects (see Fig. 5), the pattern of loss follows closely that observed in the 500-ppm subjects with the exception of 16 kHz. Here, a 15-dB increase in sound intensity is needed to produce a 3-dB CDP amplitude. For the lower concentrations, 250 and 500 ppm at 16 kHz, a 3-dB CDP is still generated at or near the lowest sound stimulation level employed. For the other middle frequencies, a 5- to 10-dB loss in sensitivity occurred across all sound intensities in subjects receiving 1000 ppm toluene. The maximal CDP amplitude that resulted from stimulation by tones of 70 dB was reduced by 5 to 15 dB from preexposure baseline depending on frequency.

All data were subjected to repeated-measures ANOVA to

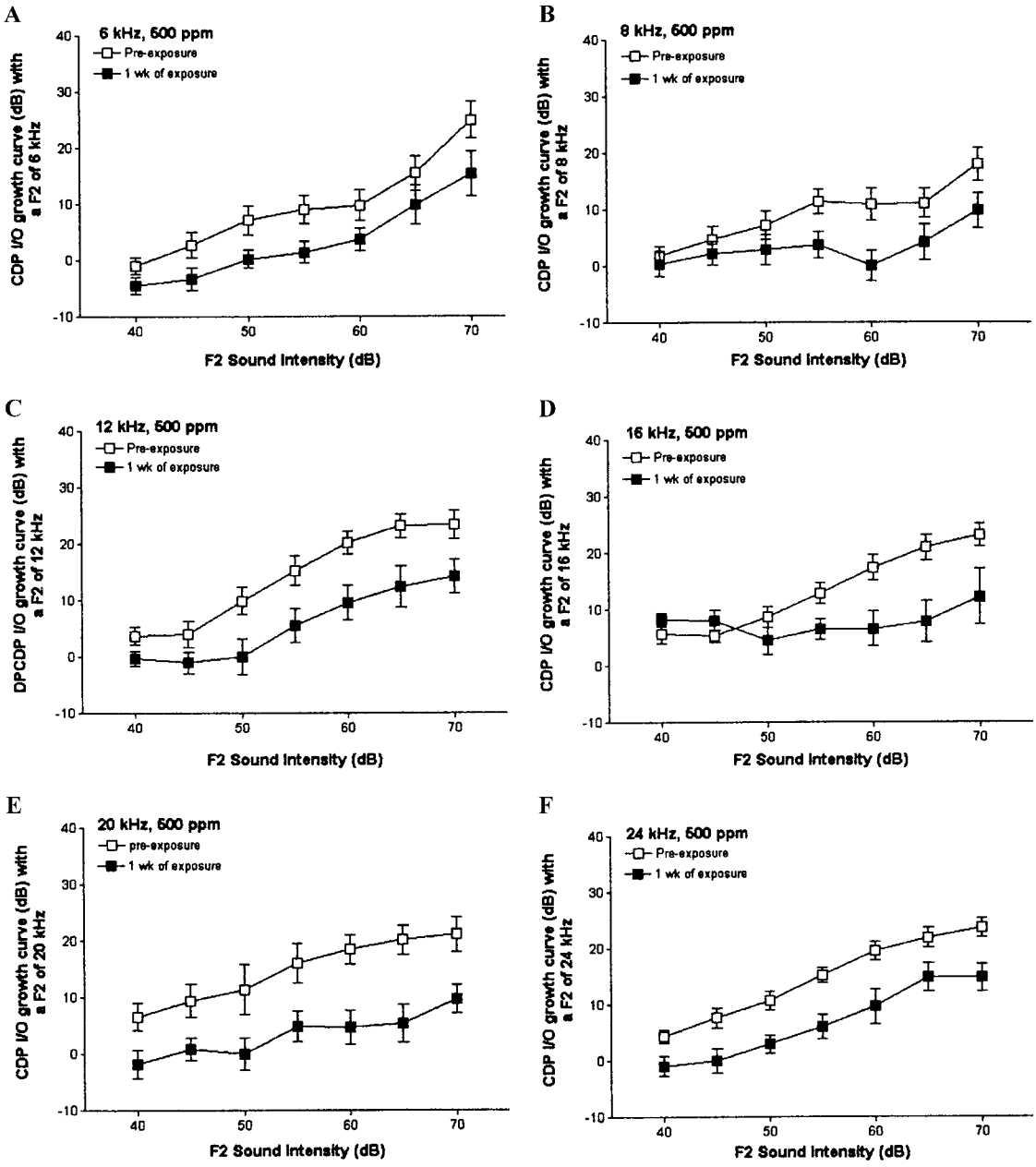

FIG. 4. CDP input/output (growth) functions for animals exposed to 500 ppm toluene with stimulation from F_2 = 40 to 70 dB and at six frequencies between 6 and 24 kHz. Measurements were obtained preexposure (open symbol) and immediately after (filled symbol) 8 h/day, 5-day toluene exposure (means ± SE). (A) 6 kHz, (B) 8 kHz, (C) 12 kHz, (D) 16 kHz, (E) 20 kHz, and (F) 24 kHz.

determine whether toluene exposure produced significant impairment of CDP amplitude. The treatment × test day interaction was significant ($F_{[3,29]} = 4.54$, $p = 0.001$), with Scheffe's multiple comparison showing all three toluene treatments to be significantly different from controls ($p < 0.02$). The treatment × sound intensity interaction was also significant ($F_{[18,167]} = 3.31$, $p < 0.001$). In addition, the treatment × day of exposure × sound intensity interaction was found to be significant ($F_{[18,167]} = 1.76$, $p = 0.034$).

Recovery of 5-Day Toluene-Induced Auditory Dysfunction after 3-Day Rest Period

CDP amplitude returned to or near control levels in all three exposure groups (250 and 500 ppm not shown), after 3 days of recovery from toluene inhalation. Figure 6 shows the recovery of function for the 1000-ppm subjects ($n = 4$), demonstrating full recovery of the CDP at all test frequencies except, perhaps, 20 kHz. Statistical analysis did not demonstrate a significant

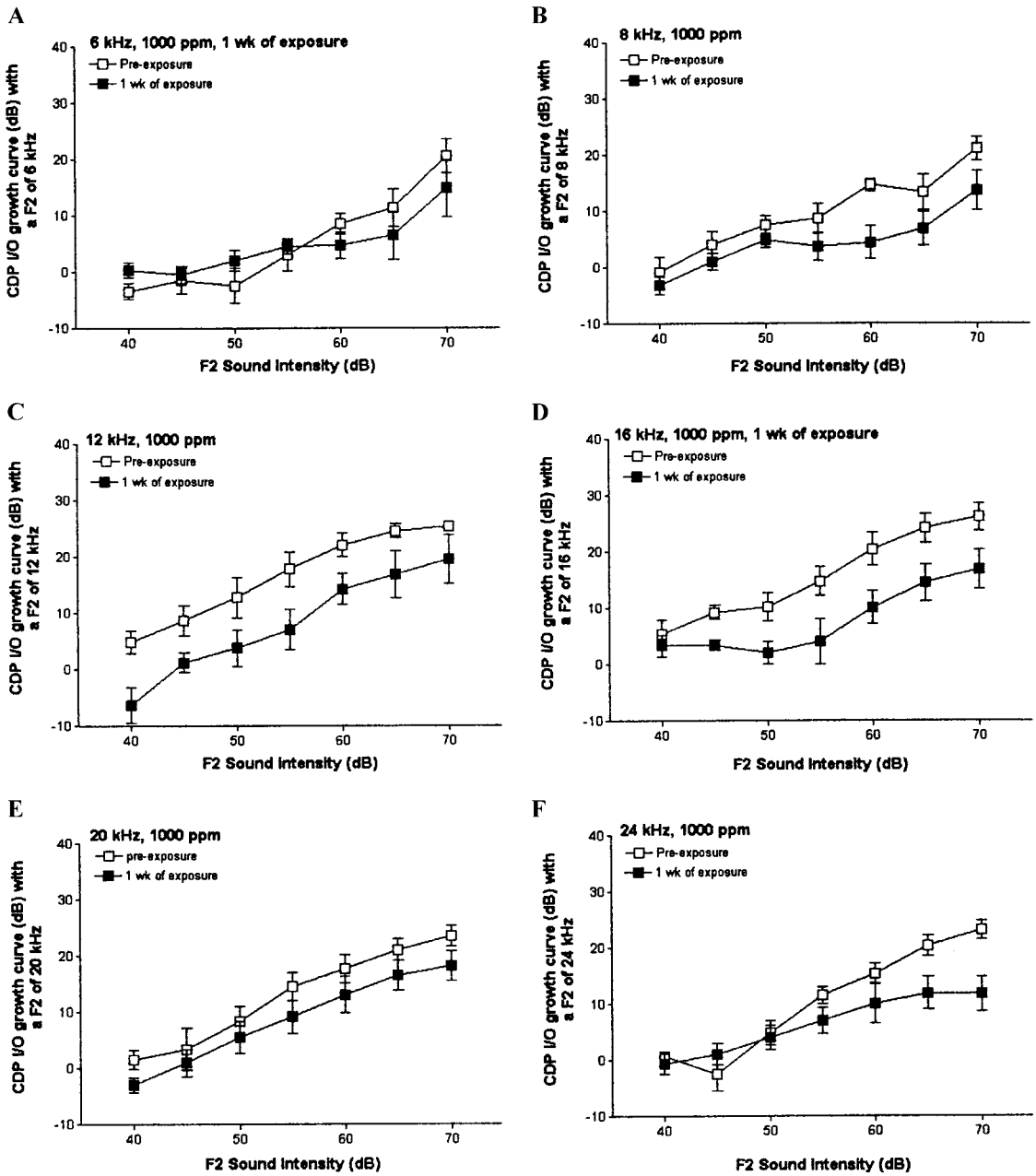

FIG. 5. CDP input/output (growth) functions for animals exposed to 1000 ppm toluene with stimulation from F_2 = 40 to 70 dB and at six frequencies between 6 and 24 kHz. Measurements were obtained preexposure (open symbol) and immediately after (filled symbol) 8 h/day, 5-day toluene exposure (means ± SE). (A) 6 kHz, (B) 8 kHz, (C) 12 kHz, (D) 16 kHz, (E) 20 kHz, and (F) 24 kHz.

difference between CDP amplitudes prior to toluene exposure and that seen 3 days following toluene exposure ($F_{[1,2]} = 0.24$, $p = 0.67$).

Development of Acute Loss in CDP Amplitude with 4-Week Toluene Inhalation Exposures

Continued exposure to 500 ppm toluene for 4 weeks, 5 day/week, 8 h/day resulted in greater reduction in CDP output than that seen after 1 week. Figure 7 presents the depression in acute CDP output resulting from exposure to toluene compared to nonexposed controls. Four-week inhalation exposure increased auditory dysfunction of the 500-ppm group (see Fig. 7D) relative to the losses seen in the same subjects at the conclusion of 1 week of exposure (see Fig. 7B). Whereas the 1-week exposure produced the greatest dysfunction at frequencies above 16 kHz, as the exposure continues, the loss of CDP became equally evident across all test frequencies. CDP amplitudes were reduced to between 3 and 7 dB across test

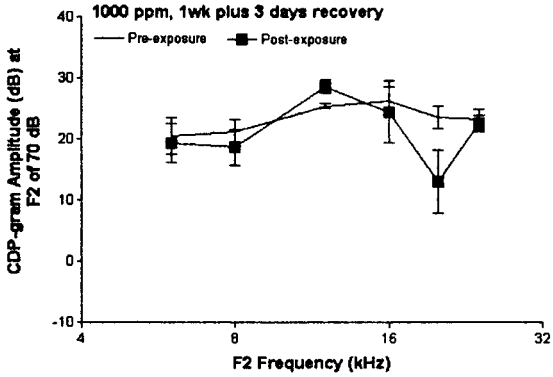

FIG. 6. CDP-gram showing the recovery of temporary threshold shift induced by 1000 ppm toluene (8 h/day, 5 days) after 3 days of rest with the possible exception of 20 kHz. Preexposure (no symbol) and postexposure (■) are shown for six animals (means ± SE).

frequency following 4 weeks compared to levels of 14 and 23 dB prior to toluene and 6 and 16 dB following 1 week of toluene exposure. Although the loss of function increased as exposures continued from 1 to 4 weeks of exposure, a permanent CDP loss was not observed (Fig. 7F). Recovery of the CDP amplitude was observed when the animals were screened 3 days following the 4-week exposure. Control animals were run concurrently and their CDPs over the 4-week experimental procedure are shown (see Figs. 7A–7C).

Analysis of CDP-gram amplitudes between treatment groups and across the pretest and two subsequent exposure times shows a significant treatment effect ($F_{[1,6]} = 18.21$, $p = 0.005$). Statistical significance was also found for the frequency × test day interaction ($F_{[10,60]} = 2.32$, $p = 0.022$).

SDH Histological Sections of Toluene-Exposed Cochleae

Figure 8 shows relative SDH staining density in histological sections of the cochlea from a representative control subject and a subject exposed to 500 ppm (8 h/day, 5 days/week) for 4 weeks and a control subject. This toluene subject showed an average loss of CDP output of 18 dB, with the control subject showing a change in CDP output of ±3 dB. Using a published cochlear map of the guinea pig (see Wada *et al.*, 1998), an approximation of frequency correlation was made. A distinct reduction in SDH staining density was seen in regions of the cochlea corresponding to frequencies above 8 kHz, suggesting that toluene preferentially impairs metabolic activity in this region of the cochlea. For the cochlea taken immediately following toluene exposure, the cells that were located toward the base (and high frequency) showed loss of SDH (see Figs. 8A–8E) staining (i.e., reduced SDH enzyme activity) compared to the control. Figure 8F shows the relative change in inner and outer hair cell SDH staining density averaged across sections representing 5% of cochlear length from the apex. The frequency corresponding to each locus is specified. At the cochlear location corresponding to 8 kHz or below, the SDH staining was relatively even across all turns (see Figs. 8A and 8F). Around 12 kHz, inner and outer hair cells start to show signs of lighter staining (see Figs. 8B and 8F). At 16 kHz, hair cells show signs of altered SDH staining (see Figs. 8C and 8F). This lack of staining continues around 20 and 24 kHz, with a few outer hair cells starting to show heavier staining (see Figs. 8D—8F). At 40 kHz (a frequency above our functional testing range), SDH activity returns to that of the control (see Fig. 8F). The control cochlea shows an even level of staining across all frequencies except for the most basal turn, which seems to drop off by 25%.

DISCUSSION

An important step in considering the potential risk of solvent-induced ototoxicity in humans is determining this effect across species. This study demonstrates the ototoxicity of toluene in the guinea pig, using exposure levels ranging from a level slightly above the human PEL to a level close to the ototoxic concentration in the rat. The current findings demonstrate that significantly lower toluene concentrations are able to produce equivalent auditory dysfunction in the guinea pig as measured by the loss of CDP amplitude or threshold shift. Second, while no hair cell loss resulted from the exposure schedule used here, a change in hair cell metabolism was detected. Last, a permanent auditory deficit could not be generated after 4 weeks of exposure.

Distortion product otoacoustic emissions are acoustic signals that can be recorded from the ear canal in many species, including humans (Brown and Kemp, 1984; Norton and Widen, 1990) and most rodents. These emissions are believed to arise predominantly from the outer hair cells of the cochlea in response to the simultaneous presentation of two different tone frequencies (F_1 and F_2) (Harel *et al.*, 1997). One such emission is the CDP, represented at $2F_1 - F_2$. A loss in CDP amplitude results when auditory periphery is impaired (Johnson and Canlon, 1994a; McFadden and Pasanen, 1994; Subramaniam *et al.*, 1994; Trautwein *et al.*, 1996; Hofstetter *et al.*, 1997; Mom *et al.*, 1999). The CDP can be characterized both by the maximal output of all functional outer hair cells within the region of the cochlea responding to the stimulating tones or by the growth of the emission as the tone intensity increases from sound levels at or below the auditory threshold to levels that saturate the DP response. It can be anticipated that very limited OHC impairment might shift the minimal sound level required to produce a just-detectable DP response (i.e., a signal of 3 dB above the noise floor) but that sufficient units might remain functional such that the maximal CDP amplitude elicited at sound levels well above a detection threshold might be normal. With more severe damage, both a reduction in the maximal CDP amplitude and a shift in the minimal sound level required to produce a CDP would be anticipated. The former can be estimated using a CDP-gram while the latter can be observed using a growth or input/output function.

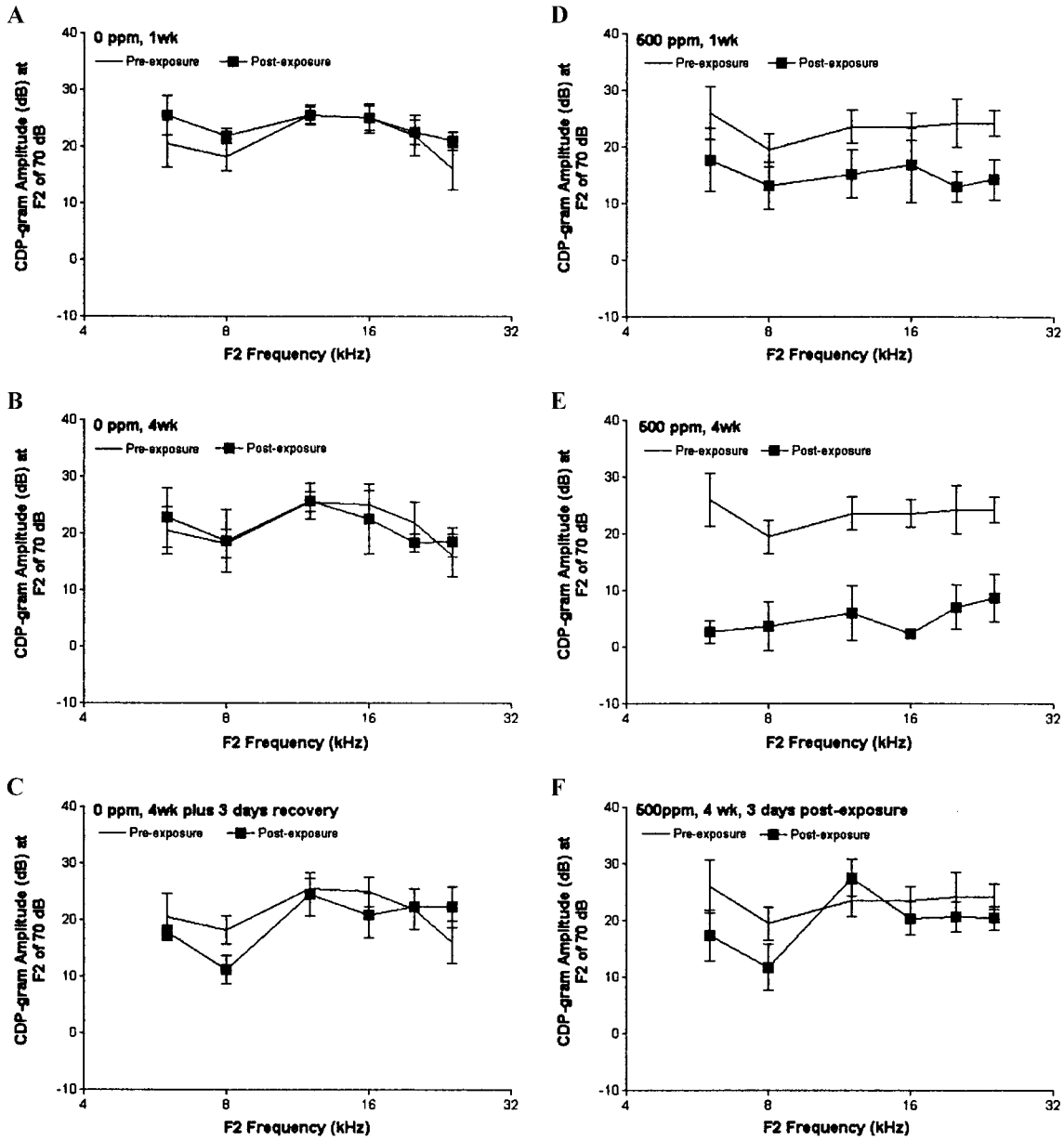

FIG. 7. CDP-grams comparing different stages of toluene inhalation exposure. Measurement made at F_2 = 6, 8, 12, 16, 20, and 24 kHz at F_1 = 80 dB and F_2 = 70 dB in guinea pigs (n = 4) (means ± SE). (A) Shows the change in CDP output from baseline (no symbol) immediately after 1 week of 0 ppm (■) 8 h/day, 5 day/week exposure. (B) Shows the change in CDP output from baseline (no symbol) immediately after 4 weeks of 0 ppm (■) 8 h/day, 5 day/week exposure. (C) Shows the recovery of CDP output from baseline (no symbol) after a 3-day rest period from a 4 week 0 ppm (■) 8 h/day, 5 day/week exposure. (D) Shows the change in CDP output from baseline (no symbol) immediately after 1 week of 500 ppm (■) 8 h/day, 5 day/week exposure. (E) Shows the change in CDP output from baseline (no symbol) immediately after 4 weeks of 500 ppm (■) 8 h/day, 5 day/week exposure. (F) Shows the recovery of CDP output from baseline (no symbol) after a 3-day rest period from a 4 week 500 ppm (■) 8 h/day, 5 day/week exposure.

When the change in CDP is closely evaluated against a comparable study performed by Johnson and Canlon (1994a) in the rat, it is evident that very different conclusions regarding an effective dose can be drawn. Johnson and Canlon reported that 5 days of 1400 ppm toluene for 16 h/day was needed to produce a 5- to 10-dB loss of CDP in the rat. To obtain this same auditory deficit in the guinea pig, only 5 days of 250 ppm toluene for 8 h/day was needed. Even with this significant difference in dosage, both studies revealed a preferential effect of toluene at frequencies between 8 and 24 kHz. Granted, comparison of functional output at different exposure concentrations is less than ideal. To appropriately compare toluene sensitivity between the two species, similar dosing regimens are needed. Campo *et al.* (1997) found that rats had no change in auditory function with in the first 24–48 h after exposure to 1000 and 1250 ppm toluene for 6 h/day, 5days/week for 4

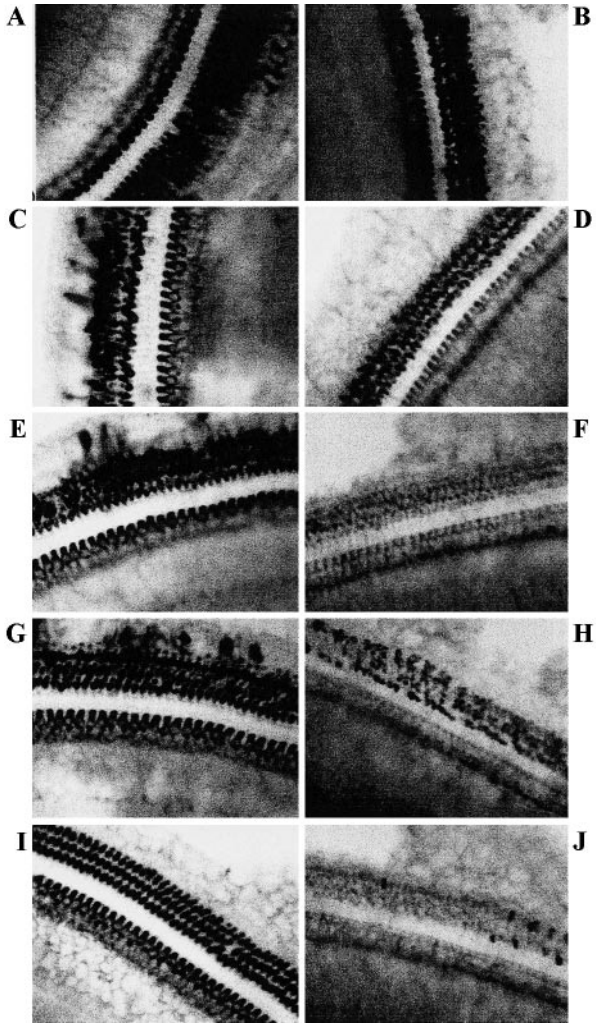

FIG. 8. SDH surface preparations of a control subject and a 500-ppm 8 h/day, 5 day/week, 4 week toluene-exposed subject. Cochleae were taken immediately following CDP recording. (A and B) Section of cochlea that corresponds with approximately 8 kHz for the control subject and 500-ppm toluene subject. All turns located closer to the apex (lower frequencies) showed the same even staining pattern. (C and D) At approximately 12 kHz, the control subject remains "normal" while the SDH staining for the 500 ppm toluene subject for both inner and outer hair cells starts to lighten and becomes uneven. (E and F) At approximately 16 kHz, the control subject shows no change in staining density or hair cell loss while the 500-ppm toluene subject's inner and outer hair cells are still visible but the staining has become undistinguishable from the background. (G and H) At approximately 20 kHz, the control subject remain evenly stained with the 500-ppm toluene subject having a few outer hair cells showing heavier staining but no inner hair cells have returned to normal staining. (I and J) At approximately 24 kHz, the control subject retain the dark staining density and the 500-ppm toluene subject's outer hair cell staining is returning to "normal" but still the inner hair cells are not visibly stained.

weeks. The same study determined that a dosage of 1500 ppm toluene for 6 h/day, 5 days/week for 4 weeks was needed to generate a significant threshold shift in the high-frequency region of the cochlea.

While equivalent loss in CDP amplitude was demonstrated in rat and guinea pig, albeit at very different dose levels, no hair cell loss was found in the guinea pig even with our most severe exposure. This again is contradictory to existing data in the rat. In the same Johnson and Canlon study (1994b), the exposure producing equivalent functional CDP loss resulted in outer hair cell loss in the third row. Interestingly, even thought this study did not find hair cell loss, our histological data revealed that an acute change in metabolism occurred as determined by SDH activity. The loss of relative SDH staining density corresponded to the frequencies showing the greatest functional impairment. For the 250-ppm subjects, loss of SDH staining density was located almost exclusively in the most basal turns of the cochlea (data not shown). For the 1000-ppm subjects, the loss of SDH staining density showed a identical pattern (data not shown) to that of the 500-ppm subjects. When the subjects were allowed to recover for 3 days (independent of exposure duration), a strong and even staining pattern was seen (similar to the control line on Fig. 8F).

Consistent with the lack of hair cell loss, a permanent auditory dysfunction could not be generated in the guinea pig. Using the same study as a comparison once more, a significant difference is yet again revealed. After a 3-day recovery period, all subjects in each exposure condition revealed normal functional output. CDP amplitudes returned to those measured before exposure. When the Johnson and Canlon (1994a) subjects were allowed to recover for 4 days, a further decrease in CDP amplitudes resulted for all frequencies.

Even though a permanent ototoxic effect was not found, the current findings are nonetheless important. This study has established that exposure to toluene, without additional factors such as noise, can produce temporary auditory dysfunction in the guinea pig at concentrations much closer to the human than previously reported in the rat. This is important, because it establishes a difference between rodent species, suggesting that a model based solely on the rat may not provide an accurate picture of toluene ototoxicity. While guinea pigs show recovery of auditory function, even a transient loss of auditory function represents a significant acute effect. Moreover, repeated temporary threshold shift (TTS) may predispose individuals to a permanent threshold shift (PTS) with further insult. Support for this hypothesis has been shown in a study by Macrae (1995) in which the PTS resulting from excessive amplification by hearing aids was predictable based upon the TTS that resulted after a day of use by hearing-impaired children. Additionally, Lonsbury-Martin *et al.* (1987) reported that, in monkeys, repeated exposure to 100 dB sound pressure level (SPL), 3-min pure tones, generated a TTS that lasted about 20 min and lead to a PTS after 18 months. If TTS is predictive of susceptibility to PTS for chemical ototoxicants, then repeated exposure to toluene concentrations close to the human PEL may result in significant permanent auditory dysfunction. In addition, the current report provides a hypothesis that may partially explain toluene ototoxicity. Namely, toluene may interfere with oxidative metabolism at the level of the

sensory cells. Whether such an action can also explain the neurotoxic consequence of toluene exposure remains to be tested.

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