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ORIGINAL ARTICLE

Lack of protection against gentamicin ototoxicity by auditory conditioning with noise☆,☆☆

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KEYWORDS
Inner ear;
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Abstract
Introduction: Auditory conditioning consists of the pre-exposure to low levels of a potential harmful agent to protect against a subsequent harmful exposure.
Objective: To confirm if conditioning with an agent different from that used to cause the trauma can also be effective.
Methods: This was an experimental study with 17 guinea pigs, divided into three groups: an ototoxic control group (Cont) that received intramuscular administration of gentamicin 160 mg/kg/day for ten consecutive days, but no sound exposure; a sound control group (Sound) that was exposed to 85 dB broadband noise centered at 4 kHz, 30 min each day for ten consecutive days, but received no ototoxic medications; and an experimental group (Expt) that received sound exposure identical to the Sound group and after each noise presentation, received gentamicin similarly to Cont group. The animals were evaluated by distortion product otoacoustic emissions (DPOAEs), brainstem auditory evoked potentials (BAEPs), and scanning electron microscopy.
Results: The animals that were conditioned with noise did not show any protective effect compared with the ones that received only the ototoxic gentamicin administration. This lack of protection was observed functionally and morphologically.
Conclusion: Conditioning with 85 dB broadband noises, 30 min a day for ten consecutive days does not protect against an ototoxic gentamicin administration of 160 mg/kg/day for ten consecutive days in the guinea pig.

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PALAVRAS-CHAVE
Orelha interna;
Perda auditiva neurossensorial;
Surdez;
Ruído;
Estimulação acústica;
Toxicidade

Falta de proteção contra a ototoxicidade da gentamicina pelo condicionamento auditivo com ruído

Resumo
Introdução: O condicionamento auditivo consiste da pré-exposição de um agente lesivo em baixos níveis para proteger contra uma posterior apresentação lesiva.
Objetivo: Confirmar se o condicionamento com um agente diferente do utilizado para causar o trauma pode ser efetivo.
Método: Estudo experimental com 17 cobaias albinas divididas como a seguir - grupo Som: exposto a um ruído branco de 85 dB centrado em 4 kHz, 30 minutos por dia por 10 dias consecutivos; grupo Controle (Cont): administração intramuscular de gentamicina 160 mg/kg por dia por 10 dias consecutivos; grupo Experimental (Expt): condicionado com ruído como o grupo Som. Após cada exposição ao ruído, recebeu gentamicina similarmente ao grupo Cont. Os animais foram avaliados por emissões otoacústicas - produto de distorção (EOAPDs), potencial evocado auditivo de tronco encefálico (PEATE) e microscopia eletrônica de varredura (MEV).
Resultados: Os animais que foram condicionados com ruído não mostraram qualquer efeito protetor, em comparação com os que receberam apenas a gentamicina em doses ototóxicas. Esta ausência de proteção foi observada tanto funcionalmente quanto morfologicamente.
Conclusão: Os autores concluem que o condicionamento com ruído branco a 85 dB por 30 minutos por dia por 10 dias consecutivos não protege contra uma administração de gentamicina 160 mg/kg/dia por 10 dias consecutivos.

Introduction

Auditory conditioning consists of a pre-exposure of a potentially harmful agent to the auditory system at low or non-toxic doses to stimulate intrinsic mechanisms that increase the resistance of the auditory structures, thus protecting them against a subsequent exposure to the same agent used in pre-exposure, or against other agents potentially harmful to the organ of Corti. The agent first tested was noise,[1,2] used both for conditioning as well as to cause auditory trauma. Since then, this phenomenon has been tested in different animals, such as guinea pigs, chinchillas, Mongolian gerbils, and even in humans. The results have shown a reduction in morphologic and functional damage in conditioned animals, compared with those exposed only to traumatic stimuli.[1,3–5] Many conditioning paradigms, using different frequencies, intensities, and patterns of presentation, such as continuous or intermittent exposures, have proven to be effective.[1,3–6] Conditioning with agents other than noise has also been shown to be effective. Pre-exposure to low doses of amikacin has been shown to be effective in protecting against subsequent administration of the same drug in harmful doses.[7] The same effect was shown with gentamicin.[8] It has been suggested that the stress caused by conditioning, and not the action of a particular agent, is responsible for stimulating protective mechanisms.[9] These researchers hypothesized that this stress can stimulate the release of corticosteroids, known to attenuate cochlear damage.

However, the vast majority of studies use the same agent for conditioning and causing trauma. More recently, the effectiveness of conditioning with a different agent from that used to cause trauma was tested. Theneshkumar et al. exposed rats to an 8 kHz noise with an intensity of 85 dB for 15 min, in order to reduce the damage caused by cisplatin.[10] Their study demonstrated that animals that were noise-conditioned showed a smaller change in their hearing thresholds, compared to those that received only the drug. Another study conditioned mice with kanamycin and then exposed the animals to a broadband noise (4–45 kHz) at 110 dB intensity for 30 s.[11] The animals that received the drug before the noise exhibited less cochlear damage, demonstrated by morphological and functional assessments conducted during the study. A third study conditioned guinea pigs by administering ototoxic doses of gentamicin, in an attempt to protect them against a subsequent acoustic trauma. Animals conditioned with gentamicin exhibited less cochlear damage than those who were treated only with acoustic trauma.[12] Yet another study conditioned Mongolian gerbils with noise, in an attempt to protect the animals against a topical administration of gentamicin applied across the round window. The authors used a conditioning protocol consisting of a continuous noise with frequency between 1410 Hz and 5650 Hz at 81 dB for 3 weeks, and observed that the group treated with this stimulus showed fewer injuries to the inner and outer hair cells.[13] Another study tested whether conditioning mice with a noise able to cause a temporary shift in the threshold would be effective in protecting the animals against gentamicin and cisplatin ototoxicity. The researchers observed that the presentation of a noise of 8–16 kHz at an intensity of 91 dB for 2 h caused a temporary threshold shift and also protected against the ototoxicity of these substances.[14] The purpose of the present study was to test whether conditioning animals with nontraumatic noise levels may protect the cochlea against gentamicin administration in ototoxic doses.

Materials and methods

This was an experimental study in which a total of 17 albino guinea pigs were used; they weighed between 350 and 500 g and had no middle ear infection, as determined by otoscopy. The sample size and its distribution were statistically scaled, in order to meet the requirements of the Ethics Committee, aiming at the use of the lowest possible number of animals. All procedures were approved by the Ethics Committee on Animal Experimentation of the institution (Document No. 058/2008). The animals were selected by presence of the Preyer reflex. Only animals with distortion product otoacoustic emissions (DPOAEs) and electrophysiological hearing thresholds of 25 dB estimated by brainstem auditory evoked potential (BAEP) were included in the study.

The animals were divided into three groups: the first group (Cont) acted as control for gentamicin lesion and consisted of five animals that received intramuscular gentamicin in an ototoxic dose, 160 mg/kg/day for ten consecutive days. The second group (Sound) acted as control for noise conditioning and consisting of six animals treated with noise centered at 4 kHz at an intensity of 85 dB during 30 min per day for ten consecutive days. This frequency was chosen because the functional methods of assessment used in this study are effective for detecting changes in this frequency band; and, in addition, to test if conditioning with this frequency would also be effective. The third group (Expt), the experimental group, consisted of six animals which were conditioned in the same way as the Sound group; however, after 30 min of noise exposure, the animals received a potentially harmful gentamicin dose of 160 mg/kg/day for 10 days, in the same way as the Cont group. The injury protocol by gentamicin, as mentioned above, had been successfully used in previous studies.[8]

Functional assessment

The functional assessment was performed by examination of distortion product otoacoustic emissions (DPOAEs) and brainstem auditory evoked potential (BAEP). The equipment used was the SMART DPOEA/EP and SMART EP (Intelligent Hearing Systems - Miami, Florida, United States).

To perform the tests, the animals were anesthetized with intramuscular ketamine at a dose of 65 mg/kg; this is an anesthetic drug without significant effects on the functioning of the auditory system.[15]

BAEP and DPOAE

Functional assessment by BAEP and DPOAE tests was performed at the following time points: before any treatment in all animals; one hour after noise conditioning to assess whether this procedure was harmless to the auditory system; and 1 day after administration of the last dose of gentamicin, both in the group treated only with this medication (Cont), and in the group with noise conditioning before the administration of gentamicin (Expt).

For evaluation of auditory electrophysiological potentials (BAEP), surface electrodes were positioned on the cranial vertex (positive) and the posterior portion of the pinna bilaterally (negative), and a reference electrode was placed on the forehead (ground). The response of the auditory potentials was obtained from the average of 2048 stimuli for each intensity. A click-type stimulus was used, presented at a repetition rate of 11.1/s. Hearing threshold was defined as the lowest intensity at which it was possible to identify a wave II in two records.

DPOAEs were performed according to the 2F1-F2 relation, with respect to an F1:F2 ratio = 1.22. The intensity of both frequencies was 70 dB. Responses from a low frequency of 1–4 kHz were evaluated. The presence or absence of response according to the signal/noise ratio was determined. In addition, the values obtained before and after each treatment for each group were statistically analyzed.

Morphological assessment

Scanning electron microscopy (SEM)

All guinea pigs were euthanized at the scheduled time, one hour after the last functional assessment, after intramuscular anesthesia with ketamine hydrochloride (65 mg/kg) and xylazine (6.5 mg/kg). The animals were then decapitated and their cochleae were removed from the bulla.

For fixation, a solution of 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 h at 4 °C, was injected through the round window. The cochleae were rinsed three times for 5 min with the same buffer, then fixed with 1% osmium tetroxide for 2 h at 4 °C and subjected to dehydration at room temperature in a battery of increasing ethanol concentrations (50%, 70%, 90% and 95%, 1×/10 min for each concentration) and in absolute ethanol 3×/15 min. When the dehydration was over, the drying phase was completed using the critical point method in CO_2, in which the material is devoid of water. After being fixed on an appropriate specimen plate, the material was coated with gold vapour deposition in a vacuum camera and examined with a scanning electron microscope.

For analysis and photography of SEM specimens, an electron microscope JEOL SCANNING MICROSCOPE - JSM 5200 was used.

For the statistical analysis, an injured hair cell was defined as a cell exhibiting total absence of cilia; any other change in these structures was not taken into account for statistical calculation. Only lesions to the outer hair cells were considered. The cochlea of the guinea pig is formed by three-and-a-half turns, i.e. the first, second and third turns, and an apical portion formed by a half turn. Each turn may be divided into three thirds, i.e. the first, middle, and final third. The percentage of damaged outer hair cells from the total number of hair cells was calculated for the middle third of each turn, except the apical portion. This calculation was obtained from a cochleogram. The cochleogram was defined as the graphical representation of a SEM photograph of the middle third of the first, second, and third cochlear turns in a field of 500× magnification. The apical region was not included in the statistical calculation since there is a natural breakdown of cilia, making differentiating normal aging from injured structures difficult. Additional calculations were performed to determine the total percentage of damaged hair cells and the percentage of damaged hair cells per row of outer hair cells.

Table 1 Values of DPOAE pre-conditioning with noise and post-exposure to an ototoxic dose of gentamicin – Expt group.

Expt group	F 1	F 1.5	F 2	F 3	F 4
Pre-	24.08	25.33	20.33	18[a]	26.33[a]
Post-	8.75	9.17	12.58	3.5[a]	12.92[a]

[a] Statistically significant difference.

Table 2 Values of DPOAE pre- and post-exposure to conditioning noise – Sound group.

Sound group	F 1	F 1.5	F 2	F 3	F 4
Pre-	23.58[a]	26.08	20.17	23.58	25.08[a]
Post-	36.33[a]	35.58	24.67	24.17	37.75[a]

[a] Statistically significant difference.

Noise conditioning

The guinea pigs were conditioned with a white noise centered at 4 kHz, at an intensity of 85 dB, 30 min per day for 10 consecutive days. The exposure was performed with the animals inside a soundproof box (EP-125 Audio Signal Generator; Insight – Ribeirão Preto, SP, Brazil). The dimensions of the device were 760 mm × 485 mm × 705 mm. Inside the box, the animals were placed in a cage with three equal divisions, with three guinea pigs at a time. The animals had free access to food and water and were kept in a 12 hour light-dark cycle. Sound amplifiers were attached to the top of the box, at a distance of 25 cm from the top of the cage. The variation of noise intensity was less than 2 dB at any point inside the box.

Statistical analysis

Statistical analyses were performed using nonparametric Mann–Whitney *U* and Wilcoxon tests. A significance level of 5% ($p = 0.05$) was adopted. The analyses were performed with SPSS, Minitab, and Excel programs.

Results

DPOAE results revealed that the Cont group that received only gentamicin in ototoxic doses, and the Expt group that was noise conditioned prior to the administration of the aminoglycoside, exhibited no response at any of the test frequencies in the assessments performed after drug administration. Evaluating these results in more detail, it was also observed that, both groups (Cont and Expt), had no responses after the administration of gentamicin and, in addition, the group that was noise conditioned prior to this medication (Expt group) exhibited significantly worse values at frequencies of 3 kHz and 4 kHz versus the group treated only with the aminoglycoside ($p = 0.009$ and $p = 0.007$, respectively) (Table 1), suggesting a potentiation of the deleterious effects of the drug by pre-exposure to noise. The Sound group, treated only with noise conditioning, exhibited intriguing results. Responses were observed in post-noise DPOAEs in all frequencies of this group and, additionally, there was improvement in post-treatment values of DPOAEs, compared to pre-treatment baseline values. This improvement was observed at all frequencies, but the difference was significant only at the frequencies of 1 kHz ($p = 0.006$) and 4 kHz ($p = 0.023$) (Table 2). With respect to electrophysiological hearing thresholds obtained by BAEP, it was observed that only the noise-conditioned Sound group animals showed no worsening of their thresholds after the respective treatment (noise conditioning). Their pre- and post-thresholds were 17.9 dB and 15.8 dB, respectively. The

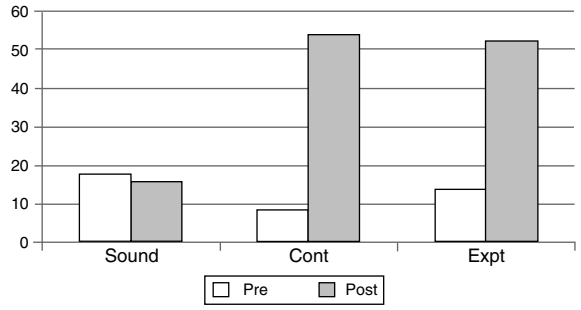

Figure 1 Hearing thresholds (in dB) before and after respective treatments for Sound, Cont, and Expt groups.

Cont and Expt groups exhibited similar deterioration in their hearing thresholds in pre- and post-comparisons. The hearing thresholds of the animals of these groups had a mean value, before any treatment, of 8.5 dB and 13.7 dB, respectively. The Cont and Expt groups showed similar worsening of their hearing thresholds in the pre-and post-comparison. Before any treatment, the hearing thresholds of the animals of these groups had average values of 8.5 dB and 13.7 dB, respectively. After administration of the aminoglycoside, the thresholds of Cont and Expt groups became, on average, 53.5 dB and 52 dB respectively, with no statistically significant difference (Fig. 1).

The histological evaluation revealed that the Sound group had not been damaged as a result of the auditory conditioning with a white noise of 4 kHz at the intensity of 85 dB, 30 min per day for ten consecutive days (Fig. 2). The histological analysis and the cochleograms showed the following

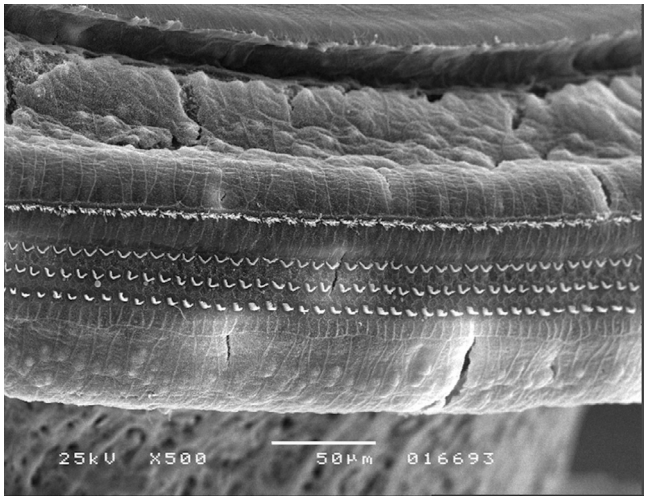

Figure 2 Photo from Sound group (scanning electronic microscopy), depicting no injuries to outer hair cells, 500× magnification.

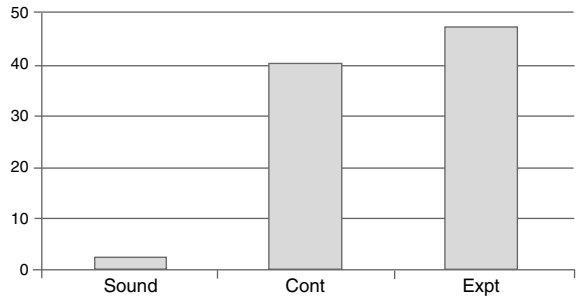

Figure 3 Percentage of outer hair cell damage in each group after their respective treatments.

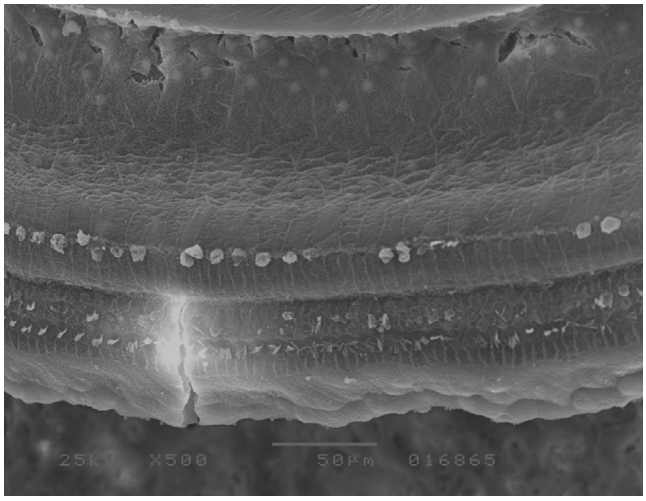

Figure 4 Photo from Cont group (scanning electronic microscopy), 500× magnification.

mean percentage of injury for each group: Sound: 2.2%; Cont: 39.9%; and Expt: 47.3% (Figs. 3–5). By detailing these results and evaluating the mean percentage of damage per each turn, it was perceived that the Cont group, which received only gentamicin in ototoxic doses, and Expt group, which was noise-conditioned prior to the administration of this drug, showed similar values for injury in first and second cochlear turns – Cont (first turn): 52.7% and Expt (first turn): 53.2%; and Cont (second turn): 32.8% and Expt (second turn): 36.9%. However, when comparing the mean values for outer hair cells damage between these two groups, it was found that the Expt group had a greater number of lesions, compared with the Cont group: 47.3% and 39.9%, respectively. However, this difference was not statistically significant.

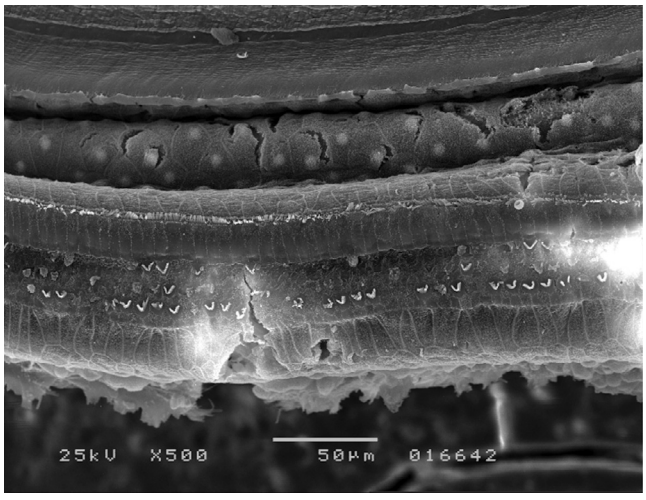

Figure 5 Photo from Expt group (scanning electron microscopy), 500× magnification.

Discussion

The vast majority of studies testing auditory conditioning have demonstrated good results. Many types of noise and drugs have been tested successfully. Almost all of these studies used the same stimulus in the conditioning and traumatic phases. The first study evidencing this phenomenon conditioned guinea pigs with noise, with the aim of stimulating the body to develop protection mechanisms, not yet fully understood, against a subsequent presentation of an acoustic trauma.[1] Several other subsequent studies also used noise, both as a protective agent and as a traumatic factor, all of them with positive results.[1–6] Another very common cause of hearing loss is induced ototoxicity by aminoglycoside drugs. Based on the same principle of auditory noise conditioning, other studies tested whether pre-exposure to non-traumatic doses of aminoglycosides also avoid or at least reduce the damage caused by an ototoxic administration of these drugs. The results showed that conditioning was also effective with drugs.[7,8] The injury protocol for gentamicin used in this study was the same as the aforementioned work.[8] Furthermore, the use of different drugs in the conditioning and trauma phase, e.g., conditioning with salicylate for protection against cisplatin or gentamicin, was tested.[16,17] These studies also showed positive results. Only one study showed no protection by cross-conditioning with different drugs. Non-ototoxic doses of gentamicin were administered for protection against further ototoxicity with amikacin. The results showed that the animals previously treated with gentamicin (i.e., conditioned) showed no signs of protection, compared with those treated only with amikacin.[18]

It has been suggested that the stress resulting from the auditory conditioning procedure, rather than the action of any agent in particular, promotes the observed protection. This could occur by the release of corticosteroids in response to stress.[9] This hypothesis was recently challenged by a study that tested whether conditioning with kanamycin was effective in protecting against acoustic trauma. To determine if the conditioning process itself was responsible for stimulating protective mechanisms, one of the study groups received saline solution (rather than kanamycin), using the same scheme of administration as those animals that were conditioned with kanamycin. Both groups were exposed to acoustic trauma. The animals that received saline solution showed no protective effect, which led the authors to conclude that the protection resulting from the stress of animal handling and from the injections was not significant.[11]

The authors reviewed several studies that tested the conditioning with a physical agent (sound) in an attempt to prevent subsequent ototoxic trauma by drugs (aminoglycoside antibiotics or antineoplastic agents) or vice versa. In one study, rats were exposed to a white noise centered

at 8 kHz at an intensity of 85 dB for 15 min. After 45 min, cisplatin was administered intravenously at a single dose of 14 mg/kg. The evaluation was made by determining the electrophysiological thresholds by BAEP before and after administration of cisplatin. The researchers found that noise-conditioned animals showed a significantly lower change in their auditory threshold than in those receiving cisplatin alone.[10] In another study, which did not have the study of auditory conditioning as its primary objective, but rather studied the synergy between amikacin and noise in a population of young CBA/J mice, the authors were led to the ''startling conclusion'' that administration of the antibiotic before the acoustic trauma not only did not cause potentiation of the damage caused by the drug, but also protected the outer hair cells, which, in animals receiving amikacin prior to acoustic trauma, showed less expressive injury.[11]

Another study tested the conditioning with gentamicin in nontraumatic doses for protection against acoustic trauma. The authors used albino guinea pigs medicated with intramuscular gentamicin 30 mg/kg/day for 30 consecutive days and subsequently exposed to a continuous acoustic trauma centered on 4 kHz at an intensity of 110 dB for 72 h. It was found that the animals conditioned with gentamicin showed less morphological damage, assessed by scanning electron microscopy. However, no functional protection was observed, since the change in thresholds was similar in both conditioned and unconditioned animals.[12] A continuous noise conditioning with a frequency between 1410 Hz and 5650 Hz at 81 dB for 3 weeks was used in another study to test whether this procedure would promote protection against a topical administration of intratympanic gentamicin.[14] The researchers found that the animals conditioned with this stimulus had less injury to their inner and outer hair cells. No functional assessments were made. A conditioning protocol with the use of a traumatic noise, leading to a temporary decrease of the thresholds, was also tested for protection against cisplatin and gentamicin ototoxicity. This conditioning was effective against the two drugs, both morphologically and functionally.[13]

In the present study, the noise-conditioned animals showed no protection compared with animals that were treated with ototoxic doses of gentamicin. This lack of protection could be observed both functionally and morphologically. The noise-conditioned animals showed absence of DPOAE, change in electrophysiological thresholds, and outer hair cell damage similar to that observed in animals that received only the drug. There were no other studies found with the same manner of conditioning protocol as used in this work. Most studies testing auditory conditioning used noises for longer periods than those used in the present work. In some, the noise conditioning was performed consecutively for days.[14] The choice of a conditioning protocol with use of noise for a shorter period was based on an abovementioned study that showed good results against cisplatin ototoxicity.[10] However, it was decided to test with a slightly longer protocol than that used in the aforementioned work (i.e. only one presentation of noise for 15 min).

Other studies have already achieved positive results with noise conditioning as a prevention against cisplatin ototoxicity.[10,14] One of them performed the conditioning with white noise centered at 8 kHz at an intensity of 85 dB for 15 min, to protect against a single dose of cisplatin 14 mg/kg. The other study used a protocol of noise at 8–16 kHz, 91 dB for 2 h, which caused a temporary threshold shift; this latter study also observed protection of animals thus noise conditioned against both cisplatin and gentamicin ototoxicity. This protocol of ototoxic injury with gentamicin was the same used in previous works, consisting of 160 mg/kg/day for ten consecutive days.[8] In the present study, the presentation of the conditioning noise was made over a period of 30 min before each dose of the antibiotic. The hypothesis for the absence of protection triggered by this conditioning protocol is that it was not strong enough to trigger the intrinsic mechanisms responsible for the protection; or at least not strong enough to protect against injury of the magnitude caused by 160 mg/kg/day for ten consecutive days. One hypothesis for the mechanism responsible for the effectiveness of noise conditioning is that the stress triggered by stimulation results in release of heat shock protein.[14] Therefore, it seems important that the conditioning stimulus be able to generate some kind of stress in cochlear structures. Previous studies have shown that this dose of gentamicin promotes extensive damage to outer hair cells.[8] Knowing that a conditioning protocol had been effective with a pre-exposure of white noise centered at 8 kHz at an intensity of 85 dB for only 15 min, as in the abovementioned study, the authors intended to test whether a conditioning protocol using a short exposure period would also be effective. It would be very worthwhile to define simpler and less traumatic possible conditioning protocols, when considering possible future clinical applications of this paradigm. It is also important to underscore some differences between the study mentioned above, which achieved positive results with noise conditioning for 15 min, and the present study. The animal studied by these authors was different and, in addition, the ototoxicity was achieved with a single dose of cisplatin. Another important point is that the assessment performed by them was only functional, estimating the electrophysiological thresholds, and studies have been published that show disagreement between auditive thresholds and morphological changes.[19] The DPOAE findings reveal that the present study's conditioning seems to have stimulated outer hair cells (OHC), since the values obtained by this post-noise exposure examination, in the Sound group, showed an improved response, and this response was statistically significant for frequencies 1 and 4 kHz, as shown in the present results. Thus, a noise conditioning protocol at 85 dB for 30 min for ten consecutive days seems to have stimulated the outer hair cells, but not enough to protect them against ototoxic injury; or perhaps this change was just a sign of early injury to cochlear structures. In view of the authors' personal experience and also considering the information obtained by other studies, it is believed that the conditioning protocol should not have an intensity so detrimental as to cause great cochlear damage, nor so benign as to not promote any change in cochlear structures - which appears to have occurred in the present conditioning protocol.

The potential synergistic effect between noise and aminoglycoside antibiotics must be taken into consideration, since these agents are potentially harmful to the auditory system. There are studies showing that simultaneously-exposed animals exhibit greater injury than that presented by the simple sum of injuries of each agent separately.[20] The present results show a trend of synergism

between noise and gentamicin, considering that the animals receiving both factors had worse outcomes than those who received each factor alone. However, considering the results obtained by this study, it cannot be stated that there was an effective synergism between these factors. As mentioned above, those animals treated only with noise exhibited virtually no injury. Conversely, the animals of Expt group, that received gentamicin after noise, exhibited greater changes than those treated only with gentamicin. Functionally, the variation in auditory thresholds was very similar, with no statistically significant difference. However, the DPOAE results showed, at frequencies of 3 and 4 kHz, that the animals treated with both factors showed statistically significant worse results, compared to animals treated only with gentamicin. Histologically, the animals of Expt group, which were treated with both factors, also had a greater number of outer hair cells injuries; however this difference was not statistically significant.

Conclusion

The present study demonstrated that the conditioning protocol with a white noise centered at 4 kHz at an intensity of 85 dB for 30 min per day for ten consecutive days was not effective in preventing cochlear damage caused by intramuscular administration of gentamicin in a dose of 160 mg/kg/day for ten consecutive days.

Conflicts of interest

The authors declare no conflicts of interest.

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