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Effects of hyperbaric oxygen treatment on auditory hair cells after acute noise damage

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Abstract Acute acoustic trauma (AAT) is a sudden sensorineural hearing loss caused by exposure of the hearing organ to acoustic overstimulation, typically an intense sound impulse, hyperbaric oxygen therapy (HOT), which favors repair of the microcirculation, can be potentially used to treat it. Hence, this study aimed to assess the effects of HOT on guinea pigs exposed to acoustic trauma. Fifteen guinea pigs were exposed to noise in the 4-kHz range with intensity of 110 dB sound level pressure for 72 h. They were assessed by brainstem auditory evoked potential (BAEP) and by distortion product otoacoustic emission (DPOAE) before and after exposure and after HOT at 2.0 absolute atmospheres for 1 h. The cochleae were then analyzed using scanning electron microscopy (SEM). There was a statistically significant difference in the signal-to-noise ratio of the DPOAE amplitudes for the 1- to 4-kHz frequencies and the SEM findings revealed damaged outer hair cells (OHC) after exposure to noise, with recovery after HOT ($p = 0.0159$), which did not occur on thresholds and amplitudes to BAEP ($p = 0.1593$). The electrophysiological BAEP data did not demonstrate effectiveness of HOT against AAT damage. However, there was improvement of the anatomical pattern of damage detected by SEM, with a significant reduction of the number of injured cochlear OHC and their functionality detected by DPOAE.

Keywords Acute acoustic trauma · Noise damage · Hyperbaric oxygen therapy · Otoprotection

Abbreviations

AAT	Acute acoustic trauma
ATA	Absolute atmospheric pressure
OHC	Outer hair cells
HOT	Hyperbaric oxygen therapy
FMRP	Faculty of Medicine of Ribeirão Preto
USP	University of São Paulo
OAE	Otoacoustic emission
DPOAE	Distortion product otoacoustic emission
BEAP	Brainstem auditory evoked potential
dB SPL	Decibel sound pressure level
SEM	Scanning electron microscopy
T1	Basal turn
T2	Second turn
T3	Third turn
SOD	Superoxide dismutase
SOD1	Copper/zinc-superoxide dismutase
R1	First row
R2	Second row
R3	Third row

Introduction

Acute acoustic trauma (AAT) is a sudden sensorineural hearing loss due to exposure of the hearing organ to acoustic overstimulation, typically an intense sound

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impulse [26]. AAT results in structural changes such as the rupture of cell membranes, which reduces cochlear blood flow and causes destruction of cochlear hair cells and/or dendrites of primary auditory neurons [3, 7–9, 13].

Mitochondrial biogenesis is involved in the cell response to injury and can regulate the probability of cell survival after metabolic challenges to hair cell integrity [11]. The reduction of cochlear blood flow causes a reduction of oxygen in the fluids of the inner ear, reducing the various cell activities depending on blood flow [13].

Reversibility of hearing loss has been reported in animals and humans. Some studies have demonstrated improvement of the electrophysiological measurements of action, summation, and microphonic cochlear potentials, as well as the formation of new outer hair cells (OHC) and/or repair of existing cells indicating ability for spontaneous recovery and suggesting a self-defense mechanism. A synaptic repair may occur within a few days, but damage to hair cells that cause a loss of hearing acuity may persist [3, 5, 8, 24].

Many drugs have been used for the treatment of acoustic trauma. In general, all of them are directed at the repair of the microcirculation [3, 8, 14].

Hyperbaric oxygen therapy (HOT) favors repair of the microcirculation and is used for the treatment of various diseases, including sensorineural hearing loss [2]. Also, some studies have pointed out that HOT improves corticotherapy and functions as a protective agent against cisplatin ototoxicity [8, 14, 26].

According to Kuokkanen et al. [13] and Ylikoski et al. [26], when restored, ionic equilibrium helps damaged hair cells to survive the interval of lack of perilymphatic oxygen supply. HOT actually provides an adequate oxygen supply, preventing the oxidative stress secondary to cochlear hypoxia often followed by the eventual death of initially uninjured cells. Although some studies have shown a possible injury to cochlear hair cells and danger with the application of HOT in AAT, few studies have investigated this damaging effect [3, 8].

Although HOT has been routinely utilized for the treatment of AAT in military personnel in countries of the European community, its full effect in this type of treatment has been little studied and requires further investigation [2, 3, 8, 14, 26]. Thus, the objective of the present study was to assess the effects of HOT on guinea pigs exposed to acoustic trauma.

Materials and methods

Animals

The study was conducted on 15 female albino guinea pigs. These animals were chosen because they are easy to handle, with easy cochlear dissection and manipulation and administration of anesthetic drugs by the intraperitoneal, intramuscular, and subcutaneous routes. All procedures followed the norms for the care of experimental animals recommended by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee for animal experimentation of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP) (Document no: 035/2007). The animals were kept in the animal facilities of Experimental Surgery, Department of Surgery and Anatomy, FMRP-USP, and were selected from the central animal facilities of USP, Ribeirão Preto Campus, by determining the presence of the Preyer reflex [12]. Preference was given to animals weighing 400–600 g. The animals were kept in a quiet room with constant temperature (23 ± 2°C) and humidity (50–60%) with a 12-h light/dark cycle (light on from 6 to 18 h). The noise level of this room was close to 40 dB.

After 24 h of auditory rest, the animals were re-evaluated by manual otoscopy. Animals with signs of external otitis or acute otitis media, with ear wax of difficult removal, inflammatory changes of the outer auditory canal or a too narrow auditory canal for proper insertion of the otoacoustic emission (OAE) probe were excluded. Only animals with present distortion product otoacoustic emission (DPOAE) and 5–10 dB HL threshold on brainstem auditory evoked potential (BEAP) before AAT were included in the study. Changes to at least one of the ears were a reason for exclusion from the study.

Brainstem auditory evoked potential and distortion product otoacoustic emissions

The effects of cochlear toxicity from drugs or acoustic injuries have been studied on the basis of the electrophysiological changes detected by the BAEP [20–23, 25]. OAE is a simple and rapid method for the evaluation of injuries to OHC and is used to monitor the ototoxic effects of drugs [1, 6, 10, 16, 17, 21, 22]. On this basis, in the present study the animals were submitted to BAEP and DPOAE exams using the Smart EP and Smart DPOEA equipment of Intelligent Hearing Systems (Miami, FL, USA).

For the DPOAE and BEAP tests, ketamine hydrochloride, an anesthetic drug with no effects on auditory function, was used.

The BEAP evoked responses was averaged for 1,024 sweeps at each intensity level twice. Stimuli were presented at a repetition rate of 11.1/s. The threshold was defined as the lowest intensity at which a visible five wave of BEAP was seen in two averaged runs.

The DPOAE test was performed according to the frequency relation 2F1–F2 with the ratio F1:F2 = 1.22, and a resolution of 2 points per octave.

We considered OAE starting from 1.5 kHz, since the dimensions of the external auditory canal of the guinea pig make it difficult to detect OAE below this frequency. Thus, by analyzing the 2-kHz frequency, we offer a pure tone a little above and a little below in such a way that the ratio between them will be 1.22, thus obtaining automatically a resultant frequency response according to the 2F1–F2 relation (below the frequency evaluated) and the 2F2–F1 relation (above the resultant frequency). It should also be taken into consideration that the intensities of f2 and f1 can be equal or different. In the present study were used equal intensities of 70 dB SPL and frequency range of 1,500–8,000 Hz. The intensity of the triggering stimulus can vary within the range of 0–70 dB SPL and can be measured in the range of 500–8,000 Hz. On this basis, were observed the so-called DPGram, i.e., the audiocochleogram, in which there is a sound stimulus and a response which is also a sound that corresponds to the function of the cochlear OHC responsible for the frequencies analyzed.

Acoustic trauma

The guinea pigs were exposed bilaterally to a 4-kHz octave band noise with a 110-dB SPL intensity for 72 h in an acoustically insulated reverberation chamber connected to an audio signal generator (EP—125 Audio Signal Generator—Insight, Ribeirão Preto, SP, Brazil), amplified with a power amplifier, and delivered to a loudspeaker that was suspended directly above the cage. Noise levels were measured on four points at the floor at the chamber and calibrated to a sound level meter. Three guinea pigs were exposed at the same time in one cage in a ventilated noise exposure chamber on an "on–off" 12 h cycle light–dark during 72 h, with spontaneous food and water provision. The noise level variation was <2 dB within the space with the three animals in the cage, and the background noise level in the chamber was below 40 dB SPL. Fifteen albino guinea pigs (30 ears) were divided into three groups (G1, G2 and G3) and submitted to DPOAE and BAEP exams before and 1 h and 30 min after 72-h exposure to noise.

Three animals (6 ears) not treated with HBO, were used as a control for the AAT group, and submitted to DPOAE and BAEP examinations 1 h and 30 min after 72 h of noise exposure, and then euthanized for scanning electron microscopy (SEM) examination (G1). The second group was submitted to DPOAE and BAEP exams 1 h and 30 min after 72-h exposure to noise and after 5 days of rest (G2). The third group (G3) was submitted to DPOAE and BAEP exams 1 h and 30 min after 72-h exposure to noise and after 5-day exposure to HOT. The HOT exposures occurred 24 h after noise exposure.

Hyperbaric oxygen therapy

An experimental hyperbaric chamber directly pressurized with oxygen was used. HOT sessions occurred five times (105 min a day during 5 days), including 15 min of compression time, 75 min of stable compression time with 2.3 absolute atmospheric pressure (ATA) (0.23 MPa), and 15 min of decompression time.

Scanning electron microscopy

The guinea pigs were euthanized at programmed times after anesthesia with ketamine hydrochloride (65 mg/kg) and xylazine (6.5 mg/kg). The animals were decapitated and their cochleae were removed from the bullae.

With microscopic dissection, the cochleae were perfused with 3% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4, injected through the round window for fixation at 4°C, and kept in this solution for 24 h for fixation. The subsequent procedures were performed in the Laboratory of Electron microscopy of the Department of Morphology, FMRP-USP. The cochleae were washed three times for 5 min with the same buffer and then fixed with 1% osmium tetroxide for 2 h at 4°C, and dehydrated at room temperature through a graduated ethanol series (50, 70, 90 and 95%) for 10 min at each concentration, and three times in absolute ethanol for 15 min. After dehydration, the material was dried to the critical point in CO_2. After being attached to an appropriate specimen holder, the material was sputtered with gold in a vacuum chamber and examined by SEM [4, 18] using a JEOL scanning microscope JSM 5200.

Statistical analysis

BAEP and DPOAE data, as well as data obtained by SEM by counting the number of cells per cochlear turn were analyzed and tabulated for statistical comparison. This nonparametric methodology was used because the data did not follow the normal distribution (Kolmogorov-Smirnoff test with $p < 0.001$). Thus, the hypothesis was that the values of the three groups in question would be equivalent (H_0) versus the possibility that at least one pair of groups would differ significantly (H_1).

Results

There was a statistically significant difference (*Wilcoxon test*) in the comparison of the signal-to-noise ratio of the

DPOAE amplitudes for the frequencies from 1 to 4 kHz before and after exposure to noise in G1 ($p = 0.0313$), showing functional injury to OHC.

When G2 and G3 were analyzed separately, a statistically significant difference in the signal-to-noise ratio of the DPOAE amplitudes for frequencies from 1 to 4 kHz was observed for G3 after exposure to noise, that confirms functional injury to OHC (Fig. 1) and an improvement after treatment with HOT ($p = 0.0159$) (Fig. 2). For G2 there was no significant difference between functional (DPOAE) and histopathological (SEM) OHC lesions after exposure to noise and the situation after a 5-day rest ($p = 0.1593$).

When G2 and G3 were compared by the lesions after exposure to noise and its improvement after treatment with HOT or rest by the *Mann–Whitney test*, a significant difference was observed between groups, with a better signal-to-noise ratio of the DPOAE for G3 ($p = 0.0079$) (Fig. 2).

Regarding the electrophysiological threshold variable in the situations before and after exposure to noise, a significant difference was observed between groups (G1, G2, and G3), with an increase in the electrophysiological threshold measured in decibels above normal adult hearing level (dB SPL) ($p < 0.0001$).

When the three situations of G2 (before and after exposure to noise and after a rest) were compared with the situations of G3 (before and after exposure to noise and after HOT), a significant difference to OHC injury was observed for each situation in each group separately, but no significant difference was observed between groups ($p = 0.25$) at the end of the HOT.

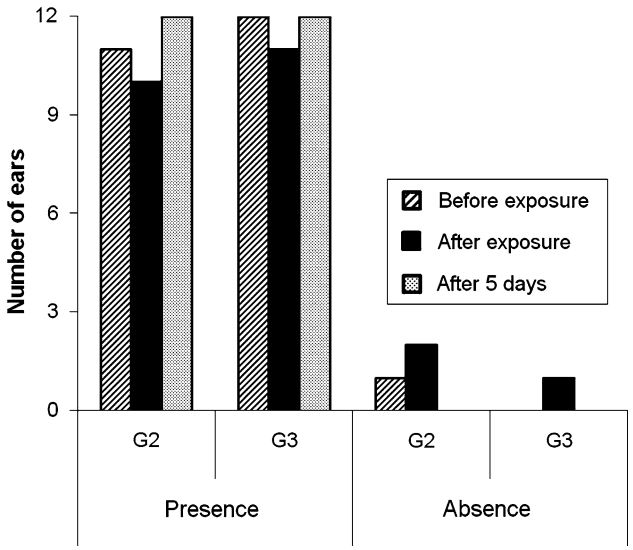

Fig. 1 Number of ears of Groups 2 and 3 with the presence or absence of responses before and after exposure to noise regarding the amplitude of the signal-to-noise ratio in the distortion product otoacoustic emissions for frequencies of 1, 1.5, 2, 3, and 4 kHz

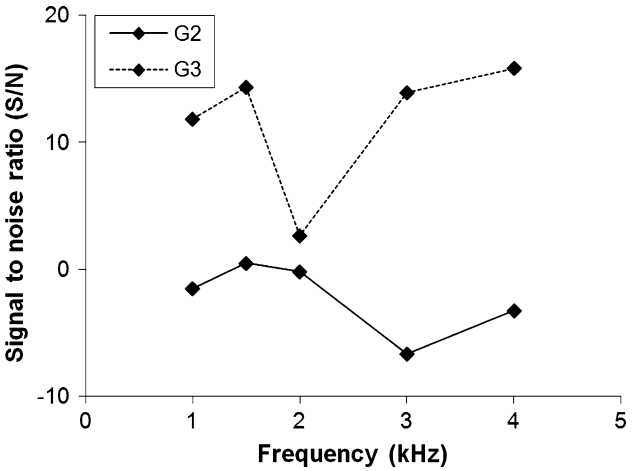

Fig. 2 Mean signal-to-noise-ratio in the amplitude of distortion product otoacoustic emissions at the frequencies of 1–4 kHz of all ears of Group 2 after 5 days of rest and Group 3 after hyperbaric oxygen therapy for 5 days

There was no significant difference ($p = 0.9639$) regarding the presence or absence of DPOAE between the pre and post-treatment situations for G2 and G3, or between the two groups (*Kruskal–Wallis test*, $p = 0.6514$).

There was a significant difference between G1, G2, and G3 regarding the mean number of OHC per row in the basal turn (T1), second turn (T2), and third turn (T3) with the greatest alteration occurring in T3. Separate comparison according to each turn revealed a significant difference between groups in all three turns T1 ($p = 0.0078$), T2 ($p = 0.04$), and T3 ($p = 0.04$), in agreement with SEM observations (Figs. 3, 4, 5).

Discussion

Acoustic trauma occurrence was detected based on statistically significant reduction of DPOAE amplitudes in G1 ($p = 0.0313$).

In the present study, after 5 days of rest the degenerative and repair processes were expressed in a more consistent manner. The brief improvement of DPOAE amplitude after the 5-day period of rest, although not statistically significant ($p = 0.1593$), indicated the possibility of spontaneous recovery, but the qualitative findings and the difference detected between G2 and G3 regarding the number of cells per OHC row suggest that the acoustic damage was not fully reversed. However, even though there was no full reversibility, the significant difference in the signal-to-noise ratio of the DPOAE amplitude in G3 after exposure to noise and after HOT ($p = 0.0159$) indicates greater recovery in G3 compared with G2, that could be attributed to the therapeutic method used.

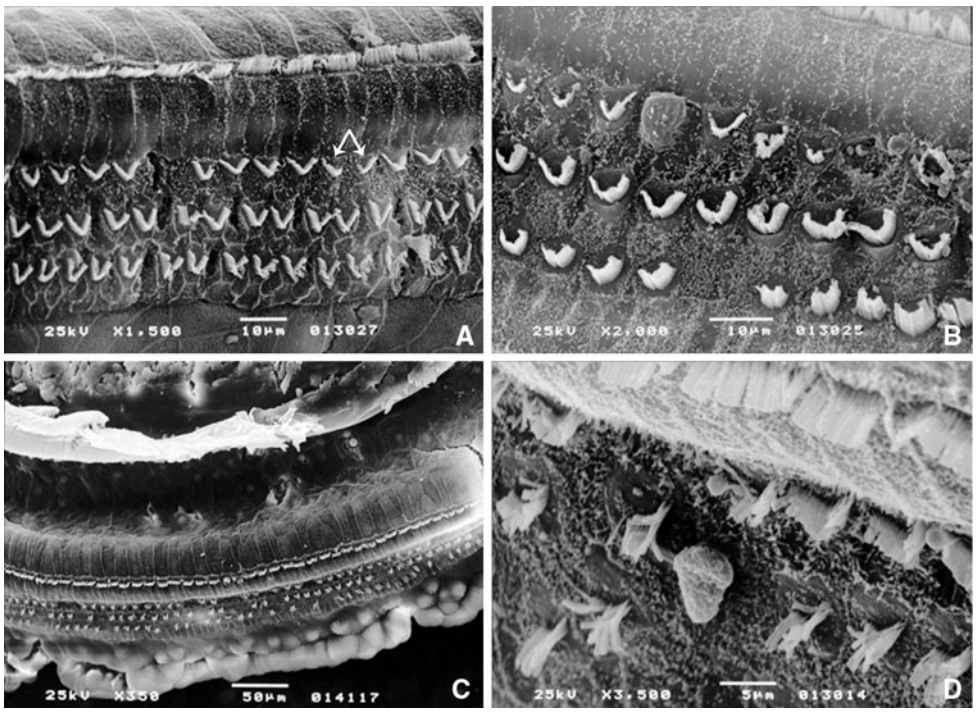

Fig. 3 **a** Basal turn of Group 1 with hair cell loss in R1 and derangements in R2 and R3; **b** second turn of Group 1, with R2 less damaged than R1 and R3; **c** damage to outer hair cells in the third turn, especially in R2 and R3, with preservation of inner hair cells; **d** Apical turn of G1 with a more preserved pattern and with only some lesions in the outer hair cells

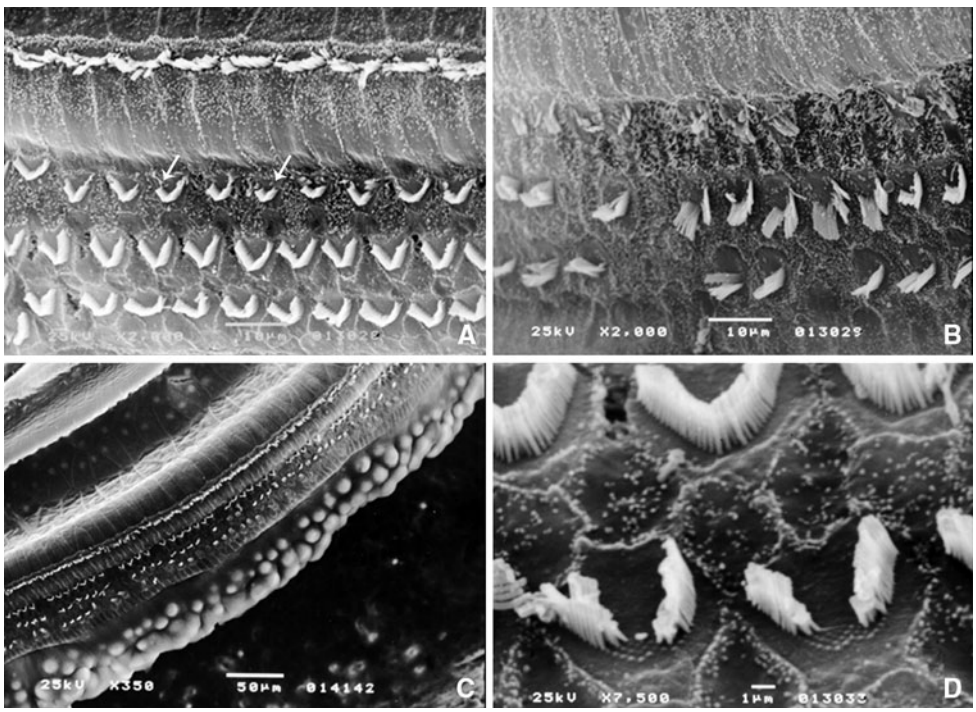

Fig. 4 Scanning electron microscope photographs of the cochlea of Group 2 after 72 h of exposure to noise of 110 dB, 4 kHz and a 5-day rest, showing injury to the outer hair cells of the basal, second, and third turns (**a**–**c**) and distortion of the ciliary pattern of outer hair cells in row 3 (**d**)

Mitochondrial biogenesis is involved in the cell response to injury, possibly regulating the probability of cell survival after metabolic challenges to hair cell integrity [11]. Restored ionic equilibrium helps damaged hair cells to survive the interval of lack of oxygen perilymph supply. Indeed, the aim of HOT is to provide an adequate oxygen supply, preventing oxidative stress secondary to the situation of cochlear hypoxia which can even be followed by the eventual death of initially uninjured cells [13, 26]. The attribution of this result to HOT is also supported by the comparison of G2 and G3 (Fig. 2).

The electrophysiological BAEP threshold has been more frequently used because of the high precision in determining the degree of hearing losses is a simple and highly

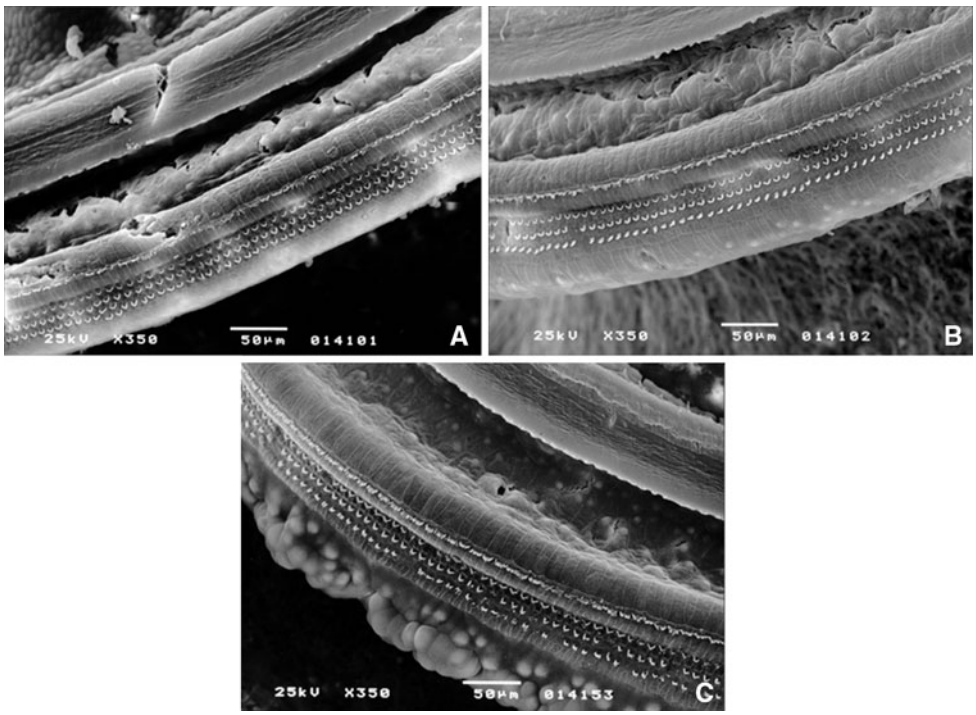

Fig. 5 Scanning electron microscope photographs of the cochlea of Group 3 after 72 h of exposure to noise of 110 dB, 4 kHz and treatment with hyperbaric oxygen therapy, showing improvement of the pattern of injury of outer hair cells in the basal (**a**), second (**b**), and third (**c**) turns

sensitive technique presenting high response reproducibility and is not altered by the use of such sedatives and anesthetics.

According to Lamm and Arnold [14], after exposure to broadband noise, the partial pressure of perilymph oxygen declines in parallel to the deterioration of BAEP amplitude. Cochlear blood flow decreases only 30 min after exposure to noise and with cochlear hypoxia deterioration of BEAP amplitude does not recover until 3 h after noise exposure [14].

Free radicals are produced in each cell as a subproduct of normal biochemical events, mainly regarding cell respiration at the mitochondrial level and in abnormal processes in the organism. Superoxide produces reactive oxygen species that are easily generated in the inner ear after acoustic overstimulation with destructive hydroxyl radicals (OH) or combined with nitric oxide radicals forming highly toxic peroxynitrite [9].

Superoxide dismutase (SOD) is an enzyme that converts superoxide to hydrogen peroxide and to three subtypes. The hearing dysfunction due to exposure to noise is attenuated by the application of SOD1, and SOD production is one of the physiological effects of hyperbaric oxygen [9].

In contrast to this probable beneficial effect, studies have demonstrated that HOT is involved in the increase of free radicals. d'Aldin et al. [8] and Çakir et al. [3], concluded that HOT has a negative effect when administered during the first hour after exposure to noise. This is explained by the increase in reactive oxygen metabolites with the application of HOT during the first hours after exposure, in addition to those already produced in the cochlea after acoustic trauma, with a consequent increased suppression of the capacity of antioxidant defense of the system.

The enzymes glutathione peroxidase, glutathione reductase, glutathione transferase, SOD, and catalase and non-enzymatic substances such as vitamins C and E act as protective substances against cell injury provoked by free radicals. Some time is needed for the enzymes responsible for antioxidant defense to be activated, as is the case for glutathione peroxidase, which reaches its highest levels after 30 min of exposure to HOT. This may explain the adverse effect of HOT when applied during the first hour after the trauma [3]. This was supported by the results obtained by d'Aldin et al. [8] which showed that HOT administered during the first hour after trauma caused damage, and by those obtained by Kuokkanen et al. [13] who demonstrated a beneficial effect of HOT when 2 or 3 h after exposure to noise started.

These factors may explain the poorly expressive improvement of the electrophysiological threshold in G3 since the first therapeutic exposure was performed 2 h after the end of the 72-h period of exposure to noise but a damaging effect to HOT was no worsening in G3 compared with G2. The results of the present study only confirm the beneficial effect of HOT when treatment started 2 or 3 h after exposure to noise, as demonstrated by Kuokkanen et al. [13].

Çakir et al. [3] conducted a detailed study on the crucial time when HOT should be started and concluded that exposure to HOT 1 h after exposure to noise may be damaging, whereas some benefit would be observed after 2 h even though full reversibility does not occur. From 2 to 6 h after exposure there is a 4-h interval whose characteristics were not determined. It would be highly useful to consider in future studies how determinant this factor could be and what the earliest time for optimum HOT treatment would be.

The application of SEM to the study of the presence or absence of hair cells is a refinement of the surface preparation. In general, only the metal on the surface of the specimen can be visualized, with the presence or absence of stereocilia being identified as a discontinuity in the surface mosaic of hair cells [19].

Experimental studies on animals have demonstrated that AAT due to cochlear exposure to intense noise provokes structural changes in the OHC of the cochlea, initially compromising those located in the first row (R1) and later in the second (R2) and third (R3) rows progreding to inner and OHC involvement [3, 8, 26], with large part of the injuries being located in the basal turn [11, 13].

SEM analysis of G1 cochleae in general revealed anatomical changes in the OHC similar to those most frequently cited in the literature, described by Saunders et al. [19] as derangements, collapses, losses, or elongation of sensory cells.

After 72 h of exposure to noise, the T1 did not present a marked number of absent OHC, but only asystematic gaps in R1. However, in its distal third there were ciliary derangements in all three rows. R1 presented ciliary loss and R3, in addition to ciliary derangement, presented distorted stereocilia. R2 was found to be less damaged than the remaining ones (Fig. 3b).Thus, this result differs somewhat from the findings most frequently cited in the literature, but, as stated by Saunders et al. [19], it is complicated to determine greater or lesser susceptibility to acoustic damage among the three rows. However, Lim and Melnick [15] observed a normal appearance in R2 and almost complete destruction of R1 and R3 in the injured area of chinchillas exposed to a sound impulse for 4 weeks. Indeed, there is variability in the anatomical injury to the OHC, as proved by this finding, which was an exception in our study.

d'Aldin et al. [8] stated that 5 days after noise injury the hair cells remained altered, as also observed in the present study (Fig. 4).

A pattern quite close to the adequate one was observed and characterized here in the first third of the T1 of G3 (Fig. 5a), presenting changes and/or absence of stereocilia in a very asystematic manner. The middle and distal thirds of the same turn showed a pattern similar to that of the initial turn. In T2 (Fig. 5b) the changes in the stereocilia were more frequent than throughout the T1, with some absences of OHC. In T3 there were more changes and absences, with an initial third showing absence in R2 and R3 and derangement of the cilia in the three rows. In the middle third there was marked alteration throughout R3, with loss of stereocilia and derangement, and this pattern was maintained in the distal third, with some improvement in the direction of the apical third (Fig. 5c).

The present functional and electrophysiological observations did not show effectiveness of the treatment after acoustic trauma; however, SEM data demonstrated that treatment with HOT improved the anatomical pattern of the injury, significantly reducing the number of injured OHC of the cochlea.

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