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Protective effect of N-acety-L-cysteine (L-NAC) against styrene-induced cochlear injuries

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Abstract

CONCLUSION—Styrene exposure causes hair cell death through both apoptotic and necrotic pathways and the N-acetyl-L-cysteine (L-NAC) treatment reduces styrene ototoxicity.

OBJECTIVE—Exposure to styrene causes hearing loss and hair cell death in the middle frequency-region in the cochlea. The current study was designed to examine the cell death pathways and the protective effect of L-NAC against styrene-induced cochlear injuries.

MATERIALS AND METHODS—Seventeen rats were exposed to styrene by gavage at 400 mg/kg 5 days per week for 3 weeks. Nine of the styrene-treated rats received L-NAC by intraperitoneal injection (325 mg/kg), and the remaining eight rats received saline injections as controls. The styrene-induced hearing loss was assessed by auditory brainstem responses (ABR). Apoptotic, necrotic and missing hair cells were quantified using combined methods, including nuclear staining with propidium iodide, F-actin staining with FITC-phalloidin, and the TUNEL assay.

RESULTS—The styrene exposure caused a threshold shift of 15 ± 4.3 dB. Both apoptosis and necrosis were involved in the pathogenesis of the cochlear lesion, but apoptosis appeared to be the major cell death pathway leading to the styrene ototoxicity. Treatment with L-NAC reduced the number of missing and dying outer hair cells (OHCs) and reduced the styrene-induced hearing loss.

Keywords

Styrene; Apoptosis; Necrosis; Antioxidant; Rat; Cochlea

Introduction

Styrene is an aromatic industrial solvent, widely used in the production of polystyrene, resins, rubber, reinforced material, and fiberglass boats [1]. Various toxic effects of styrene involving the liver, lung and nervous systems have been observed in experimental animals after a long-term exposure [2]. In addition to the systemic toxicity, styrene exposure is also ototoxic [3].

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Histological examinations in animals revealed that styrene exposure selectively targets Deiters cells (DCs) and outer hair cells (OHCs) in the middle-frequency region [4]. The vulnerability of the cells to styrene exposure appears to increase gradually from the third row to the first row [4–8]. In addition to hair cells and supporting cells in the organ of Corti, spiral ganglion cells are also a sensitive target of styrene, but the damage is usually subsequent to the formation of the OHC lesion and occurs at higher levels of styrene exposure [6].

In non-cochlear cells, styrene exposure has been shown to cause cell death via both apoptotic and necrotic pathways. Exposure to styrene 7,8-oxides, the major metabolite of styrene, caused apoptosis in neurons [9], but it induced necrosis in mononuclear cord blood cells [10]. The prevalence of apoptosis or necrosis is also related to the styrene dosage. For example, Dare et al. reported that exposure to a high dose of styrene 7, 8-oxide caused more necrotic cell death than the low dose [11]. In styrene-exposed cochleae, caspase-dependent apoptosis was observed in our previous study [4].

Several studies indicated that styrene toxicity is associated, in part, with an important cellular tripeptide, glutathione (GSH) [12–14]. Coccini et al. reported depletion of GSH in the respiratory tract cells in rats exposed to styrene [12]. Similarly, Dixit et al. reported a dose-dependent reduction in cytosolic glutathione-S-transferase activity and the GSH level in the rat brain after styrene exposure [13]. The reduction of the GSH level was also observed in various cell lines following exposure to styrene 7,8-oxide [14]. In addition to GSH level reduction, styrene exposure causes increased lipid peroxidation [15]. Because GSH is an important antioxidant, we suspected that reduction of the GSH level would induce oxidative stress on cells. This speculation is supported by a previous study which showed that an *in vitro* application of MnTBAP (manganese (III) tetrakis-(4-benzoic acid) porphyrin), a mimetic compound with superoxide dismutase-like activity, protected neurons from styrene damage [16].

Since styrene ototoxicity is likely to be associated with the reduction of the GSH level, we hypothesize that replenishment of GSH would reduce styrene-induced hearing loss and hair cell loss. The current study was designed to investigate cell death pathways involved in styrene ototoxicity and to determine the effect of GSH augmentation on the styrene-induced cochlear injuries. To increase the cellular GSH level, we selected a pro-GSH drug, N-acetyl-L-cysteine (L-NAC), which has been shown to reduce noise-induced hearing loss [17], to enhance the cochlear GSH capacity.

Materials and methods

Subjects

Twenty three adult Long Evans rats (450–550g) with normal hearing, as assessed by measuring auditory brainstem response (ABR) threshold, were used in this study. The animals were randomly assigned to one of three groups: control (n=6), styrene (n=8) or styrene-L-NAC (n=9). The care and use of the animals reported in this study were approved by Institutional Animal Care and Use Committee serving the State University of New York at Buffalo.

Styrene exposure

Styrene (S4972, Sigma) was administrated by gavage. The dosage of styrene was 400 mg/kg, once a day, 5 days/week, for 3 weeks. The styrene was mixed with olive oil (2 g styrene in 1 ml oil). This dosage of styrene was used in our previous study, which produced a blood concentration of 9.8 µg/ml blood and frequency-dependent ABR threshold shifts with the highest shift of about 16 dB at 20 kHz [4]. The control animals received olive oil alone.

Application of L-NAC

The L-NAC solution was freshly prepared before injection by dissolving L-NAC (A8199, Sigma) in physiological saline solution (80 mg/mL) and adjusting to pH 7.4. For the styrene-L-NAC group (n=9 rats), L-NAC (325 mg/kg) was intraperitoneally injected, starting 1 day before the styrene application and continuing once a day during the 3-week styrene treatment period (applied 5 minutes prior to each styrene application). The selection of the dosage of the L-NAC treatment was based on a previous study showing that this dose of L-NAC exerted an effective protection against noise-induced cochlear damage [17].

Audiometric evaluation

The dose-dependent and frequency-related ABR threshold shifts after styrene exposure by gavage were documented in our previous report [4]. Since this study focused on the styrene-induced pathological alterations and protective effect by L-NAC, we used only click stimuli (by 100-µs pulse) in the current study to evaluate the overall status of the cochlear function. Thresholds of ABR were recorded before and at the end of the 3-week styrene exposure. ABR threshold shifts were determined by subtraction of the thresholds of the pre-exposure from those of the post-exposure. The stimuli were presented at a rate of 20.1/s with alternating polarity. The intensities of the stimuli were varied from 90 to 0 dB SPL in 5-dB steps.

For ABR recording, the animals were anesthetized using an intramuscular injection of ketamine (30 mg/kg) and acepromazine (0.1 mg/kg). Needle electrodes were placed subdermally over the vertex (positive), the mastoids (inverting) and the midline of the neck region (common). The evoked responses were band-pass filtered (100–3000 Hz), amplified using a bioamplifier (TDT DB4), and digitized using an A/D converter (TDT AD1). A total of 1000 sweeps were averaged for each response over a 12.5-ms time epoch. The ABR thresholds were defined as the lowest intensity that elicited a visible response. The threshold shifts were calculated by subtracting the baseline ABR thresholds (before the treatment) from those of final ABR testing conducted at the end of the 3-week treatment.

Evaluation of cochlear pathology

Animals were sacrificed under CO₂ anesthesia at the end of the 3-week styrene and L-NAC treatments. The cochleae were fixed with 10% buffered formalin. The cochleae were dissected in PBS and the organs of Corti were harvested for pathological examination. A combination of morphological and biological methods was employed to identify apoptotic, necrotic and missing OHCs.

Propidium iodide (PI) labeling

PI, a DNA intercalating fluorescent probe, was used to visualize the morphological changes of hair cell nuclei. The animals were sacrificed and the cochleae were fixed with 10 % buffered formalin. After dissection, the organs of Corti were stained first with one of the complementary labels (see the following sections for details) and then with the PI solution (Invitrogen USA, 5 µg/ml in 10 mM PBS) for 10 min. The specimens were rinsed with PBS and mounted on slides containing ProLong™ antifade medium (Molecular Probes Inc.).

TUNEL assay

TUNEL assay (Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling) was performed to confirm the nature of cell death illustrated by PI staining. Following fixation, the organs of Corti were dissected. The organs of Corti were incubated in ice-cold 70% (v/v) ethanol overnight and then in freshly prepared DNA-labeling solution containing 10 µl of reaction buffer, 0.75 µl of TdT enzyme, 8.0 µl of BrdUTP and 31.25 µl of dH₂O for 16 hrs at room temperature. The tissue was then stained with Alexa Fluor 488 dye-labeled anti BrdU antibody (5µl of antibody plus 95 µl of the rinse buffer) at room temperature for 1 hr. The organs of Corti were double stained with PI with the procedure described above.

F-actin staining

F-actin staining was conducted to visualize the cuticular plates of hair cells in order to inspect the integrity of the cuticular plates for quantification of missing hair cells following the styrene exposure. The staining solution contained FITC-phalloidin (1:250, Sigma), 0.25% Triton-X-100 and 1% bovine serum albumin in PBS. Upon completion of cochlear dissection, the organs of Corti were transferred to the freshly prepared staining solution and incubated at room temperature for 30 min. The specimens were double stained with PI and then mounted on slides with the procedure described above.

Inspection of the organs of Corti

Each organ of Corti was thoroughly examined from base to apex using fluorescence microscopy. The numbers of apoptotic, necrotic and missing OHCs were documented and the cochleograms were constructed for each class of pathology. The cochlear sections showing the signs of hair cell damage were further examined and photographed with confocal microscopy (Bio-Rad, MRC 1024). The use of confocal microscopy in cochlear inspection reduced the occasional ambiguity in distinguishing between OHC nuclei and supporting cell nuclei.

Criteria for identification of apoptotic, necrotic, and missing hair cells

Three types of hair cell pathology, apoptosis, necrosis and missing, were documented using the following criteria:

Apoptotic hair cells: the hair cells showing condensed nuclei illustrated by PI staining were counted as apoptotic cells. The condensed nuclei appeared smaller in size than the normal nuclei of viable cells, and had an increased PI fluorescence. The nature of apoptosis was

further confirmed by TUNEL staining. All the hair cells with condensed nuclei exhibited strong TUNEL fluorescence.

Necrotic hair cells—The principal criterion for necrotic hair cells was nuclear swelling illustrated by PI staining. The feature morphologies of nuclear swelling included enlarged sizes, malformed shapes and loss of normal texture of nuclear staining. In TUNEL stained specimens, the hair cells having swollen nuclei often exhibited a positive but weak TUNEL staining.

Missing OHCs—The missing OHCs were recognized in the area of the cuticular plates with absent F-actin labeling. In certain apoptotic cells, particularly those at the early stage of the death process, F-actin fluorescence in the cuticular plates could be significantly decreased, but the condensed nuclei were present. In order to prevent double counting of the same cell as both missing and apoptotic, examinations of the cuticular plates and the nuclei were always carried out simultaneously. Priority was given to the presence of a condensed nucleus and that cell would be scored as apoptotic, regardless of the status of F-actin.

Statistical analysis

The statistical means of ABR threshold shifts between the styrene-treated group and the styrene-plus-L-NAC-treated group were compared using standard Student's *t* tests. The comparison of overall cochlear damage (sum of apoptotic, necrotic and missing hair cells) between groups was performed with chi-square analysis. The statistical method for comparison of the numbers of apoptotic and necrotic OHCs between groups was also chi-square.

Results

The styrene-treated animals appeared to tolerate the styrene exposure well (400 mg/kg for 3 weeks). There was no recognizable sign of systemic toxicity, such as weight loss (except for the first few days after the beginning of styrene application), changes in attitude, or any gastrointestinal symptoms such as vomiting or diarrhea during the course of the styrene treatment.

Styrene ototoxicity

The styrene exposure for three weeks induced significant damage to the organ of Corti. ABR testing revealed an average of 15 ± 4.3 dB of threshold shifts to click stimulus after the styrene exposure ($n=8$). Pathological examination of the organ of Corti revealed an average of $51.4 \pm 4.2\%$ of OHC damage (the sum of missing and dying cells) in the middle cochlear region (30–70% from the apex). Fig. 1A presents a typical pattern of OHC loss visualized by F-actin staining in the cuticular plates. In general, the maximal damage occurred in the third row of OHCs, followed by the second and first rows of OHCs. Fig. 1B is the cochleogram showing mean percentages of OHC damage as a function of the distance from the apex to the base of the cochlea. The middle portion of the cochlea exhibits the maximal level of hair cell damage.

The apoptotic and necrotic cell death pathways were assessed with PI and TUNEL staining in the cochleae exposed to styrene. Fig. 2A shows a section of the organ of Corti stained with PI following the 3-week styrene treatment. The arrows point to condensed nuclei and the arrowhead points to a swollen nucleus. Fig. 2B shows the same cochlear section superimposed with the TUNEL fluorescence. Note that the condensed nuclei exhibit a strong TUNEL fluorescence (appears yellowish because of the combination of red PI and green TUNEL fluorescence), indicating that these cells were dying through the process of apoptosis. The swollen nucleus shows positive but weak TUNEL staining. The numbers of OHCs with apoptotic or necrotic phenotypes were quantified and the results are presented in Fig. 2C. There were approximately 3-fold more apoptotic hair cells than necrotic hair cells ($p<0.01$). The result suggests that apoptosis is the primary cell death pathway leading to generation of the cochlear lesion following the styrene exposure.

As seen in our previous report [4], styrene also targets Deiters cells. Fig. 2D shows a double stained (PI and TUNEL) cochlear section following exposure to styrene. In addition to the condensed/fragmented (arrowheads) and swelling (asterisks) nuclei of OHCs, a few condensed Deiters cell nuclei were seen in the area immediately lateral to the OHC region (arrows). These condensed nuclei were TUNEL positive, suggesting that they were dying through apoptosis.

Protection of hearing and hair cells against styrene ototoxicity with L-NAC treatment

Auditory functions were compared between the styrene-plus-L-NAC-treated and styrene-alone-treated animals. As described above the styrene-treated animals showed an average of threshold shift of 15 ± 4.3 dB. In contrast, the styrene-plus-L-NAC-treated animals exhibited virtually no hearing loss (0.6 ± 1.6) after exposure to styrene for 3 weeks ($p<0.05$).

Consistent with the physiological measurement, pathological observation revealed a significant reduction in the extent of hair cell loss in the styrene-plus-L-NAC-treated animals compared with styrene exposure alone group. Fig. 3A presents an example of cochlear surface preparation showing styrene-induced OHC loss. All OHCs in the third row, the majority of OHCs in the second row and a few OHCs in the first row were missing. In contrast, only a few OHCs in the third row in a styrene-plus-L-NAC-treated rat showing in Figure 3B were missing. The quantitative analyses of the total number of the damaged OHCs (the sum of missing OHCs and the OHCs that were undergoing apoptosis and necrosis) are illustrated in Figure 3C. The average percentage of OHC damage in the styrene-treated animals was 28.3% (filled circles). The data are the same as Figure 1B. In contrast, the average percentage of OHC damage in the styrene-plus-L-NAC-treated group was only 10.6% (open circles). The difference between the two groups was statistically significant ($p<0.0001$).

Figure 4 presents styrene-induced apoptotic and necrotic OHCs (filled bars, same as that in Figure 2C) and apoptotic and necrotic OHCs obtained in rats that received treatment with styrene plus L-NAC (open bars). The data showed a difference in L-NAC protection against apoptotic and necrotic OHC death. The mean reduction of apoptotic cells by the L-NAC treatment was 78%, which was statistically less than that in the unprotected group ($p<0.001$). However, the mean reduction of necrotic cells was only 23%, which was not

significantly different from the unprotected group ($p>0.05$). These results suggest that the L-NAC treatment preferentially reduces the number of apoptotic OHCs.

Discussion

As seen in our previous report [4], the styrene exposure by gavage caused both pathological and physiological damage to the cochleae. The injury occurred mainly in the middle frequency region. The third row of OHCs appeared to be the initial target, followed by the second and first rows. This pattern of cochlear damage is consistent with previous reports that used gavage and inhalation exposures [4–8]. The current study revealed that cell death resulting from styrene exposure was primarily apoptotic. The main finding of this study is that the styrene-induced hearing loss and hair cell damage can be prevented by L-NAC treatment.

In the current study, we found that exposure to styrene at 400 mg/kg for three weeks caused significant OHC loss in the cochlea. In addition, active dying OHCs were observed. The cell death appeared to be either apoptosis, as evidenced by the presence of nuclear condensation with strong TUNEL activity, or necrosis as suggested by the presence of nuclear swelling. These results indicate that styrene exposure causes generation of both apoptosis and necrosis in the cochlea. Further quantitative analysis of the numbers of apoptotic and necrotic OHCs revealed that the average number of apoptotic OHCs was approximately three times greater than the number of necrotic cells, suggesting that apoptosis is the major driving force underlying the generation of the cochlear lesion following the styrene exposure.

The current report provided experimental evidence showing the dominant role of apoptosis in the styrene-induced cochlear pathogenesis. However, it should be pointed out that the ratio of apoptosis and necrosis obtained from the current study reflects only the cochlear pathology at the time point when the cochleae were examined. It is difficult to accurately postulate the total amount of contribution of apoptosis and necrosis to the overall damage to the organ of Corti from the current snapshot observation. Our previous observation on noise-induced hair cell damage demonstrated a changing ratio of apoptosis and necrosis with the passage of time following exposure to intense noise [18]. In noise-damaged cochleae, apoptosis is the dominant cell death pathway at the early stage of cochlear pathogenesis, whereas apoptosis and necrosis contribute equally to the cochlear lesion at the later stage. In order to understand the overall contribution of apoptosis and necrosis, an observation of multiple time points during and after styrene exposure should be carried out.

L-NAC, an agent that delivers exogenous cysteine to cells and raises intracellular glutathione [19], was used in the current study to enhance the cellular GSH level. Based on the biological effect of L-NAC, we speculate the presence of two possible cellular mechanisms by which L-NAC exerts its protective effect. The first is through its antioxidant property by increasing the GSH level of the cells [19]. GSH deficiency has been observed following exposure to styrene or styrene metabolites [20], which is mainly caused by the consumption of GSH in styrene detoxification, a reaction catalyzed by glutathione S-transferase to generate GSH-styrene conjugates. By increasing the cellular GSH level to counteract the GSH consumption, L-NAC can exert its protection. Previous studies have

shown that application of L-NAC protects hair cells against oxidative stress-associated damage, including noise-induced and ototoxic drug-induced hair cell death. For example, Kopke et al reported that application of L-NAC with salicylate reduced both the temporary threshold shift and the permanent threshold shift in the chinchilla following exposure to traumatic noise [17]. The current study showed L-NAC protection against styrene ototoxicity, hair cell loss and hearing loss.

In the current study, application of L-NAC reduced both apoptosis and necrosis with greater suppression of apoptosis. The preferential reduction of apoptosis in the L-NAC treated ears suggests involvement of oxidative stress in generation of apoptosis following styrene exposure. Although the precise mechanisms by which oxidative stress initiates apoptosis are still elusive, the linkage of oxidative stress and apoptosis provides a rationale for the use of antioxidant therapy in protection against styrene ototoxicity. The results of the current study may suggest that augmentation of intracellular antioxidant capacity is a pharmacological strategy with significant clinic potential for reduction of styrene ototoxicity.

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Figure 1.

(A) An example image from the cochlear location about 30% from the apex showing the typical pattern of styrene damage to the structure of the organ of Corti stained with FITC-phalloidin. Notice that the third row of OHCs (OHC3) exhibits the maximal level of damage (arrows), followed by the second (OHC2) and the first row of OHCs (OHC1); (B) Cochleogram showing the average percentage of damaged OHCs (sum of apoptotic, necrotic and missing) after styrene exposure. The animals were exposed to styrene by gavage (400 mg/kg, once a day, 5 days per week for 3 weeks). The horizontal bar in A represents 15 μm. Vertical bars in B are standard errors. N=16 cochleae.

Figure 2.

(A) An example image showing condensed (arrows) and swollen OHC nuclei (arrowhead). The specimen was stained with PI. The location of the image in the cochlea was about 50% from the apex; (B) The same image as in (A), superimposed with TUNEL fluorescence, indicating DNA fragment in the OHCs with condensed or swollen nucleus. (C) Comparison of the average numbers of apoptotic and necrotic OHCs in each cochlea following exposure to styrene for 3 weeks. The number of apoptotic OHCs is about three times greater than that of necrotic OHCs ($p < 0.01$). $N = 16$ cochleae. The vertical bars are standard errors; (D) An example image showing condensed and TUNEL positive stained nuclei in the Deiters cells (third row) following styrene exposure (arrows). Condensed/fragmented (arrowheads) and swollen OHC nuclei (asterisks) are also shown. The location of the image in the cochlea is about 40% from the apex. The horizontal bars represent 15 μm .

Figure 3.

(A) a representative image showing severe styrene-induced OHC loss; (B) a representative image showing less OHC loss in the cochlea treated with styrene plus L-NAC. The specimens (about 30% from the apex) were stained with FITC-phalloidin. The horizontal bar represents 15 μm; (C) cochleograms comparing the average percentage of damaged OHCs (sum of apoptotic, necrotic and missing) between the styrene-treated rats (n=16 cochleae, filled circles) and the styrene-plus-L-NAC-treated rats (n=18 cochleae, open circles). The L-NAC treatment showed significant reduction of OHC damage ($p<0.0001$). The vertical bars are standard errors.

Figure 4. Comparison of numbers of apoptotic and necrotic OHCs in the styrene-treated (n=16) and the styrene-plus-L-NAC-treated cochleae (n=18). Notice that the number of apoptotic OHCs was significantly reduced with L-NAC treatment ($p<0.001$). In contrast, the number of necrotic cells was only slightly reduced ($p>0.05$). The vertical bars are standard errors.