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Review Article

Styrene enhances the noise induced oxidative stress in the cochlea and affects differently mechanosensory and supporting cells

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ABSTRACT

Experimental and human investigations have raised the level of concern about the potential ototoxicity of organic solvents and their interaction with noise. The main objective of this study was to characterize the effects of the combined noise and styrene exposure on hearing focusing on the mechanism of damage on the sensorineural cells and supporting cells of the organ of Corti and neurons of the ganglion of Corti. The impact of single and combined exposures on hearing was evaluated by auditory functional testing and histological analyses of cochlear specimens. The mechanism of damage was studied by analyzing superoxide anion and lipid peroxidation expression and by computational analyses of immunofluorescence data to evaluate and compare the oxidative stress pattern in outer hair cells versus the supporting epithelial cells of the organ of Corti. The oxidative stress hypothesis was further analyzed by evaluating the protective effect of a Coenzyme Q_{10} analogue, the water soluble Q_{ter}, molecule known to have protective antioxidant properties against noise induced hearing loss and by the analysis of the expression of the endogenous defense enzymes.

This study provides evidence of a reciprocal noise-styrene synergism based on a redox imbalance mechanism affecting, although with a different intensity of damage, the outer hair cell (OHC) sensory epithelium. Moreover, these two damaging agents address preferentially different cochlear targets: noise mainly the sensory epithelium, styrene the supporting epithelial cells. Namely, the increase pattern of lipid peroxidation in the organ of Corti matched the cell damage distribution, involving predominantly OHC layer in noise exposed cochleae and both OHC and Deiters' cell layers in the styrene or combined exposed cochleae. The antioxidant treatment reduced the lipid peroxidation increase, potentiated the endogenous antioxidant defense system at OHC level in both exposures but it failed to ameliorate the oxidative imbalance and cell death of Deiters' cells in the styrene and combined exposures. Current antioxidant therapeutic approaches to preventing sensory loss focus on hair cells alone. It remains to be seen whether targeting supporting cells, in addition to hair cells, might be an effective approach to protecting exposed subjects.

1. Introduction

Aging, exposure to noise trauma, ototoxic drugs, and to organic solvents are all causes of hearing loss [1–3]. Focusing on the risk factors related to occupational exposures to organic solvents, the health effects caused by these compounds assume a relevant significance and have long been investigated [4–6]. Among the organic compounds used in the chemical processing industry, styrene is widely used in the production of plastics, resins, fiberglass, synthetic rubbers; currently it is classified as a possible carcinogen and an ototoxic agent in humans [7–9]. Small amounts of styrene are naturally present in foods such as legumes, beef, clams, eggs, nectarines, spices and in packaged foods by migration from polystyrene containers and packaging materials. Consequently, styrene may be widely absorbed by oral, inhalation, percutaneous and subcutaneous exposure [10]. Hearing loss induced by chronic exposure to styrene has long been investigated and debated [11–15]. Additional concern for industrial workers is represented by the concomitant exposure to noise (i.e. solvents plus machine noise). In

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fact, noise-induced hearing loss (NIHL) caused by single exposure to very loud sounds or repeated exposures to lower intensity sounds over an extended period is a major source of hearing disability worldwide [16]. According to literature [11,12,17,18], the combined exposure to noise and styrene induces greater cochlear functional loss and hair cell loss than the summated losses caused by styrene and noise alone.

What is mostly debated is the mechanism of cochlear damage of styrene versus noise overstimulation. In fact, most studies aimed at understanding the mechanisms underlying hearing loss in NIHL and ototoxicity due to organic solvents have been focused on the sensory receptor cells of the auditory systems, mainly the outer hair cells (OHC) [12], whereas fewer studies have examined the biology and functions of the supporting cells that surround hair cell. The nonsensory supporting cells provide trophic [19,20] and structural support for the hair cells [21], trophic support for spiral ganglion neurons [22,23], and are essential in maintaining proper ionic concentrations in the organ of Corti [24,25]. Indeed, indications on the role in styrene ototoxicity of supporting cells, namely Deiters' cells, has been reported indicating that Deiters' cells represent another highly vulnerable target [11,12], however, the relationship between OHC and supporting cells in styrene cochlear damage is still unclear.

In addition as regards noise mechanism of damage, accumulating evidence suggests that noise exposure drives mitochondrial activity and unbalanced reactive oxygen species (ROS) production, including superoxide anion radicals, lipid peroxidation, readily initiated by hydroxyl radicals, and cell death [26,27]. Conversely the mechanism of styrene ototoxicity is still unresolved even though styrene has been suggested to trigger OHC death through a caspase-dependent apoptosis pathways [11,28], the nature of which remains to be clarified. It has also been reported that styrene major metabolite, the styrene 7,8–oxide, has a direct effect on oxidative stress imbalance suggesting a ROS dependent mechanism of damage but no experimental evidence has yet been provided in the cochlea [6,29,30].

Given the above, this research addressed the interaction between styrene and noise through functional, morphological and immunohistochemical analyses. Namely, in this paper we tested the hypothesis of the oxidative stress mechanism and we focused our study on the analysis of the role played by Deiters' cells by analyzing superoxide anion, lipid peroxidation and the expression of the endogenous antioxidant enzymes. Furthermore, the mitochondrial oxidative stress hypothesis was related to the mobile electron carrier Coenzyme Q_{10} (CoQ_{10}) considering that CoQ_{10} active form, ubiquinol, is an effective antioxidant that prevents lipid peroxidation and mitochondrial damage [31–34]. We used a water soluble CoQ_{10} analogue, Q_{ter}, since it shows enhanced bioavailability with respect to the native form [35]. Moreover, it has been suggested that Q_{ter} efficacy depends on its ability to intercept free radicals in both aqueous phases and lipid–water interfaces [36]. In fact, the mobility of the antioxidant in the membranes and lipoproteins plays a key role in determining the antioxidant activity. Furthermore, Q_{ter} promotes OHC survival in NIHL animal models [31,33,34].

2. Materials and methods

2.1. Animals

Male adult Wistar rats (Catholic University Laboratories, 200–250 gr.) were used in this study. The auditory function of each animal was tested for the presence of Preyer's reflex. The experiments were performed on 135 animals, randomized and assigned to 9 experimental groups as follows: (1) control animals (Ctrl group; n=15); (2) noise exposure (Noise group; n=15); (3) styrene (Styrene group; n=15); (4) noise and styrene administration (Noise+Styrene group; n=15); (5) noise and Q_{ter} treatment (Noise+Q_{ter} group; n=15); (6) Styrene and Q_{ter} administration (Styrene+Q_{ter} group; n=15); (7) noise exposure plus styrene and Q_{ter} administration (Noise+Styrene+Q_{ter} group; n=15); (8) animals with normal hearing treated with Q_{ter} (Ctrl+Q_{ter} group; n=15) and (9) animals with normal hearing that underwent oral gavage procedure (Ctrl-Gavage group; n=15). In all experiments, animals of the last 2 groups did not show any significant difference in functional and morphological evaluations with respect to Ctrl group (data not shown). For the whole experimental period, the animals were housed two per cage at controlled temperature (22–23 °C) and constant humidity (60 ± 5%), under a 12-h light/dark cycle, with food (Mucedola 4RF21, Italy) and water ad libitum. All efforts were made to minimize animal suffering and to reduce their number, in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC). All procedures were performed in compliance with the Laboratory of Animal Care and Use Committee of the Catholic University, School of Medicine of Rome and were approved by the Italian Department of Health (*Ministero della Salute*).

2.2. Noise and styrene exposure

The schedules of noise and styrene exposures for 5 days/week for 3 consecutive weeks were chosen in order to produce a sub-maximal cochlear damage in both single and combined exposures and to allow a better assessment of their interaction. Specifically, we applied a noise trauma at the lowest intensity (10 kHz, 98 dB SPL, 60 min/day) able to induce a permanent threshold shift and disruption of the organ of Corti [37]. As regards styrene administration by gavage we considered that styrene damage in the cochlea is dose and time dependent and we applied the least damaging concentration (400 mg/kg) to induce a permanent threshold shift [12].

2.2.1. Noise exposure

The animals were placed in the anechoic room and exposed to a 98 dB sound pressure level (SPL) for 3 weeks, 5 consecutive days/week, 60 min each day. As previously described [34], the animals were placed 2 per cage, the acoustic trauma was induced by a continuous pure tone of 10 kHz generated by a waveform generator (LAG-120B, Audio Generator; Leader Electronics Corporation), amplified by an audio amplifier (A-307R; Pioneer Electronics) and presented in an open field by a dome tweeter (TW340×0; Audax) positioned at the center of the cage. Sound level was measured using a calibrated 1/4 in. microphone (Model 7017, ACO Pacific Inc., Belmont, CA, USA) and a calibrated preamplifier (Acoustic Interface System, ACO Pacific Inc).

2.2.2. Styrene administration

As commonly used in experimental models of styrene ototoxicity [11–13], the substance (styrene > 99%, Sigma Corporation, product id: S4972) was administered by oral gavage once a day for 3 weeks, 5 days/week, at a dose of 400 mg/kg body weight, mixed in olive oil (0,4 ml/kg body weight). Dosage and time of treatment were in according to previous studies [13]. A rodent's gavage device (Instech Corp. product id: FTSS-18S-76, 76×2.0 mm) was used. Through the entire period of styrene administration, the oral gavage procedure was performed by a single specialized operator and was systematically preceded by an accurate inspection of the animal upper aero-digestive tract. The animals of group Ctrl-Gavage underwent oral procedure for a total of 3 weeks with no drug delivery.

2.3. Antioxidant administration

Q_{ter}, a soluble form of CoQ10 obtained by mechano-physical activation of CoQ_{10} (provided by Scharper Therapeutics), is manufactured by using industrially available native CoQ_{10} (Kaneka Pharma Europe). It is a terclatrate that brings different substances into supramolecular contact through the administration of energy, turning a simple mixture into a multicomposite material (proprietary technology by Asoltech). In the multicomposite Q_{ter}, the CoQ_{10} is treated in association with a suitable carrier material and a bioactivator. As

previously described [38,41], Q_{ter} is about 200 times more soluble and its antioxidant capacity is approximately five times greater than the native CoQ_{10}. Q_{ter} was dissolved in saline, administered by an intraperitoneal injection at a dose of 100 mg/kg. Q_{ter} solution was prepared and administered daily 1 h before styrene and/or noise exposure, until the end of treatment (5 days/3 weeks). The animals of group Ctrl+Q_{ter} received the same antioxidant treatment for a total of 3 weeks.

2.4. Functional evaluation

Hearing function was evaluated in all animals (n=135) by measuring otoacoustic emissions and Auditory Brainstem Responses (ABR) in order to assess the micromechanic dysfunction due to OHCs damage and neural transmission.

2.4.1. Distortion product otoacoustic emissions (DPOAEs)

As described previously [38] DPOAEs were measured unilaterally using an otoacoustic emission system (Tucker-Davis Technologies, Alachua, FL, USA). The f2/f1 ratio of the primary tones was set to 1.2. DPOAE input/output functions were measured at f2 frequencies of 6, 8, 12, 16, 20 and 24 kHz. The f1 intensity (L1) always presented +10 dB above the f2 intensity (L2). Animals were anaesthetized (ketamine, 35 mg/kg and medetomidine-domitor, 0.25 mg/kg) and placed on a heating pad in a sound-attenuating booth. The probe assembly was placed in the animal's external ear canal. Input/output functions were obtained by increasing L1 intensity from 20 to 70 dB SPL at f2 frequencies of 6, 8, 12, 16, 20 and 24 kHz (32 sweeps per frequency pair). DPOAEs were recorded before and 7, 14 and 21 days after the onset of treatment.

2.4.2. Auditory Brainstem responses (ABR)

Hearing function was estimated by ABR recordings, in order to identify acoustic threshold of each animal for each group. ABRs were measured at low (6 kHz), mid (12, 16, and 20 kHz), and high (24 and 32 kHz) frequencies at several time points including treatment onset (day 0) and at 7, 14 and 21 days from treatment onset. In all animals, ABRs were assessed bilaterally before treatment to assure normal hearing and reassessed at all-time points to evaluate the effect of treatments on hearing. All animals were mildly anesthetized (ketamine, 35 mg/kg and medetomidine-domitor, 0.25 mg/kg) and placed in the anechoic room. As described previously [34,38], 3 stainless steel recording electrodes were subcutaneously inserted posterior to the tested pinna (active), vertex (reference), and contralateral pinna (ground). A PC-controlled TDT System 3 (Tucker Davis Technologies, Alachua, FL, USA) data acquisition system with real-time digital signal processing was used for ABR recording and auditory stimulus generation. Tone bursts of pure tones from 6 to 32 kHz (1 ms rise/fall time, 10 ms total duration, 20/s repetition rate) were presented monaurally. Responses were filtered (0.3–3 kHz), digitized, and averaged (across 500 discrete samples at each frequency-level combination). Threshold value was defined as the lowest stimulus level that yielded a repeatable waveform-based onset.

2.5. Morphological evaluations

2.5.1. Rh–pH staining

Rh–pH is a high-affinity F-actin probe conjugated to the red–orange fluorescent dye tetramethylrhodamine (TRITC). This stain is used to visualize the stereociliary arrays and cuticular plate of hair cells (HCs). Surface preparations of the basilar membrane with the organ of Corti were processed. After the end of treatment and after functional analyses (day 21) animals were scarified with a lethal dose of anesthetic, than the right cochleae of 5 animals/group were quickly removed and processed as described below, to perform hair cell count on surface preparation of the organ of Corti. The removed cochleae were fixed with 10% buffered formalin for 4 h. After removal of the bony capsule and the lateral wall tissues, the epithelium of the organ of Corti was separated from the bony modiolus and dissected in half-turns in 0.1 M PBS under a dissecting microscope. The specimens were incubated with a solution containing 0.5% Triton X-100 and stained for F-actin with rhodamine-conjugated phalloidin (1:100 dilution; Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature protected from light. At the end of the incubation, all specimens were washed twice in PBS. Afterward, the stained specimens were mounted on slides containing an antifade medium (ProLong, Invitrogen). Quantification of HCs survival was performed with the aid of Leica Microsystems confocal microscope using filters with an excitation of 516 nm and an emission of 543 nm. Z-stack series of 3–5 μm thickness were acquired as images of 1024×1024 pixels recorded at intervals of 0.5 μm [34]. Images were taken at 40×. Positive cells were counted in segments of ~250 μm in length each along the basilar membrane. The criterion to assess HCs loss was either a dark spot and/or the typical phalangeal scar of supporting cells in the spaces previously filled by OHC [39,40]. The results are expressed as percentage of remaining HCs over the entire length of the basilar membrane [34].

2.5.2. Nissl staining

To determine SGN viability, left cochleae of 5 animals/group were used. As described previously [34], cochleae were quickly removed, and the samples were fixed with 4% paraformaldehyde in PBS at 4 °C and a pH 7.5. Next, the cochleae were decalcified for 15 d in EDTA (10%), incubated for 48 h in sucrose (30%), embedded in OCT, and cryosectioned at a thickness of 12 μm (Cryostat CM 1950; SLEE). The sections were stained with cresyl violet (0.1% for 5 min) for the histological assessment of ganglion neuronal cell damage, which is dependent on viable and nonviable stained cells. Spiral ganglion neurons (SGN) densities for each of the three cochlear turns (basal, middle and apical) were measured from five representative mid-modiolar sections. Each cochlear turn was identified, and the cross-sectional area of Rosenthal's canal within each turn was measured using NIH Image. Viable neurons with a clear round nucleus and homogeneous cytoplasm were then counted in each turn. The SGN density (cells per square millimeters) was calculated using NIH ImageJ 1.43u (Image Processing and Analysis in Java) in a 500×500 μm area. The mean number of stained cells was obtained by two researchers blinded to the experimental conditions. The cell viability from each group is presented as percentage of survival cells, normalized whit control.

2.6. Viability of supporting cells: myosin 7A staining

To perform Deiters' cells count we performed a Myosin 7A staining in the right cochleae of 5 animals/group sacrificed at day 21. Since myosin 7A expression is restricted to inner hair cells (IHCs) and OHCs, where it is found in the apical stereocilia as well as in the cytoplasm [41], we were able to distinguish clearly hair cells from Deiters' cells in surface preparations. After cochlear dissection, as described above, the specimens were incubated in a blocking solution containing 1% fatty acid-free bovine serum albumin (BSA), 0.5% Triton X-100%, and 10% normal goat serum in PBS for 1 h at room temperature. The surface preparations were then incubated overnight at 4 °C with a solution containing rabbit polyclonal anti-myosin 7A primary antibody (Proteus Biosciences, Ramona, CA, USA) diluted 1:100 in PBS. The rabbit polyclonal myosin 7A primary antibody cross-reacted with the rat tissue. At the end of the incubation, all specimens were washed twice in PBS and incubated at room temperature for 2 h, light-protected, in labeled conjugated goat anti-rabbit secondary antibody (Alexa Fluor 633, IgG; Invitrogen) diluted 1:400 in 0.1 M PBS. After another wash in PBS, samples were double stained with DAPI (blue fluorescence, 1:500) for 20 min in the dark at room temperature. DAPI labeling was used to identify cell nuclei. Then, specimens were mounted on slides

containing an antifade medium (ProLong, Invitrogen). Fluorescence images (40×) were obtained with the aid of a confocal laser scanning system. As regard cell count, Deiters' cells were considered missing when DAPI staining was absent. The results were expressed as a percentage of remaining cells.

2.7. Oxidative stress evaluation

Dihydroethidium (DHE) and 8-isoprostane immunostainings were used to assess superoxide anion and lipid peroxidation production respectively. At day 21, the animals were scarified with a lethal dose of anesthetic and the cochleae were quickly removed. The samples were fixed with 4% paraformaldehyde in PBS at 4 °C and a pH 7.5. Next, the cochleae were decalcified for 15 d in EDTA (10% EDTA, changed daily), incubated for 48 h in sucrose (30%), embedded in OCT, and cryosectioned at a thickness of 12 μm (Cryostat CM 1950; SLEE). Right cochleae were used for DHE assay, whereas the left cochleae of the same animals were used for 8-Isoprostane immunofluorescence in a total of 5 animals/group. The optical density analysis was performed to quantify DHE and 8-Isoprostane fluorescence signals (LCSLite, Leica Confocal Software) in the organ of Corti, SGNs and *stria vascularis*.

2.7.1. Detection of superoxide anion formation (DHE assay)

DHE is a lipophilic cell-permeable dye that is rapidly oxidized to ethidium in the presence of free radical superoxide. In theory, the produced ethidium is fixed by intercalation into nDNA, it gives an indication of oxidant stress within cells undergoing investigation [34]. However, the specificity of ethidium for superoxide anion in vivo is limited by auto-oxidation as well as by non superoxide-dependent cellular processes that can oxidize ethidium probes. Conventional fluorescence techniques exciting at 510 nm cannot overcome this bias [42]. However, in order to obtain minimal interference from other nonspecific oxidation products we imaged the staining by two-photon excitation (792 nm, < 140 fs, 90 MHz) performed by ultrafast tunable mode locked titanium: sapphire laser (Chameleon; Coherent). The cochlear specimens were incubated with 1 μm DHE (Invitrogen D23107, Carlsbad, CA, USA) in PBS for 30 min at 37 °C and then cover slipped with an antifade medium (ProLong Gold, Invitrogen P36930). Images were taken at 20×.

2.7.2. 8-Isoprostane immunostaining

In order to detect the level of lipid peroxidation, the slides were incubated in a blocking solution containing 1% fatty acid-free bovine serum albumin (BSA), 0.5% Triton X-100, and 10% normal goat serum in PBS for 1 h at room temperature. The specimens were then incubated overnight at 4 °C with a solution containing rabbit polyclonal anti-8-Isoprostane primary antibody (Oxford Biomedical Research, product id: IS20) diluted 1:100 in PBS. The rabbit polyclonal anti-8-Isoprostane primary antibody cross-reacted with the rat tissue. At the end of the incubation, all slides were washed twice in PBS and incubated at room temperature for 2 h, light-protected, in labeled conjugated goat anti-rabbit secondary antibody (Alexa Fluor 488, IgG; Invitrogen) diluted 1:400 in 0.1 M PBS. After another wash in PBS, samples were double stained with DAPI (blue fluorescence, 1:500) for 20 min in the dark at room temperature. DAPI labeling was used to identify cell nuclei. The slides were cover-slipped with an antifade medium (ProLong Gold; Invitrogen). Images of 8-Isoprostane immunolabeled specimens (20×) were taken by a confocal laser scanning microscope (Leica Microsystems) equipped with an argon/argon–krypton and a helium/neon lasers for 488 and 519 excitation. DAPI staining was imaged by two-photon excitation (740 nm, < 140 fs, 90 MHz) performed by an ultrafast tunable mode-locked titanium: sapphire laser. The 8-Isoprostane positive cells were identified by green fluorescence. No spontaneous fluorescence was observed in control experiments performed omitting the primary antibody, indicating the antibody specify. Tissues from all groups were always processed together during the procedures to limit variability related to antibody penetration, incubation time, post-sectioning age, and condition of tissue.

2.8. Measurement of endogenous antioxidant defenses: SODs immunostaining

The analyses were performed on the left cochleae of the same 5 animals/group (Control and Q_{ter} treaded groups) used for myosin 7A staining. As previously reported [43], the specimens were incubated with a blocking solution (1% BSA, 0.5% Tritonx-100, and 10% normal goat serum in PBS 0.1 M) and then the slices were incubated overnight at 4 °C with a solution containing anti-SOD1 and anti-SOD2 (anti-SOD1: Abcam, Cambridge, UK; anti-SOD2: Upstate, NY, USA) diluted1:100 in PBS. All specimens were incubated at room temperature for 2 h in labeled conjugated goat anti-rabbit or donkey anti-mouse secondary antibody (Alexa Fluor 488 and 546, IgG, Invitrogen) diluted 1:400 in 0.1 M PBS and DAPI stained (1:500 in 0.1 M PBS). Images were obtained with a confocal microscope system (Nikon Ti-E, Confocal Head A1 MP, Japan).

2.9. Fluorescence spectrum intensity analysis

In order to assess separately cell membrane lipid peroxidation and SOD activation in OHCs and Deiters' cells, we analyzed cryosections of the organ of Corti immunolabeled, with DAPI, 8-Isoprostane, SOD1 and SOD2 as above described. A square-region of interest (ROI) was defined so as to include the entire organ of Corti. Lastly a sub-ROI was defined to include a single Deiters' cell and OHC on the same longitudinal axis. The confocal acquisition frames were rotated in order to orientate OHCs and Deiters' cells on the same longitudinal axes. A vertical readout of fluorescence signals was performed for each OHC and underlying Deiters' cell, minimizing the effects of an erratic side-summation of the immunofluorescence signal. The analysis was performed on 3 separate ROI including a single OHC and underlying Deiters' cell on each cryosection (n=10), with an interval trimming of 15 μm, in 5 cochleae/group. The separate fluorescence readouts for detectable OHCs/Deiters' cells were mediated in order to draw a vertical Corti's optical density plot profile. On the Corti-focused ROI, a fluorescence spectrum intensity analysis was performed for 8-Isoprostane, SOD1, SOD2 and DAPI labeled specimens, to confirm the vertical readout data and better relate fluorescence signal variation with the position of OHC and Deiters' cell nuclei.

2.10. Statistical analyses

Results are presented as means ± standard error of the mean (SEM) and differences were assessed by using variance analysis (ABR data: group×frequency×time point, three-way ANOVA with repeated measures; DPOAE data: group×level×time point, three-way ANOVA with repeated measures; cochleogram data and DC count: groups×cochlear turn, two-way ANOVA with repeated measures. *Post-hoc* comparisons were assessed with Tukey's test (Statistica, Statsoft, Tulsa, OK, USA); p value < 0.05 was considered significant.

3. Results

3.1. Functional analysis

3.1.1. DPOAEs

To evaluate the effect of noise and/or styrene administration and the protective effect of Q_{ter} on OHCs function, DPOAEs were tested in all animals at 4, 8, 12, 16 and 20 kHz and before and 7, 14 and 21 days after treatments. However, only 16 kHz data, the most affected frequency, are shown in Fig. 1. Baseline DPOAE values were not altered in Ctrl+Q_{ter} and in Ctrl-Gavage group (data not shown). In all

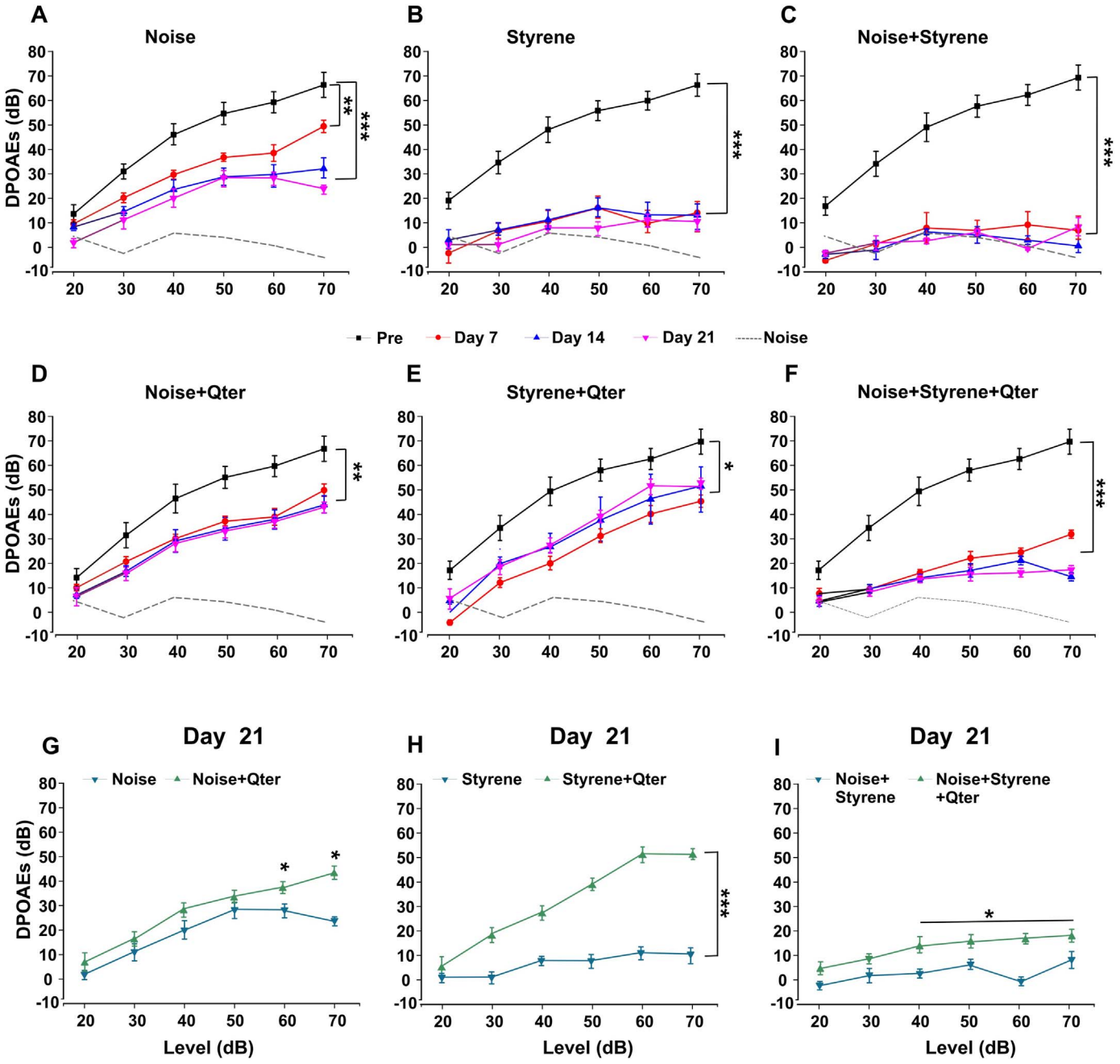

Fig. 1. OHC function altered by noise and styrene is protected by Q_{ter} supplementation. DPOAE amplitude trend over 4 time points (pre exposure and at days 7,14 and 21 after the onset of exposures, 16 kHz frequency shown). In noise exposed animals, progressive DPOAE amplitude decrease (A); in Styrene and Noise+Styrene groups (B,C), a rapid and drastic DPOAE decrease was observed (background noise level already at day 7). The functional impairment is partially counteracted by Q_{ter} administration (D,E,F). Comparison between DPOAE values in Noise and Noise+ Q_{ter} (G), Styrene and Styrene+Q_{ter} (H), as well as Noise+Styrene and Noise+Styrene+Q_{ter} (I) at the end of treatment day 21 are shown. ***$p < 0.0001$, **$p < 0.001$, *$p < 0.05$.

treated groups there was a decrease of DPOAE amplitude suggesting a micromechanic cochlear dysfunction. Specifically, in noise exposed animals, DPOAE amplitudes decreased progressively from day 7 to day 21, reaching about 15–20 dB (Fig. 1A) whereas in the Styrene group, a drastic drop in amplitude was observed already at day 7, without a further worsening over the subsequent time points (Fig. 1B). The combined exposure of noise and styrene induced a pronounced decrease in DPOAE responses as the amplitudes were not different from background noise (Fig. 1C). Specifically, in the combined condition, DPOAE amplitudes were slightly worsen compared to styrene exposure, indicating that styrene impaired mostly OHCs, while noise exposure produced an additional sensitization to styrene. Q_{ter} treatment significantly attenuated DPOAE drop in Noise+Q_{ter} and Styrene +Q_{ter} groups (Fig. 1D-E,G-H). Specifically, in Styrene+Q_{ter} animals, the antioxidant protection was more evident at days 14 and 21 of treatment (Fig. 1E,H). In animals exposed to noise and styrene, Q_{ter} administration induced a slight attenuation (about 10–15 dB) of DPOAE level, already after 7 days of treatment (Fig. 1F). Apparently, in styrene exposed cochleae, a part of the functional impairment was not solely related to a loss of vital OHC, but also due to a non-lethal functional impairment of the remaining OHCs. This phenomenon was not observed in the combined exposure where the highest degree of

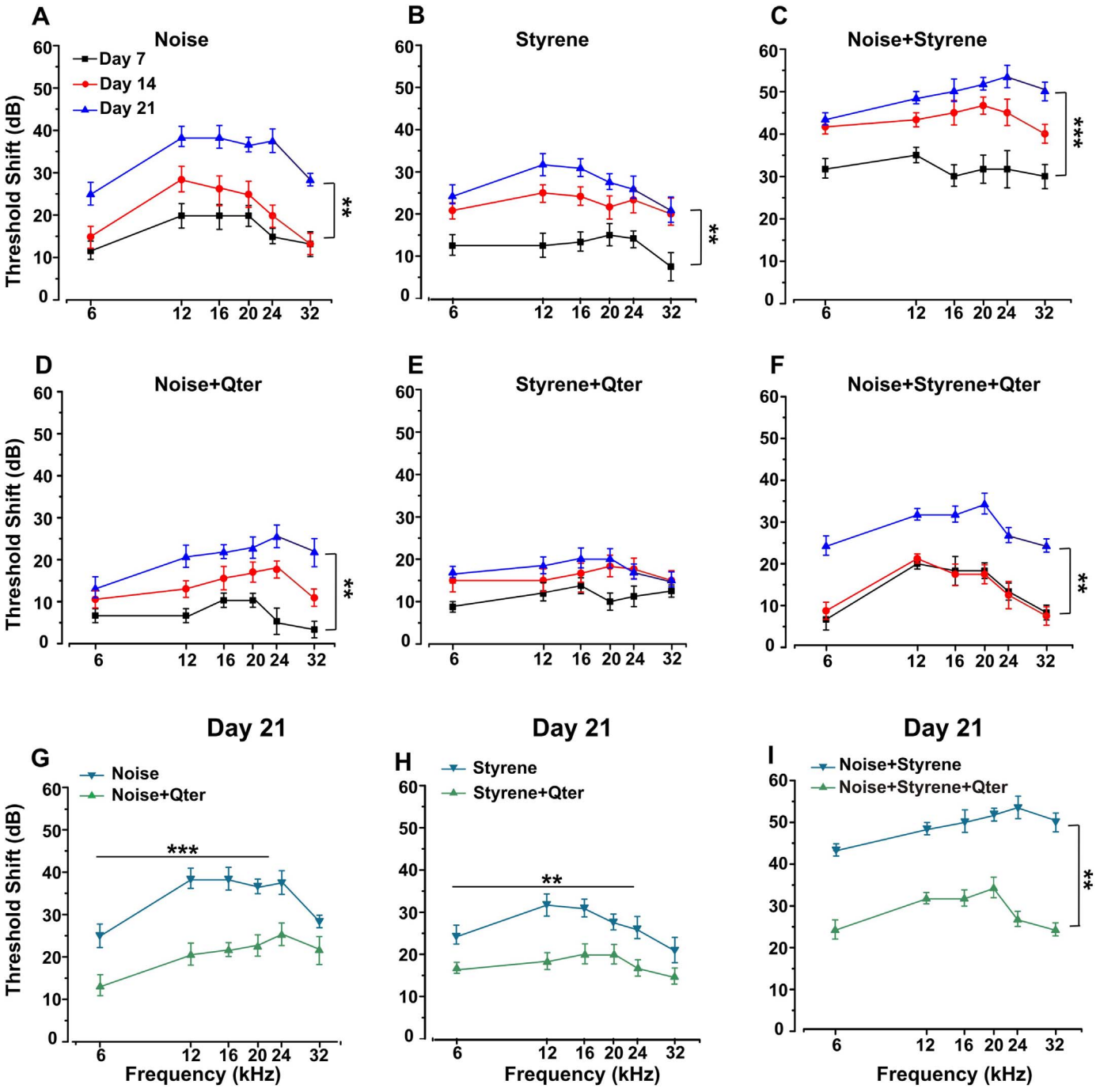

Fig. 2. Noise and styrene functional impairment is attenuated by the antioxidant treatment. ABR functional evaluation, measured at the same time points as above (data expressed as mean threshold shift values ± SEM). Results indicate a similar threshold shift impairment following noise (A) and styrene (B) exposures; a greater threshold shift impairment in Noise +Styrene group (C) was observed. Q_{ter} attenuates the functional impairment in all experimental groups (D-F). Specifically, a comparison between Noise, Styrene, Noise+Styrene groups and animals treated with antioxidant at day 21 is shown in lower panels (G-I). ***$p < 0.0001$, **$p < 0.001$, *$p < 0.05$.

protection did not fluctuate over time. These findings could indicate that DPOAE amelioration in the Noise+Styrene+Q_{ter} group, was due to an early antioxidant protection provided by Q_{ter} against the noise sensitization.

3.1.2. ABRs

In order to determine hearing loss induced by styrene and/or noise exposure, ABRs were recorded before noise exposure and/or styrene administration and 7, 14 and 21 days after the onset of treatment. Baseline ABR values were altered neither in Ctrl+Q_{ter} nor in Ctrl-Gavage group (data not shown). The results are shown as threshold shift values (Fig. 2). In the noise-exposed animals, ABR thresholds were elevated at day 7 and progressively increased at day 14 and day 21 (Fig. 2A), indicating a worsening threshold shift pattern with repeated noise exposures. Specifically, in the Noise group, the ABR threshold shift, across mid and high frequencies (12–24 kHz), measured about 15 dB at day 7 and progressively worsened with time, reaching about 20–25 dB at day 14 and about 40 dB at day 21 (Fig. 2A,G). In the Styrene group at day 7, ABR threshold shift was about 15 dB across mid and high frequencies (Fig. 2B). At day 14 threshold shift worsened

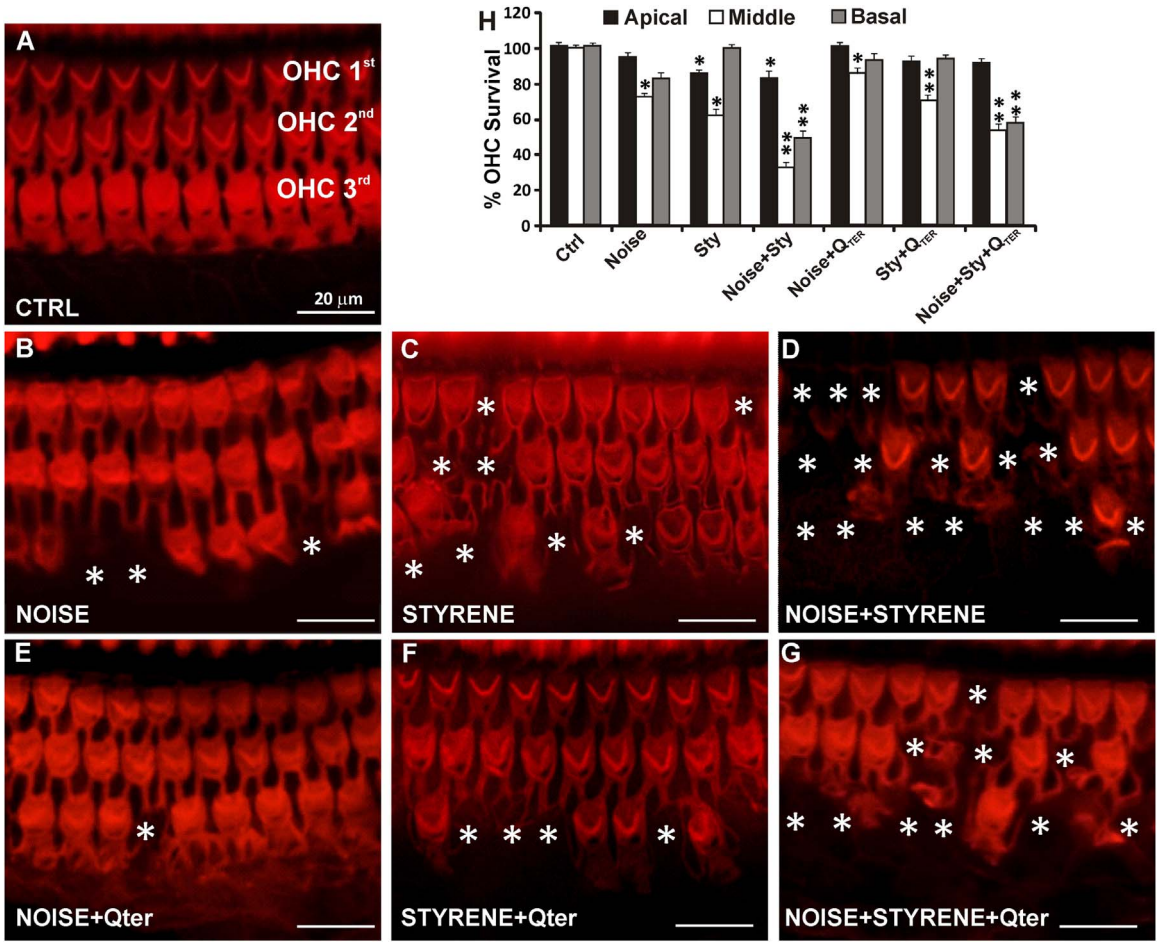

Fig. 3. Q_{ter} prevents OHC loss in both noise and styrene exposures. Representative images of surface preparation of the organ of Corti showing F-actin distribution in the middle cochlear turn. Normal cochlear organization is represented in control image A (Ctrl): OHC three rows are well aligned. In Noise group (B) a severe OHC loss was observed as indicated in OHC cochleogram (H; means ± SEM) and mainly distributed in the third OHC row. A greater OHC loss was produced by styrene exposure (C). A massive OHC loss, up to 65–70%, was detected in combined exposure (D,H). In all groups, the antioxidant treatment counteracts significantly the effects of exposures (E,F,G,H). White asterisks indicate missing OHCs (dark spots). In H, asterisks are referred to significant differences vs control group. ***p < 0.0001, **p < 0.001, *p < 0.05; Scale bar: (A-G), 20 μm.

slightly, reaching about 25 dB. Lastly, at the end of treatment (day 21), the ABR threshold shift reached about 30 dB (Fig. 2 B,H). The combined exposure in Noise+Styrene group, caused an early noticeably higher ABR threshold shift of about 30–35 dB at day 7 (Fig. 2C). At day 14, ABR threshold shift was about 45 dB, followed by a further slightly worsening at day 21, when the threshold shift value reached 50–55 dB (Fig. 2C,I).

Q_{ter} treatment significantly reduced functional damage (Fig. 2D-I). In Noise+Q_{ter} group threshold shift worsened over time but the functional damage was attenuated at all time points with respect to Noise group, showing at the end of treatment a threshold shift of about 20 dB (Fig. 2D,G). In Styrene+Q_{ter} group threshold shift decreased of about 10–15 dB after 14 and 21 days from the onset of treatment with respect to Styrene group (Fig. 2E,H). Interestingly, in Noise+Styrene +Q_{ter} group the antioxidant administration showed the highest protection at days 7 and 14 (Fig. 2F); at day 21 the threshold shift attenuation was about 20 dB as compared to Noise+Styrene group (Fig. 2I). Q_{ter} treatment produced a significant threshold shift amelioration in the Noise group and a slight improvement in the Styrene group. In combined exposure thresholds were ameliorated during the entire period, similarly to what was observed in the Noise group. Considering this impairment trend, we speculate that threshold alteration was predominantly induced by noise and increased, during the entire trial period, by the concomitant styrene exposure.

3.2. Rh-pH staining and hair cell count

In order to evaluate OHCs survival we performed Rh–pH staining (Fig. 3). In Ctrl animals, OHC surface was characterized by an orderly arrangement of the three rows of OHCs and one row of IHCs (Fig. 3A). In Noise group cell loss was characterized by dark spots (asterisks), phalangeal scars and disappearance of cuticular plate (Fig. 3B). After noise exposure, the morphological damage involved mainly OHCs of the outer row. Specifically, cell loss was about 25% in the middle turn, 15% in the basal turn and less than 5% in the apical turn (Fig. 3H). In the Styrene group a marked OHC loss was observed (Fig. 3C). The damage was more pronounced in the cochlear middle turn, where cell loss was about 40%, whereas in the apical and basal turns cell death was about 15% and 5% respectively (Fig. 3H). Notably, cell loss showed an irregular distribution between the three rows of OHCs: the damage was more evident in the outer row and it was progressively less pronounced in the middle and inner rows (Fig. 3C). In the Noise +Styrene group OHC outer row was completely destroyed and the middle and inner rows were also markedly damaged (Fig. 3D). OHC damage affected not only the middle turn, where the OHC loss was about 70%, but also the basal and apical turns, where the OHC loss was about 50% and 20% respectively (Fig. 3H). Thus, cell death in Noise +Styrene group exceeded the sum of OHC loss measured in Noise and Styrene groups, indicating a synergistic effect in the combined exposure. Consistent with functional data, Q_{ter} administration signifi-

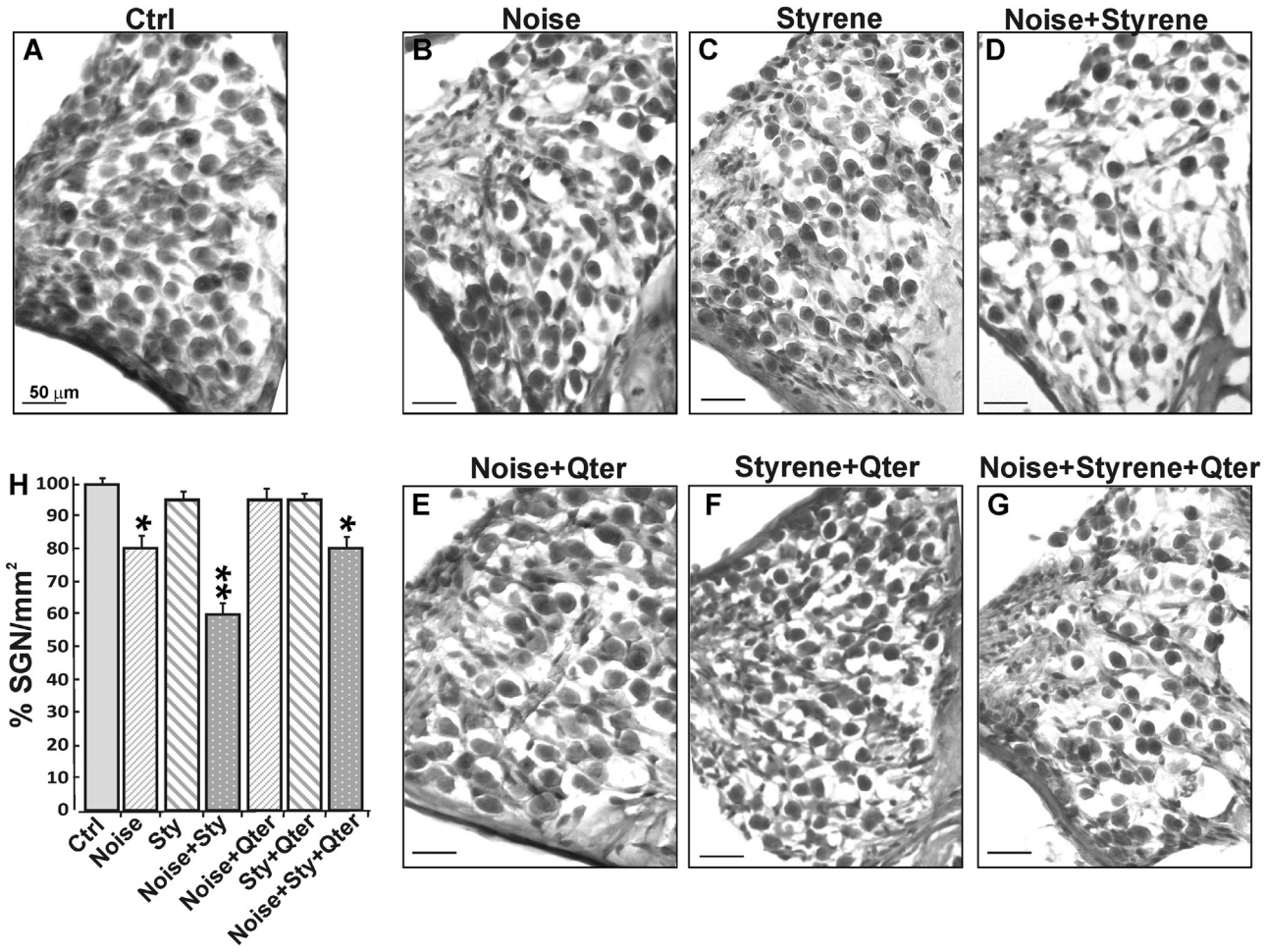

Fig. 4. SGN loss caused by noise exposure is increased by styrene sensitization. Representative Nissl images of SGN cryo-sections at middle cochlear turn level (the most affected cochlear region). A: normal morphology of SGNs in Ctrl group. B,C and D show representative SGN loss in Noise, Styrene and Noise+Styrene groups respectively. As shown in SGN cochleogram (H; means ± SEM), noise exposure causes a greater SGN loss compared to styrene exposure. Panels E,F,G refer to Noise+Q_{ter}, Styrene+Q_{ter} and Noise+Styrene+Q_{ter} groups. The combined exposure produces a greater SGN loss than the sum of single exposures. Q_{ter} administration counteracts significantly SGN loss, especially in noise and combined exposures (H). Asterisks are referred to significant differences vs control group.**$p < 0.001$, *$p < 0.05$. Scale bar: (A-G), 50 μm.

cantly attenuated cell loss in all groups. As shown in Fig. 3 (E-H), in Q_{ter} treated groups OHC loss was significantly reduced and the hair bundle arrangement was primarily restored. In the cochlear middle turn, OHC loss was about 15% in Noise+Q_{ter} group, about 30% in Styrene+Q_{ter} group and about 40% in Noise+Styrene+Q_{ter} group (Fig. 3H), indicating that cell loss was significantly reduced by the antioxidant protection. Overall, Styrene and noise exposures produced a similar OHC loss in both cases, mainly located in the middle cochlear turn, the most affected cochlear region. The location-dependency of the disruption can be explained by the frequency specificity of noise exposure [32] and the distribution gradient of styrene in the cochlea [11]. Nevertheless, noise targeted only the third OHC row; in fact, these cells show a more severe metabolic impairment with respect to the inner rows in noise exposed animals, as shown by the different spatial distributions of NAD(P)H oxidation [44]. On the contrary, following styrene damage, all OHC rows were implicated, from the middle up to the apical turn (although to a lesser extent). In the combined exposure, the damage was more severe (up to 70% OHC loss) but not along the apical turn, confirming that this cochlear region is targeted only by styrene.

3.3. SGN viability

Fig. 4 shows the Nissl staining and SGN viability. In Ctrl group, the Rosenthal's canal was densely packed with SGNs and fascicles of auditory nerve fibers (Fig. 4A). After noise exposure, SGN density was reduced (Fig. 4B). SGN number was reduced of about 20%. After styrene administration SGN number decreased (Fig. 4C), however a smaller number of SGNs was lost (about 5%) as compared to Noise group (Fig. 4H). The most extensive reductions in SGN density occurred after combined exposure to noise and styrene (Fig. 4D), where a cell loss of about 40% was observed (Fig. 4H). Interestingly, Q_{ter} administration increased the number of preserved SGNs in all experimental groups (Fig. 4E-G), specifically in Noise+Styrene+Q_{ter} group, where cell survival reached about 80% (Fig. 4H). In summary, the antioxidant treatment provided a greater protection in the combined group. These findings confirm the hypothesis of a synergistic amplifying interaction between noise and styrene.

3.4. Myosin 7A staining and DC count

In order to identify Deiters' cells and to perform precisely cell count, we used, on surface preparations of the organ of Corti, both myosin 7A and DAPI staining. Myosin 7A staining is specific for OHCs and not for Deiters' cells. In this way, we analyzed separately Deiters' cell and OHC planes, overcoming a risk of erratic cell loss count due to the possibility of surface preparation geometry irregularities. Representative images of surface preparation of the organ of Corti

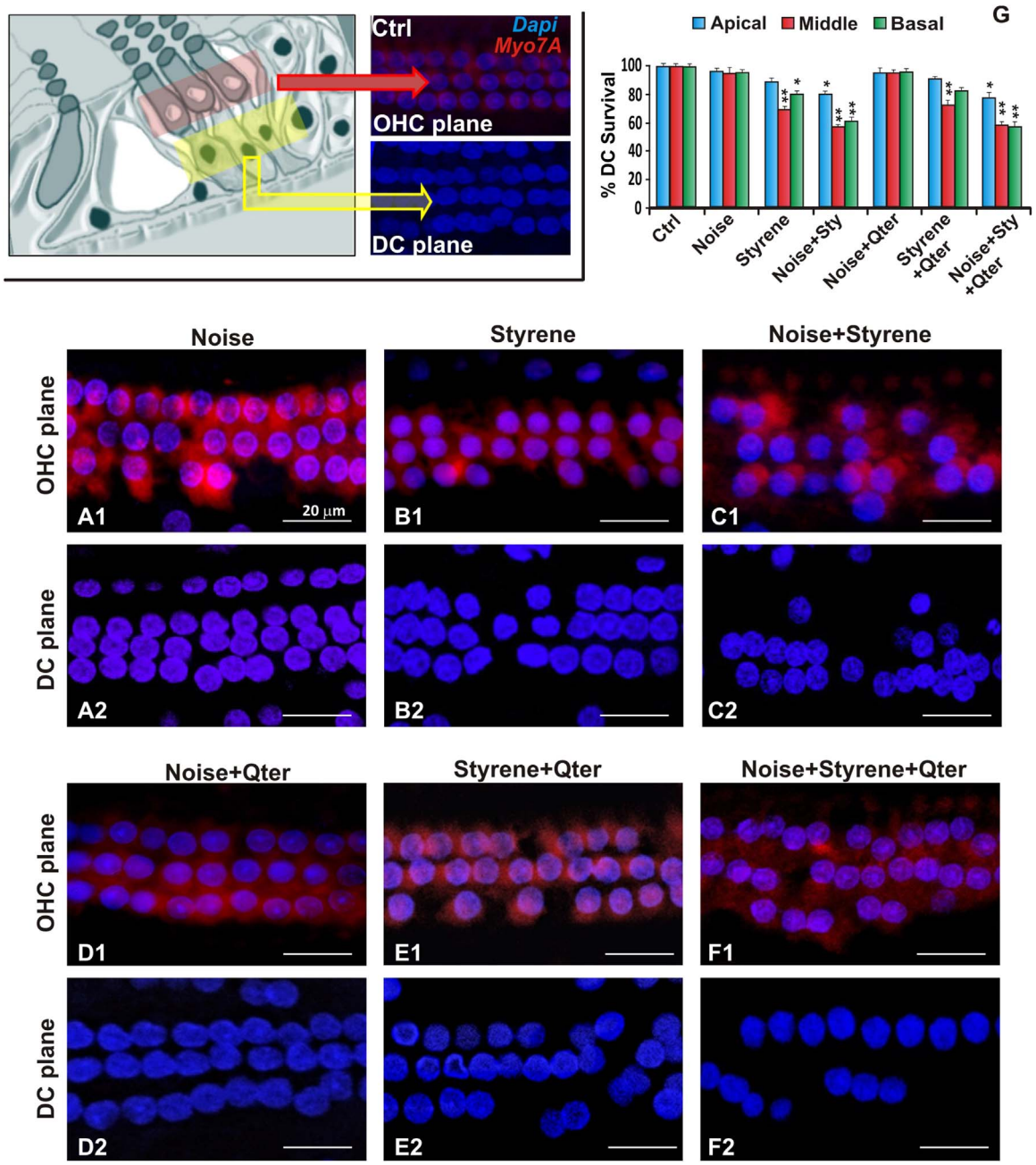

Fig. 5. Styrene affects Deiters' cell survival. Schematic representation of OHC-Deiters' units (upper left panel) and representative images of surface preparations of OHCs and Deiters' cells (DC) double stained with DAPI (blue) and Myosin 7A (red, as a marker of cuticular plane) in Ctrl group. A-C: representative images of OHC (A1, B1, C1) and Deiters' layer (A2, B2, C2) for Noise (A), Styrene (B) and Noise+Styrene (C) groups. In noise exposed specimens Deiters' cells are not affected (A2), while in Styrene group a significant Deiters' loss is detected (B2). A further increase in Deiters'loss is present in the combined exposure (C2). D-F: Confocal acquisition frames scanned at OHC (D1, E1, F1) and Deiters' level (D2, E2, F2). In all groups Q_{ter} fails to restore Deiters' loss, as shown by cell count (O; means ± SEM). In O, asterisks are referred to significant differences vs control group.**$p < 0.001$, *$p < 0.05$. Scale bar: (A-F), 20 μm.

showing OHC and Deiters' cell plane are shown in Fig. 5. Noise exposure induced OHC loss (Fig. 5A1), without affecting significantly Deiters' cell viability (Fig. 5A2): sporadic Deiters' cell loss was observed in the apical, middle and basal turns of noise-exposed cochleae (Fig. 5G). Q_{ter} administration attenuated OHC loss (compare in Fig. 5: A1 with D1) without affecting the noise induced Deiters' cell slight damage (compare in Fig. 5: A2 with D2). Styrene exposure affected both OHC and Deiters' cell viability (Fig. 5B1-B2). After styrene exposure Deiters' cell loss reached about 10% in the apical turn, 30% in the middle turn and 20% in the basal turn (Fig. 5G). Notably, the antioxidant treatment with Q_{ter} failed to restore Deiters' cell loss in styrene exposed cochleae (Fig. 5E2) and the percentage of Deiters' cell survival remained unchanged with respect to styrene group, as shown in Deiters' cell cochleogram (Fig. 5G). The combined exposure of noise and styrene caused a great OHC injury in conjunction with a severe loss of the supporting cells (Fig. 5C1-C2). Deiters' cell count showed a cell loss percentage of about 20% in the apical and about 40% in the middle and basal turns. Once again, Q_{ter} supplementation did not counteract styrene+noise cytotoxic effect on Deiters' cells (compare Fig. 5C2 and F2).

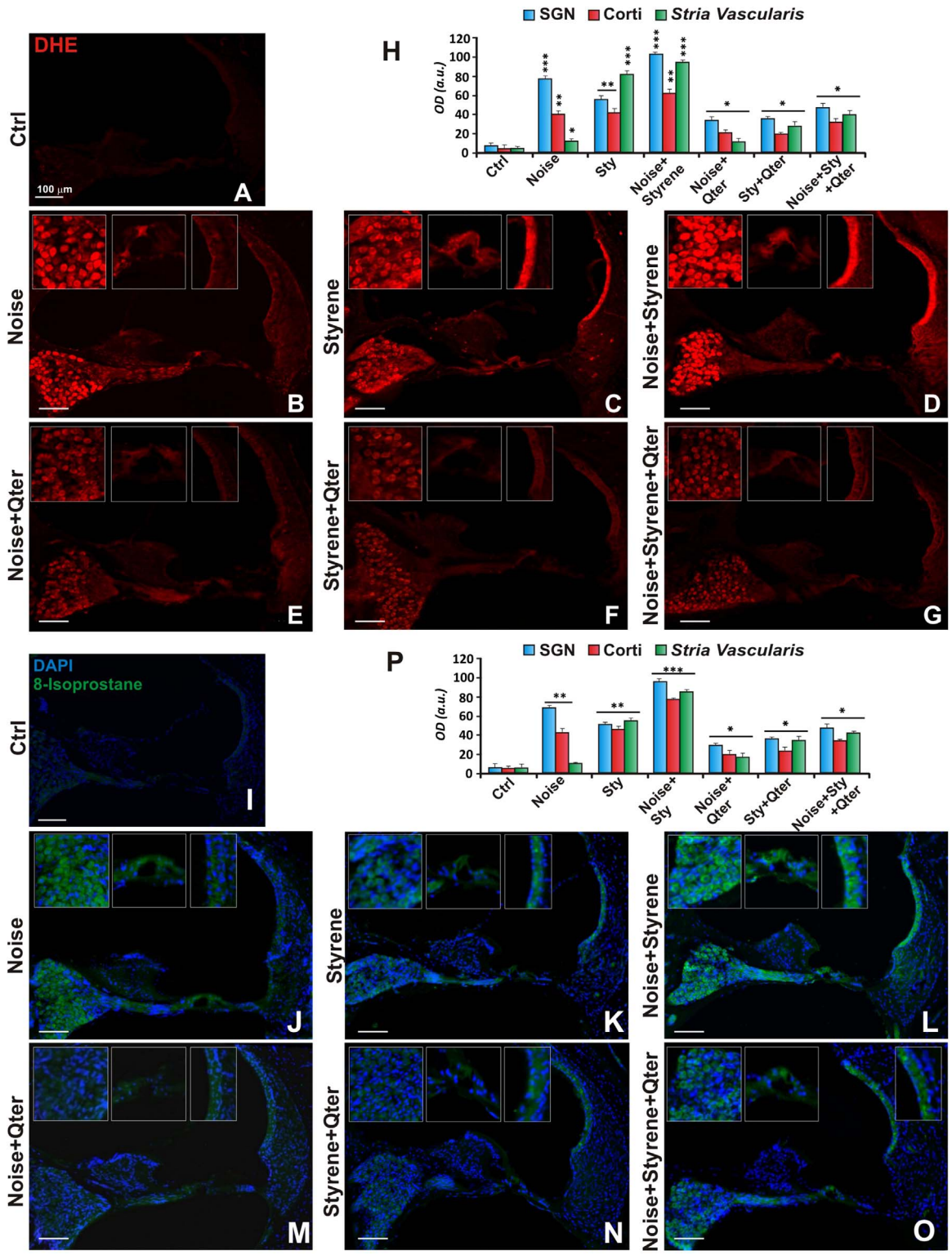

Fig. 6. Q_{ter} supplementation reduces superoxide amount and lipid peroxidation in both noise and styrene exposures. Representative images of cochlear cryo-section stained with DHE (A-G) or 8-Isoprostane and DAPI staining (I-O). DHE staining (red) is faint in the cytoplasm of control cochleae (A). Noise exposure increases superoxide amount, mainly in SGNs and organ of Corti (B). In styrene exposed cochleae (C) and, even more in combined exposure (D), a greater increase of superoxide expression involves SNGs, the organ of Corti and *stria vascularis*. Q_{ter} treatment decreases significantly superoxide expression in all main cochlear structures (E,F,G). H: optical density (OD) analysis (means ± SEM) is reported. An increase of lipid peroxidation (green fluorescence) is observed in Noise (J), Styrene (K) and Noise+Styrene (L) with respect to Ctrl cochleae (I). The antioxidant supplementation reduces 8-Isoproistane expression in all experimental groups (M-O). P: OD analysis for 8-Isoprostene immunostaining (means ± SEM). In H and P asterisks are referred to significant differences vs control group. ***$p < 0.0001$, **$p < 0.001$, *$p < 0.05$.

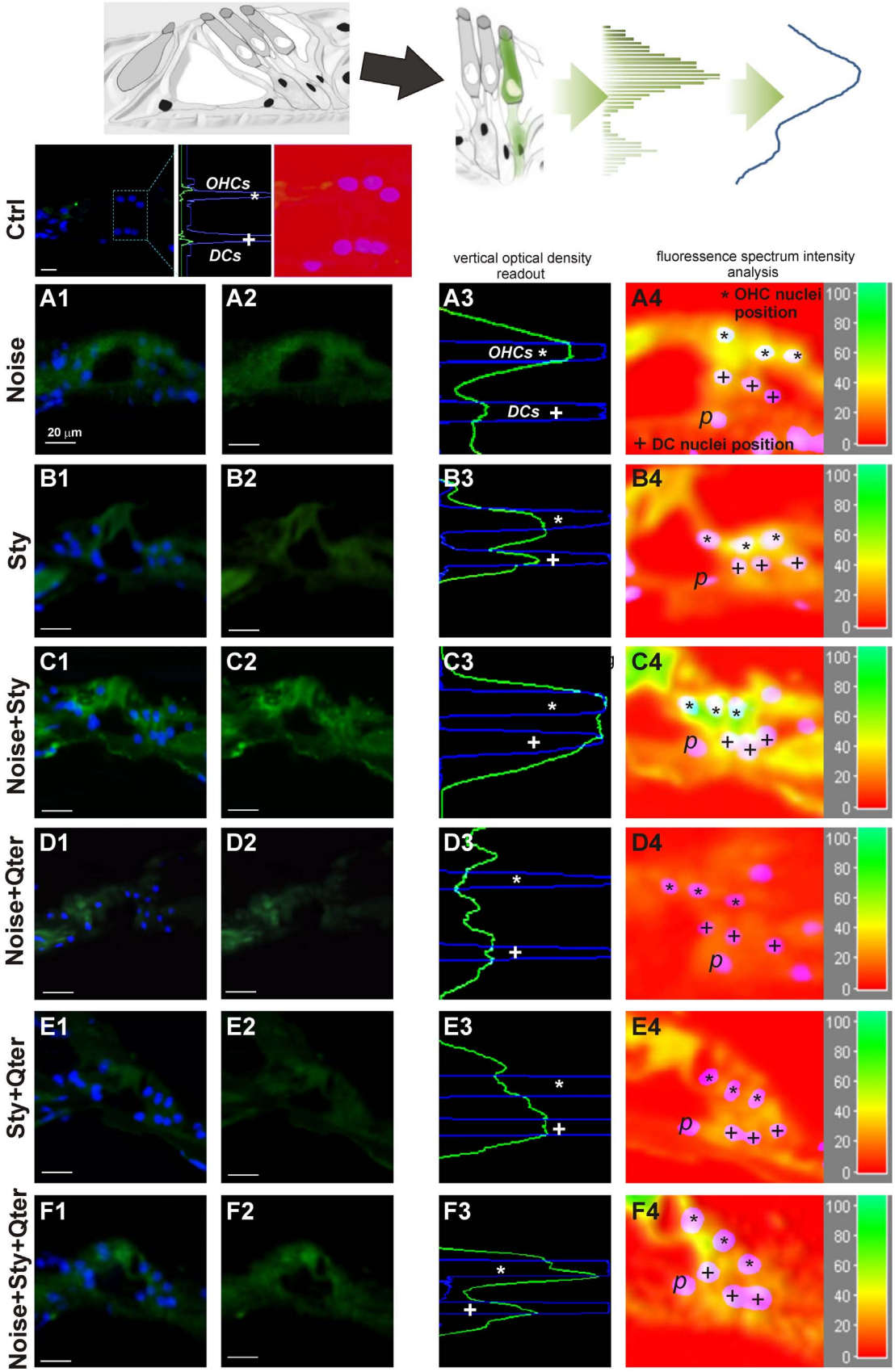

Fig. 7. Q_{ter} does not attenuate lipid peroxidation in Deiters' cell layer. Upper panel: schematic representation of OHC-DC units showing the method used to detect the vertical optical density readout and the related fluorescence spectrum intensity. Left panels: Magnification of the organ of Corti stained with 8-Isoprostane and DAPI labeling in Noise (A1-A2), Styrene (B1-B2), Noise+Styrene (C1-C2), Noise+Q_{ter} (D1-D2), Styrene+Q_{ter} (E1-E2) and Noise+Styrene+Q_{ter} (F1-F2). Right panels: Vertical readout of fluorescence matched with the fluorescence spectrum intensity analysis (A3-F3) and a pseudo-colored rainbow scale spanning from red (low fluorescence intensity) to green (high fluorescence intensity) in OHC and DC plane (A4-F4) are shown. In the Noise group (A1-A4) lipid peroxidation involves mainly OHC layer and only slightly DC layer (compare marker * with marker + in A3-A4). A greater involvement of DC layer in Styrene group (B1-B2) compared to Noise specimens is shown (compare marker + in B3-B4 with marker + in A3-A4). Q_{ter} treatment reduced lipid peroxidation amount in all treated groups (D1-F4). However, the antioxidant failed to counteract lipid peroxidation in DC layer (compare marker + in E3-E4; F3-F4 with marker + in B3-B4 and C1-C4). p= Pillar Cells. A1-F2: scale bar 20 μm.

3.5. Level of Oxidative stress

3.5.1. Superoxide detection

Fig. 6 illustrates the oxidative damage in cochlear cryosections using DHE assay (upper panel) for the superoxide detection. Red fluorescence was faint in the unexposed cochleae (Fig. 6A). In noise exposed animals the fluorescence increased, specifically in SGNs and in the organ of Corti (Fig. 6B), as also illustrated by optical density analysis (Fig. 6H). The superoxide amount was high in styrene exposed animals (Fig. 6C) and interestingly, fluorescence was evident not only in SGNs and in the organ of Corti, but also in the *stria vascularis*. A further fluorescence increase was observed in the Noise+Styrene treated animals (Fig. 6D) in all cochlear structures. The oxidative stress induced by noise and styrene exposure was counteracted by Q_{ter} administration: the specimens of all Q_{ter} treated groups exhibited a significant decrease of superoxide production (Fig. 6E-G) as compared to Noise, Styrene and Noise+Styrene groups in all cochlear structures.

3.5.2. Lipid peroxidation

To determine the level of lipid peroxidation, we performed 8-Isoprostane immunofluorescence in cochlear cryosections. Representative images and fluorescence quantification are illustrated in the lower panel of Fig. 6. Similarly to DHE, in control cochleae there was a faint green fluorescence (Fig. 6I). In noise exposed animals (Fig. 6J) green fluorescence increased in all cochlear structures, indicating a lipid peroxidative damage. Styrene administration induced an 8-Isoprostane marked expression, mainly localized in the *stria vascularis* and SGNs (Fig. 6K). A further increase of fluorescence was observed in Noise+Styrene treated animals (Fig. 6L), involving SGNs, the organ of Corti and the *stria vascularis*. Noise and styrene exposure effects were counteracted by Q_{ter} administration, that reduced significantly the total lipid peroxidation amount in all cochlear structures (Fig. 6M-O). Quantitative analyses confirmed the immunostaining observations (Fig. 6P).

3.5.3. Fluorescence spectrum intensity analysis: 8-Isoprostane

A fluorescence optical density analysis (Fig. 7), performed on the organ of Corti substructures, showed a marked increase of lipid peroxidation in both noise and styrene exposed groups and even a greater fluorescence increase in the combined exposure, as compared to control group. However, as shown by fluorescence vertical readout analysis, in the Noise group (Fig. 7A1-A4), lipid peroxidation involved mainly OHC layer with a negligible extent on Deiters' cell layer. In the Styrene group (Fig. 7B1-B4), lipid peroxidation increase showed a different pattern, with a greater involvement of Deiters' cell layer compared to noise. A further increase in lipid peroxidation was detected in the combined exposure, in both OHC and Deiters' cell layers (Fig. 7C1-C4). Q_{ter} treatment reduced lipid peroxidation amount in all treated groups (Fig. 7D1-F4). Interestingly, vertical fluorescence readout showed that the antioxidant was able to counteract lipid peroxidation in the OHC layer in Noise+Q_{ter}, Styrene+Q_{ter} and Noise +Styrene+Q_{ter} treated groups while, in contrast, the level of lipid peroxidation in Deiters' cells did not show any significant amelioration due to Q_{ter} treatment.

3.5.4. Fluorescence spectrum intensity analysis: SODs

Immunofluorescence labeling performed on the organ of Corti was faint in Ctrl group (data not shown), however, a marked increase of both SOD1 and SOD2 expression after Q_{ter} treatment was observed in all experimental groups (Fig. 8). The fluorescence optical density analysis on the organ of Corti substructures revealed an increase of SOD fluorescence signals in OHC layer, whereas only a slight labeling in Deiters' cell layer was found in animals exposed to Noise (Fig. 8A1-A4 and D1-D4), Styrene (Fig. 8B1-B4 and E1-E4) and Noise+Styrene (Fig. 8C1-C4 and F1-F4). Moreover, the vertical fluorescence readout showed a similar pattern of SOD1 and SOD2 activation in all the analyzed specimens. These results indicate that the protective properties of the antioxidant, and its ability to potentiate the endogenous antioxidant defenses, target specifically the mitochondrial impairment in the sensory epithelium (OHCs). The marked expression of SODs in OHC as compared to Deiters' layer following Q_{ter} supplementation suggests a different mechanism of ROS production in noise and styrene cochlear injury.

4. Discussion

In this study we analyzed in Wistar rats the effects on hearing of chronic exposure to loud noise, to styrene and a combination of noise plus styrene. Briefly, the salient results of this study are: a) noise, styrene and the combined exposure of noise and styrene disrupt auditory function and cochlear morphology by involving oxidative stress mechanisms, b) styrene ototoxicity targets the viability of Deiters' cells, conversely to what observed after the noise-induced damage, c) the antioxidant supplementation with Q_{ter} reduces both functional and OHC damage, decreases the redox imbalance in the main cochlear structures after noise or/and styrene exposures but does not prevent Deiters' cell loss in the animals treated with styrene and the combined noise plus styrene exposure.

4.1. Noise, Styrene and combined exposure disrupt the auditory function and cochlear morphology

Both chronic noise exposure and styrene administration by gavage induced a significant alteration of DPOAEs and ABRs however, the time course of the hearing impairment was different. Our functional data extend previous literature reports [11,12,15], inasmuch we highlight a predominant styrene disruption of OHC micromechanics with respect to noise as indicated by DPOAE analysis. Conversely, we observed a greater auditory threshold elevation by noise with respect to styrene as shown by ABRs. It can be suggested that noise induces changes in synaptic connections, considering that loss of synaptic elements is already essentially complete after a single 98 dB noise exposure as reported in literature [45,46]. In the combined exposure, the neural damage due to noise was added to the OHC micromechanic alteration induced by styrene. The functional data were confirmed by the morphological analysis. Indeed, a significant decrease of OHCs and SGNs was detected both in Noise and Styrene groups and accentuated in the combined exposure, confirming the hypothesis of a synergistic amplifying interaction between noise and styrene as indicated by the functional observations. Nevertheless, the pattern of Corti's damage was different. Interestingly and consistent with previous works [11], styrene exposure affected significantly Deiters' cell viability, whereas noise did not. Similarly to OHC and SGN viability, in the combination group, Deiters' cell loss increased (more than the sum of single exposures) suggesting, in this case, that noise may have caused a stressful condition on supporting cells able to potentiate the styrene damage on Deiters' cells. The antioxidant protection gave further insight onto the different cellular damage; namely, no significant cytoprotective effect on Deiters' cells was detected after Q_{ter} administration in the Styrene alone group, the antioxidant reduced only the susceptibility effect of noise on styrene in the combined exposure. Altogether, the antioxidant protection provided an asymmetric cytoprotective effect addressed mainly towards the sensory-neural layer, that is mostly damaged by noise, and did not affect the additional epithelial target of styrene. Thus, it is conceivable that Deiters' cells play a determinant role in styrene ototoxicity providing OHC structural support and serving as mediators of hair function, death, clearance processes [20,47,48] and, in particular, of potassium reuptake and recirculation [25,49–51]. Thus, the massive Deiters' cell loss, in styrene and combined exposure, may have produced a potassium flow disruption with endocochlear potential perturbation and functional impairment not completely recoverable through the antioxidant treatment

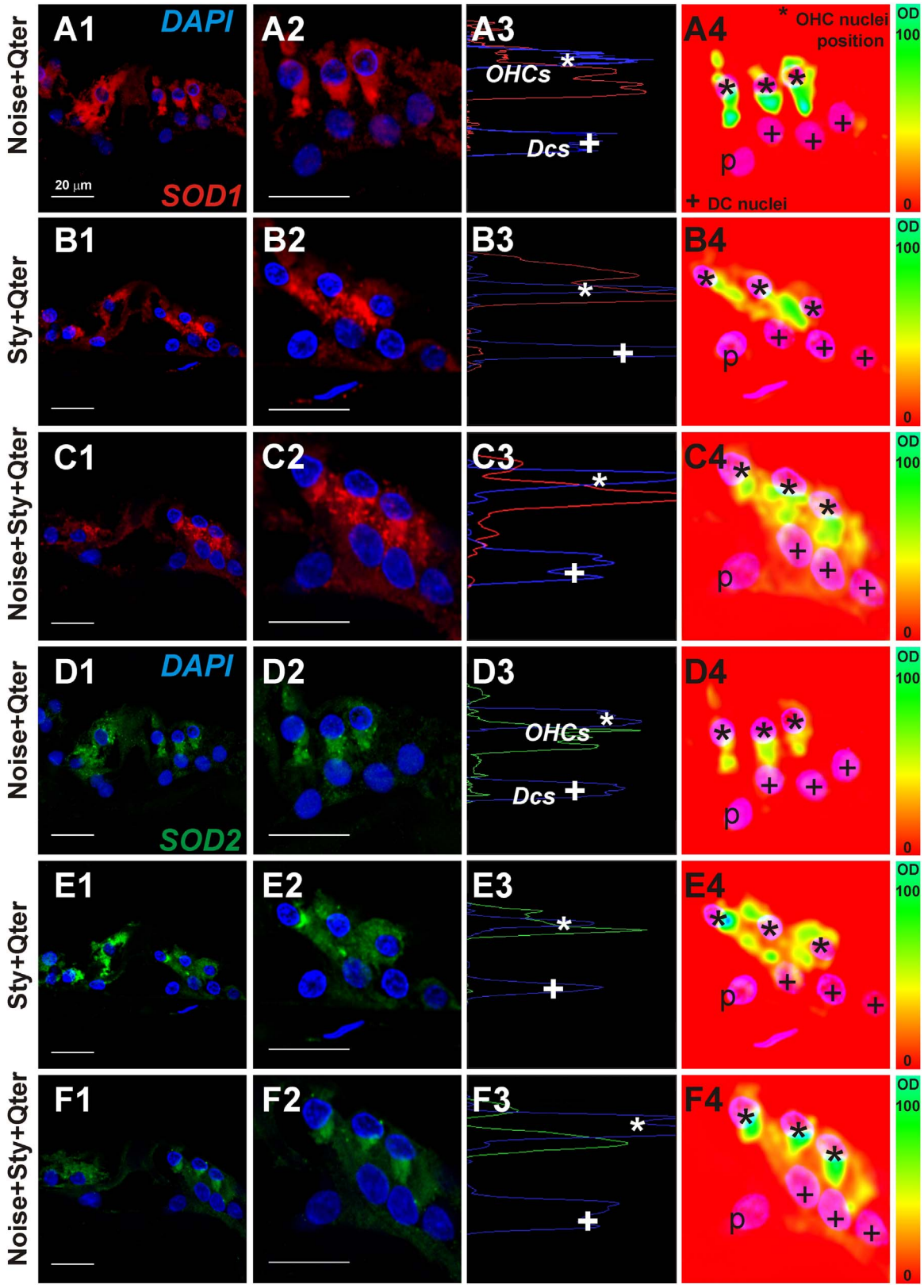

Fig. 8. Q_{ter} increases cochlear endogenous antioxidant responses in OHCs after noise and styrene insults. A1-F2: representative images of the organ of Corti stained with DAPI, SOD1 (red fluorescence, A1-C2) and SOD2 (green fluorescence, D1-F2). In the right panels (A3-F4), for each representative ROI of the organ of Corti, the vertical readout of fluorescence matched with the fluorescence spectrum intensity analysis (A4-F4) was reported: OHC-DC units are represented in a pseudo-colored rainbow scale spanning from red (low fluorescence intensity) to green (high fluorescence intensity). Q_{ter} treatment increased markedly SOD expression in the OHC layer after Noise exposure (boxes A1-A4 and D1-D4), and only slightly DC layer (compare marker * with marker + in A3-A4 and in D3-D4). In Styrene+Q_{ter} (B1-B4 and E1-E4) and Noise+Styrene+Q_{ter} groups (C1-C4 and F1-F4) SOD expression was detected mainly in OHC region (compare marker * with marker +). A4-F4: p= Pillar Cells. A1-F2: scale bar 20 μm.

[20,52,53]. In addition, Deiters' injury can be responsible for the increased SGN loss in the combined exposure since supporting cells also play a crucial role in promoting SGN survival [54] by expressing several trophic factors known to promote SGN survival [20,23,55]. Finally, supporting cells may be critical determinants of whether hair cells under stress ultimately live or die and play important roles in the clearance of dead or damaged hair cells [56].

4.2. A redox imbalance is involved in the cochlear damage by noise, styrene and combined exposure

Our data on superoxide and lipid peroxidation expression indicated a ROS dependent mechanism of damage both for noise and styrene and further amplified in the combined exposure. However, noteworthy differences concerned fluorescence increase in the supporting cell layer. Furthermore, superoxide and lipid peroxidation increase was counteracted by Q_{ter} only in the OHCs, SGNs and *stria vascularis* and not in Deiters' cells (Figs. 6 and 7). Q_{ter}, is a CoQ_{10} analogue and CoQ, in addition to its role in mitochondrial respiration and electron transport chain, acts as a potent antioxidant by either directly scavenging free radicals or recycling and regenerating other antioxidants [34,57,58]. CoQ_{10} is known for its benefits in pathologies that exhibit impaired mitochondrial bioenergetic function and/or increased oxidative damage [59,60]. Indeed, Q_{ter} antioxidant supplementation potentiated SOD1 and SOD2 expression in the organ of Corti, suggesting a strong activation of the cell stress response to face the oxidative insult. Notably, as documented by fluorescence vertical readout analysis (Fig. 8), the oxidative stress was counteracted by the antioxidant in OHC layer but not in Deiters' cells. Thus in our experimental setting, it is plausible that the exogenous CoQ_{10} was used as a ROS scavenger and incorporated into mitochondria to enhance cellular bioenergetics and the endogenous defense system upon the depletion induced by the oxidative imbalance in OHCs, SGNs and *stria vascularis* but not in Deiters' cells. The lack of the cytoprotective effect on these cells after Q_{ter} administration suggests a different mechanism of styrene toxicity between mechanosensory and supporting epithelial cells in the cochlea and, possibly, a different mechanism of apoptosis induction. ROS accumulation has been directly detected in noise-exposed cochleae [26,61] and our previous works have underlined the efficacy of selected antioxidant molecules to re-establish the redox status [31,32]. As regards styrene, our study provides a support for the free radical generation of toxicity as suggested for styrene pneumotoxicity and deduced by ROS increase and glutathione decrease in isolated Clara cells from CD-1 mice following styrene administration [62]. It is generally assumed that styrene toxic effects are mediated by binding of reactive metabolites to distinct but yet unknown molecular structures in the target cells/organs. In humans, styrene is mainly metabolized, by cytochrome P450, to styrene 7,8-oxide, which is considered to be the principal reactive and genotoxic intermediate of styrene [63,64]. Styrene 7,8-oxide forms covalent adducts to DNA, RNA and proteins [65] and it is further proposed to have a direct oxidative stress effect on cells [66], possibly through the depletion of the major cellular antioxidant glutathione as well as other non-protein sulfhydrils [67,68]. However, other studies are needed to clarify the mechanisms underlying styrene induced oxidative stress in the cochlea and its cytotoxic effect on Deiters' cells.

5. Conclusions

Our data provide further evidence on the noise and styrene driven damage inasmuch describe a reciprocal synergism based on a redox imbalance mechanism affecting, although with a different intensity, the OHC sensory epithelium. However, our data reveal as well that the two damaging agents address different targets: noise mainly against the sensory epithelium, styrene against the supporting epithelial one as well. Indeed, Deiters' injury (loss and sublethal) by styrene may represent the hallmark of OHC dysfunction as enlightened by DPOAE drop and OHC death may results from styrene insult directly and/or indirectly by its ototoxic effect on Deiters' cells, possibly by DNA adducts formation. In a translational perspective, the antioxidant therapy might be a rational approach in the environmental or occupational exposure to styrene. It remains to be seen whether targeting supporting cells, in addition to hair cells, might be an effective strategy in protecting sensory-neural hearing loss induced by noise and organic solvent exposures.

Competing financial interests statement

Authors declare no competing financial interests.

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