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MAIN ARTICLE

Prevention of gentamicin ototoxicity with N-acetylcysteine and vitamin A

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Abstract

Objective: To investigate the use of systemic N-acetylcysteine and vitamin A in the prevention of gentamicin ototoxicity in rats.

Methods: Forty-two Wistar rats were divided into four groups according to treatment: intratympanic saline, intratympanic gentamicin, intraperitoneal vitamin A after intratympanic gentamicin, and intraperitoneal N-acetylcysteine after intratympanic gentamicin. Signal-to-noise ratio and distortion product otoacoustic emissions were evaluated in all groups.

Results: N-acetylcysteine had a significant protective effect at 1.5, 2, 3, 4, 6 and 8 kHz, whilst vitamin A had a significant protective effect at 2, 3, 4 and 6 kHz, as determined by the distortion product otoacoustic emission measurements. According to the signal-to-noise measurements, N-acetylcysteine had a significant protective effect at 1.5, 2, 3, 4, 6 and 8 kHz, whilst vitamin A had a significant protective effect at 3, 6 and 8 kHz.

Conclusion: Gentamicin-induced hearing loss in rats may be prevented by the concomitant use of vitamin A and N-acetylcysteine. Specifically, N-acetylcysteine appeared to have a more protective effect than vitamin A for a greater range of noise frequencies.

Key words: Hearing Loss; Rats; Aminoglycosides; N-Acetylcysteine; Vitamin A

Introduction

Ototoxicity is a term that describes cochlear and vestibular organ damage resulting from exposure to various therapeutic agents or chemical substances.[1] More specifically, ototoxicity is the functional impairment and cellular degeneration of inner-ear tissues caused by therapeutic agents. The most common ototoxic drugs currently in clinical use include the following: aminoglycoside antibiotics, platinum-based chemotherapeutic agents (cisplatin and carboplatin), loop diuretics, macrolide antibiotics and antimalarial drugs.[2]

Aminoglycosides, such as gentamicin, are readily used as antibiotics because of their potent activity against several bacterial infections (e.g. enterococci, mycobacteria and multidrug-resistant Gram-negative bacteria), despite well-known side effects, like ototoxicity and nephrotoxicity.[3] Any solution applied to the middle-ear cavity can pass through the round window and cause side effects on the cochlear and vestibular apparatus. To date, aminoglycoside-induced hearing loss is most prevalent in developing countries where these drugs are typically the only affordable antibiotics available. However, intratympanic gentamicin has become an important treatment modality in the management of unilateral Ménière's disease over the past decade.[4] Several different modalities of gentamicin administration to the tympanic cavity have been utilised. For example, vertigo can be controlled by using local gentamicin injections to achieve a chemical labyrinthectomy. However, this regimen is known to negatively affect hearing.[5]

In 1978, Kemp reported the production of acoustic energy by the outer cochlear hair cells, which he termed 'otoacoustic emissions' (OAEs) (as cited by Tepel).[6] The energy that comes from the outer hair cells spreads through the base of the stapes, ossicles, tympanic membrane and external ear canal. This acoustic energy can be measured by a sensitive microphone placed within the ear canal. There are different types of OAEs, but distortion product OAE (DPOAE) technology has good frequency specificity and has been widely used in studies. Measurement of DPOAEs in a rat model was reported for the first time in 1996 by Khvoles *et al.*[7] and has since become a topic of interest in numerous studies.[8–10]

N-acetylcysteine has been utilised as a treatment for several diseases, ranging from chronic bronchitis to

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toxic liver damage. The antioxidant effects of N-acetylcysteine are based on the following characteristics: free radical scavenger, glutathione (γ-glutamyl-cysteinyl-glycine) production substrate, mitochondrial protectant, glutamate excitotoxicity inhibitor, lipid peroxidation inhibitor and necrosis inhibitor. Its protective effects against acoustic trauma have already been documented.[11] Several studies have addressed its mechanisms, protective doses and methods of administration in acoustic trauma. However, there are only a few *in vitro* studies focusing on the effect of N-acetylcysteine on aminoglycoside ototoxicity.[12–14]

Vitamin A acts as an anti-apoptotic agent through its active metabolite retinoic mechanism. Specifically, all-trans-retinoic acids have been shown to inhibit the apoptosis of T cells, leukaemia cells and haematopoietic cells, and to attenuate hydrogen peroxide induced apoptosis in mesangial cells and fibroblasts.[15] Vitamin A, through its metabolite agent β-carotene, prevents lipid peroxidation.[16] Various studies suggest vitamin A reduces noise-induced hearing loss via its antioxidant effects.[17]

In this study, we examined the roles of systemic N-acetylcysteine and vitamin A in the prevention of gentamicin ototoxicity. Both are readily available, safe agents already in clinical use. We administered both agents via simple transtympanic injections, with the goal of utilising a more translational approach.

Materials and methods

Animals

The protocol used in this study was approved by the Institutional Animal Care and Use Committee, and the animals were treated in accordance with this protocol. We used 58 adult male Wistar rats weighing 250–300 g. All rats had free access to food and water, and were maintained in an environment with a controlled temperature (25 °C), with 12-hour light/dark cycles.

All rats were evaluated by otoscopy. Cerumen in the ear canal was cleaned prior to distortion product OAE (DPOAE) testing.

Study design

All rats were anaesthetised intraperitoneally with 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine on days 2, 4 and 6. Rats were monitored for 30 days to ensure tympanic membrane re-epithelisation. At the end of the study period, all rats were decapitated under deep anaesthesia using intravenous sodium pentothal.

Prior to the end of the study period, nine rats from different groups died. We excluded seven rats because of a lack of re-epithelisation of the tympanic membrane. Hence, a total of 42 rats were utilised in the study.

The DPOAE measurements were obtained for left ears prior to drug administration (baseline recording) and on day 30. Rats with normal hearing, as confirmed by DPOAE testing, were divided into the following four groups: group 1 ($n = 12$) received left intratympanic injection of 0.2 cc saline (40 mg/ml); group 2 ($n = 10$) received left intratympanic injection of 0.2 cc gentamicin (40 mg/ml); group 3 ($n = 10$) received an intraperitoneal injection of 100 000 IU palmitate in the form of vitamin A after the left intratympanic injection of 0.2 cc gentamicin (40 mg/ml); and group 4 ($n = 10$) received an intraperitoneal injection of 300 mg/kg N-acetylcysteine after the left intratympanic injection of 0.2 cc gentamicin (40 mg/ml).

Otoacoustic emissions testing

We included rats with normal DPOAE measurements, which we confirmed prior to drug administration. The DPOAE recordings were performed in a quiet room, where we measured the left ears of both control and experimental rats. After administration of anaesthesia, an earphone was inserted into the external ear canal via a plastic adapter, which released the primary tones. The DPOAEs were recorded in terms of distortion product grams (DP grams). The intensity of the primary tones was constant and the DPOAE data were recorded at different frequencies (1, 1.5, 2, 3, 4, 6 and 8 kHz), with a frequency plan of F2. Both signal-to-noise ratio and distortion product levels were evaluated during DPOAE procedures. All rats were decapitated at the end of the study under deep anaesthesia with intravenous sodium pentothal.

Statistical analysis

For the data analyses, SPSS® software (version 21) and PAST software[18] were used. Kolmogorov– Smirnov and Shapiro–Wilk tests were used to assess the compatibility of single variant values with normal distribution. An omnibus test was used to assess the compatibility of multiple variant values with normal distribution. A paired-samples *t*-test was performed to measure dependent variants. A general linear model repeated analysis of variance was used to assess the interaction of repeated measurements of the variants amongst the groups. Least significant difference and Games-Howell tests were used for post hoc analyses. Data were processed in terms of 95 per cent confidence intervals, and *p*-values lower than 0.05 were accepted as statistically significant.

Results

The distortion product OAEs (DPOAEs) measured at baseline and on day 30 were recorded and assessed. In group 3 (gentamicin plus vitamin A group) and group 4 (gentamicin plus N-acetylcysteine group), the differences between DPOAE levels per frequency range obtained at baseline and on day 30 were lower than group 2 (gentamicin-only group) levels at all frequencies (Figure 1). In group 2, significant deterioration was observed at all frequencies compared to group 1 (saline injection group). Regarding group 3

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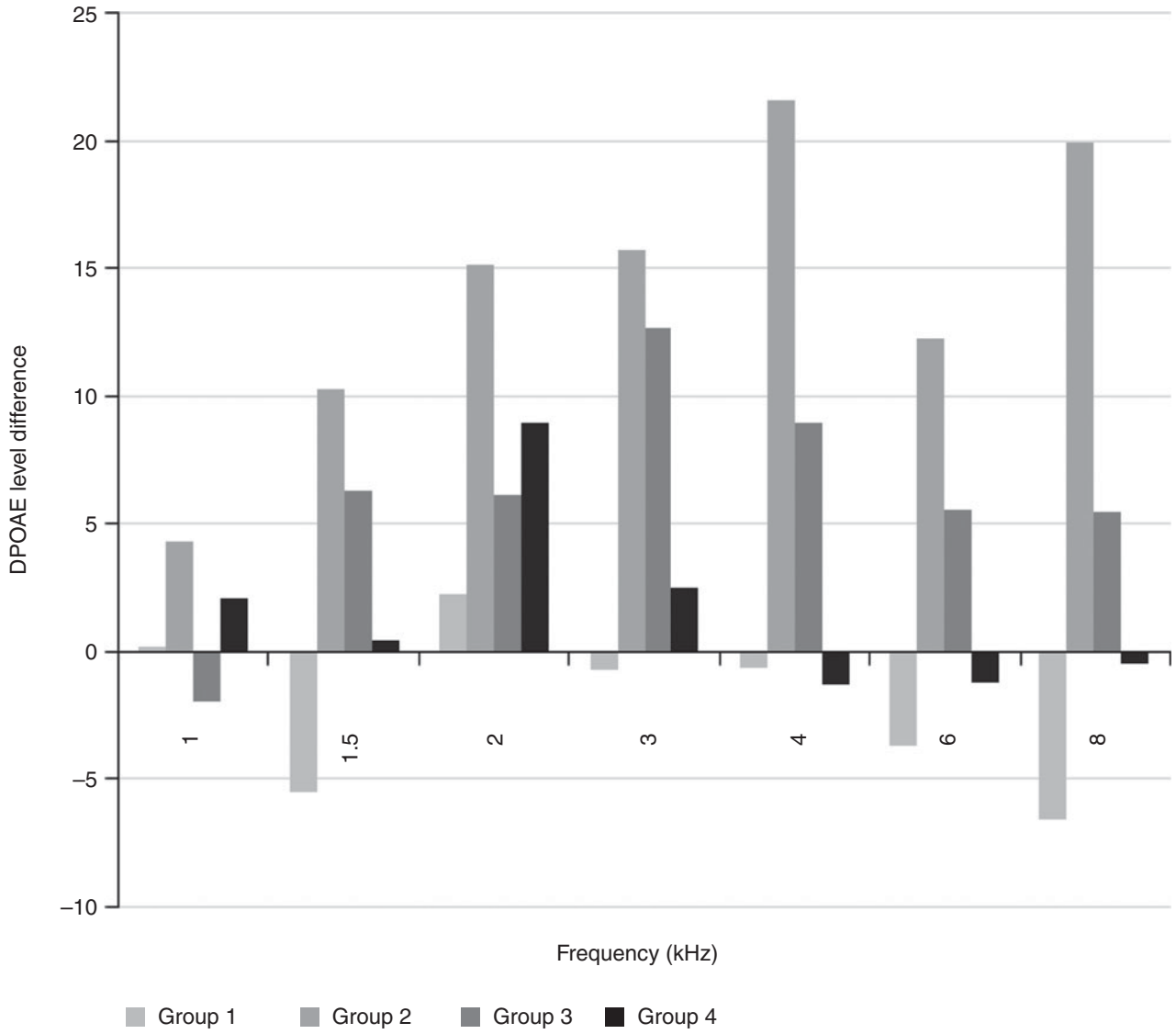

FIG. 1
Differences between distortion product otoacoustic emission (DPOAE) levels per frequency range obtained at baseline and on day 30.

and group 4, vitamin A had a better protective effect at 1 and 2 kHz, whilst N-acetylcysteine had a better protective effect at 1.5, 3, 4, 6 and 8 kHz (Figure 1).

Signal-to-noise ratio levels per frequency range obtained at baseline and on day 30 for group 3 were lower than group 2 at 3, 4, 6 and 8 kHz. Group 4 had lower signal-to-noise ratio levels than group 2 at 1.5, 2, 3, 4, 6 and 8 kHz (Figure 2). When the signal-to-noise ratio levels at 3, 4, 6 and 8 kHz were compared amongst group 3 and group 4, N-acetylcysteine was more protective for all frequencies (Figure 2).

Gentamicin and saline groups significantly differed in both DPOAEs and signal-to-noise ratio levels at all frequencies except 1 kHz (Figures 3 and 4). When group 3 and group 4 DPOAE levels were compared to the saline group levels, N-acetylcysteine had a significant protective effect at 1.5, 2, 3, 4, 6 and 8 kHz, whilst vitamin A had a significant protective effect at 2, 3, 4 and 6 kHz. Regarding signal-to-noise ratio levels, N-acetylcysteine had a significant protective effect at 1.5, 2, 3, 4, 6 and 8 kHz, whilst vitamin A had a significant protective effect at 3, 6 and 8 kHz (Figure 4).

Discussion

Eardrops containing gentamicin are commonly prescribed by otolaryngologists for treatment of external otitis, chronic suppurative otitis media and discharging tympanostomy tubes. Although topical aminoglycoside ototoxicity appears to be infrequent, there is indisputable data suggesting that this may develop if eardrops reach the middle ear in the presence of a tympanic membrane perforation or defect. Chemical labyrinthectomy achieved by local gentamicin injections, a treatment modality utilised in unilateral Ménière's disease, has become more popular over the past decade. This treatment is known to negatively affect hearing.[5]

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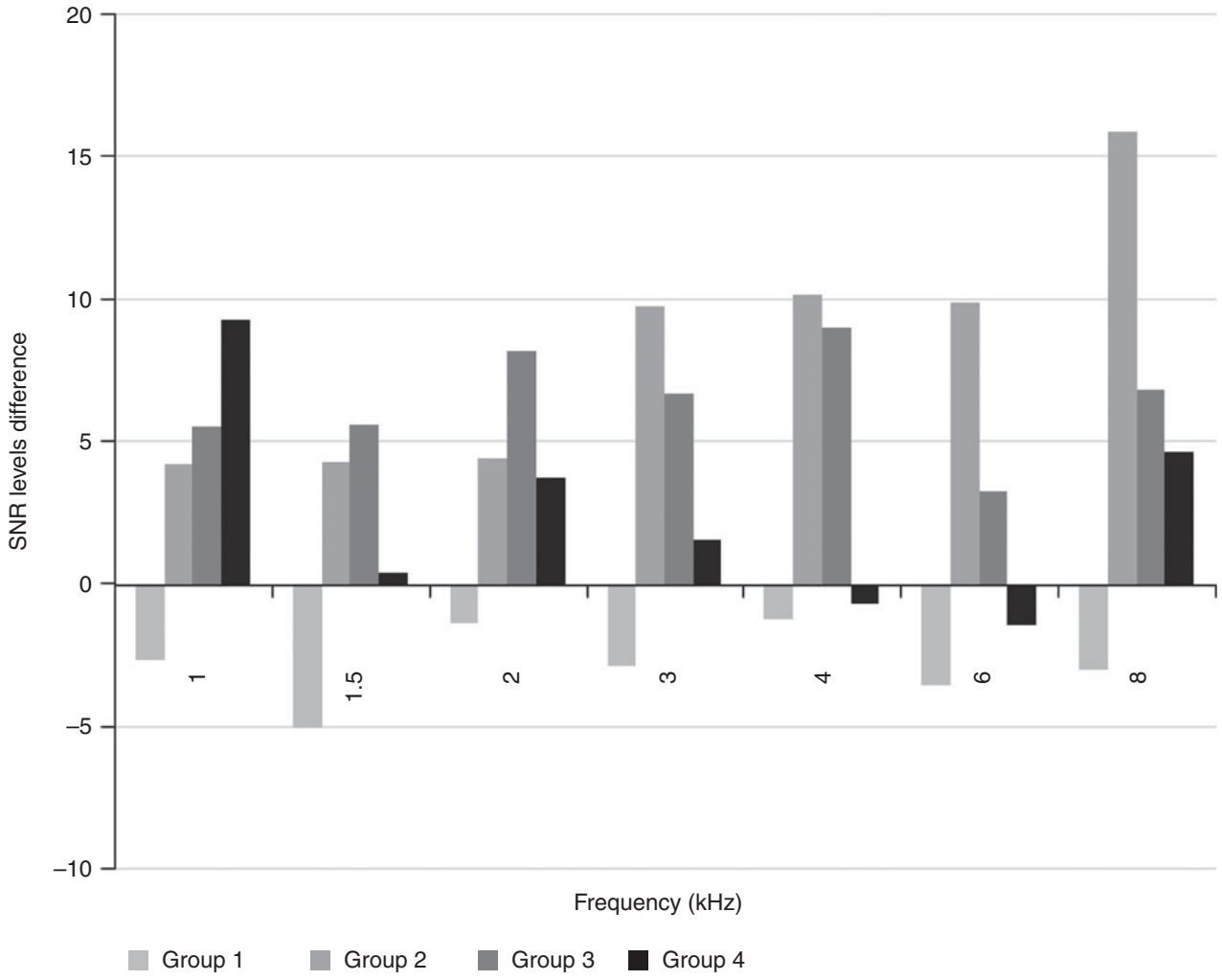

FIG. 2
Differences between signal-to-noise ratio (SNR) levels per frequency range obtained at baseline and on day 30.

Data from animal studies suggest that ototoxicity is caused by the formation of reactive oxygen species and can be attenuated by antioxidants. Much progress has been made in understanding the mechanism(s) responsible for inner-ear hair cell degeneration.[19] In this study, we utilised an animal model to investigate the roles of systemic N-acetylcysteine and vitamin A in the prevention of gentamicin ototoxicity.

Gentamicin damages the outer hair cells, starting from the basal region of the cochlea, and progressing to the middle and apical regions. As lower frequencies are affected, this may lead to permanent damage in speech discrimination scores.[20] The damage to outer hair cells is evaluated by distortion product OAE (DPOAE) testing.[10]

The *in vitro* work performed by Feghali *et al*. introduced N-acetylcysteine as a potential antioxidant for the prevention of aminoglycoside ototoxicity.[21] The drug is widely used as a nebulised mucolytic and as an antidote for acetaminophen poisoning, where it directly regenerates glutathione in hepatocytes. Recently, it has been used successfully in several models of ischaemic and toxic injuries to the heart, kidney, liver and lungs.[22,23] In each of these infections, it is thought that the activity of N-acetylcysteine is mediated, at least in part, by its antioxidant properties. The beneficial effect of N-acetylcysteine on cochlear function observed in this study may be explained by its antioxidant properties. The low molecular weight of N-acetylcysteine (163.2 g/mol) also makes it an excellent candidate for administration across the round window membrane.

Vitamin A is a family of hydrocarbons, which includes retinol, retinal, retinoic acid and several pro-vitamin A carotenoids (e.g. β-carotene).[24] Retinoic acid is an active metabolite of vitamin A that regulates a wide range of biological processes, including cell proliferation, differentiation and morphogenesis. Retinoic acid has been shown to inhibit the apoptosis of T cells, leukaemia cells and haematopoietic cells, and to attenuate hydrogen peroxide induced apoptosis in mesangial cells and fibroblasts.

In this study, N-acetylcysteine had a significant protective effect at 1.5, 2, 3, 4, 6 and 8 kHz, whilst vitamin A had a significant protective effect at 2, 3, 4 and 6 kHz, as measured by DPOAE levels. According to

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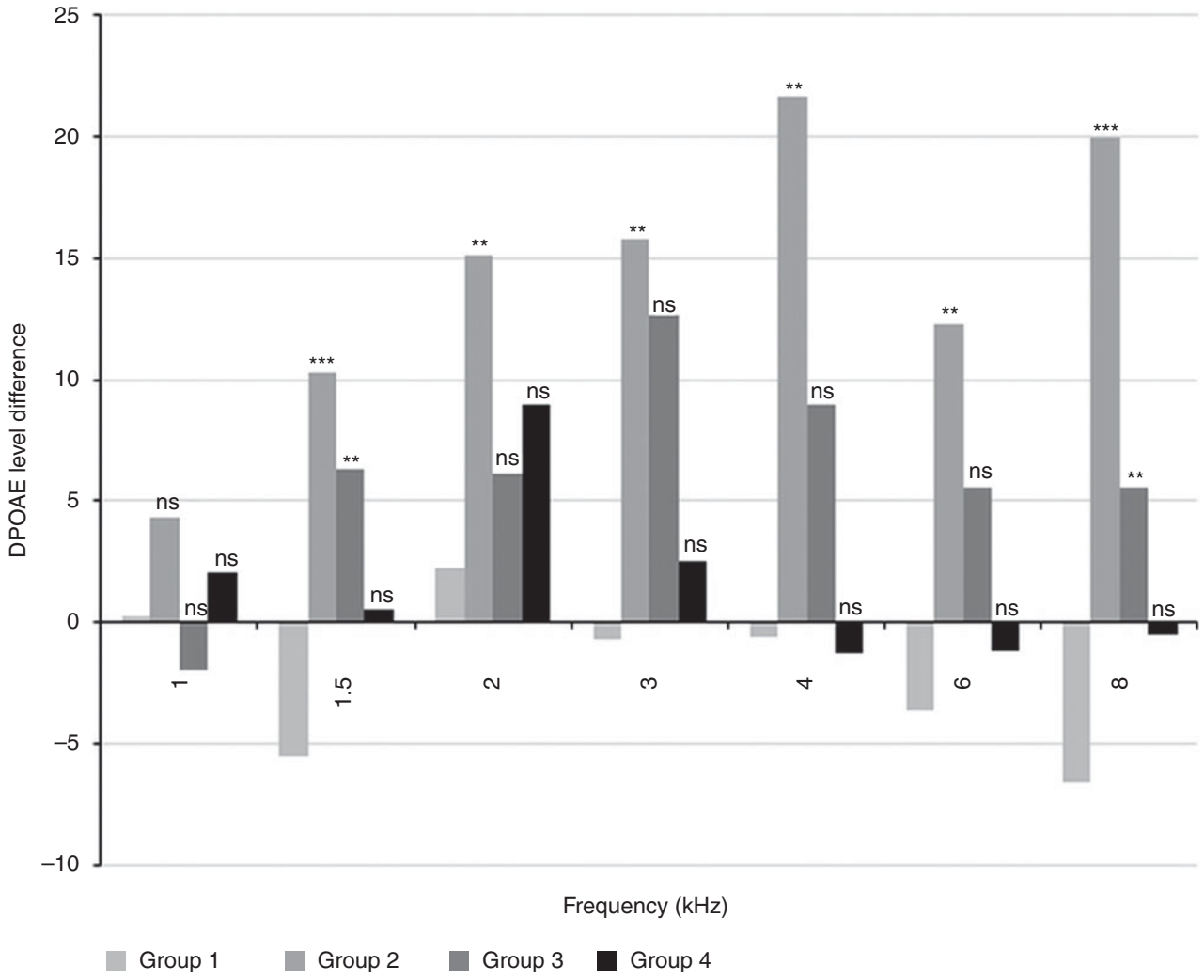

FIG. 3

Statistical comparison of differences between distortion product otoacoustic emission (DPOAE) levels per frequency range obtained at baseline and on day 30. ns = Non-significant at 0.05 level; ***significant at 0.001 level; **significant at 0.01 level

signal-to-noise ratio levels, N-acetylcysteine had a significant protective effect at 1.5, 2, 3, 4, 6 and 8 kHz, whilst vitamin A had a significant protective effect at 3, 6 and 8 kHz.

Otoacoustic emissions are an ideal, non-invasive tool to detect cochlear activity. Otoacoustic emissions are sounds that arise from the vibrations of the outer hair cells in the cochlea that are transmitted by the middle ear and tympanic membrane from the external auditory canal. These emissions can be measured and recorded by a small, sensitive microphone placed in the external auditory canal. In this study, DPOAE testing was chosen to independently evaluate the integrity of the auditory system.

The DPOAE protocol in animal experiments varies little, except with regard to the F1 and F2 stimulus intensity and the variability of the DP gram.[9,25] The range in frequencies utilised to study DPOAEs varies depending on the species of animal used in the investigation. For example, for a signal-to-noise ratio of 3 dB SPL or more: Hyppolito *et al*. determined that 1.5 kHz is necessary in guinea pigs;[26] Hatzopoulos *et al*. determined that over 4 kHz is necessary in Sprague-Dawley rats;[10] Lopez-Gonzalez *et al*. determined that 1–6 kHz is necessary in Wistar rats;[25] and Sockalingam *et al*. determined that 2–8 kHz is necessary in albino rats.[9] McAlpine and Johnstone observed cisplatin ototoxicity through DPOAE testing with input/output function shape fixing when the frequency was 8 kHz.[27] In this study, we carried out the protocol with a fixed intensity of 70 dB SPL, attaining measurable responses from 3 kHz for a signal-to-noise ratio of 6 dB SPL or more.

Increasing evidence suggests that intracellular free radical generation is the mechanism responsible for aminoglycoside-induced cochlear damage. This has led to the successful protection of the cochlea against ototoxicants via inhibition of intracellular free radical production.

This study does have some limitations. We did not measure the concentration of the intratympanic drug within the inner ear, nor did we examine the middle

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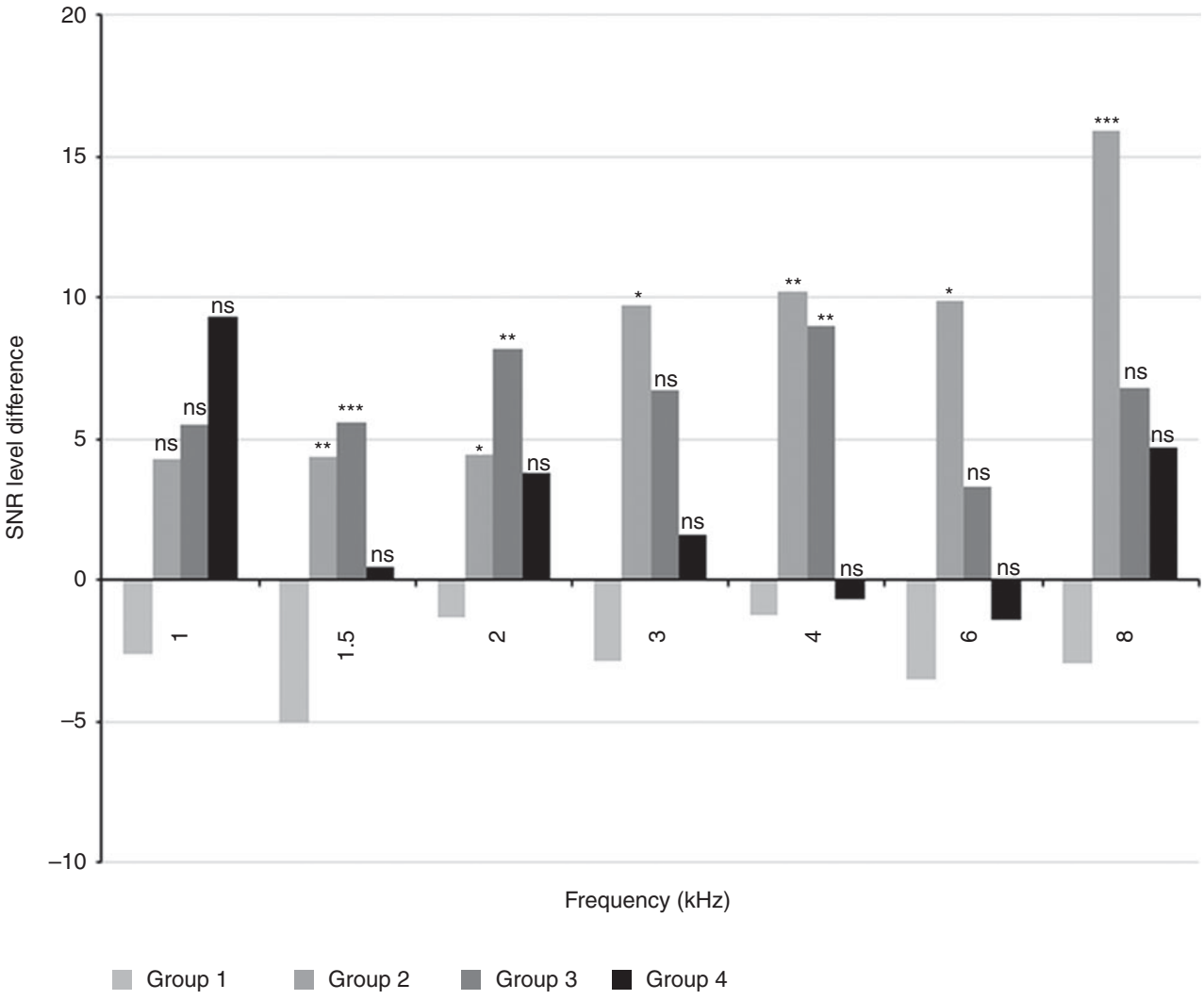

FIG. 4

Statistical comparison of differences between signal-to-noise ratio (SNR) levels per frequency range obtained at baseline and on day 30. ns = Non-significant at 0.05 level; **significant at 0.01 level; ***significant at 0.001 level; *significant at 0.05 level

ear with an electron microscope prior to gentamicin application.

- **Aminoglycosides, such as gentamicin, are antibiotics used for a number of bacterial infections**
- **Any solution applied to the middle-ear cavity can pass through the round window and may affect other ear regions**
- **This study aimed to examine systemic N-acetylcysteine and vitamin A for gentamicin ototoxicity prevention in rats**
- **Gentamicin-induced hearing loss in rats may be reduced by concomitant use of vitamin A and N-acetylcysteine**
- **N-acetylcysteine may be slightly more effective than vitamin A**

In conclusion, this study suggests that gentamicin-induced hearing loss in rats may be limited to some extent by the concomitant use of vitamin A and N-acetylcysteine. Dose-dependent changes will need to be further investigated in future studies. In addition, studies focused on the morphological changes may shed light on the protective effects of vitamin A and N-acetylcysteine.

References

1 Ahmed RM, Hannigan IP, MacDougall HG, Chan RC, Halmagyi GM. Gentamicin ototoxicity: a 23-year selected case series of 103 patients. *Med J Aust* 2012;**196**:701–4
2 Turnidge JD, Waterston JA. Gentamicin and ototoxicity: why this drug is still in use. *Med J Aust* 2012;**196**:665–6
3 Kemp DT. Stimulated acoustic emissions from within the human auditory system. *J Acoust Soc Am* 1978;**64**:1386–91
4 Cohen-Kerem R, Kisilevsky V, Einarson TR, Kozer E, Koren G, Rutka JA. Intratympanic gentamicin for Ménière's disease: a meta-analysis. *Laryngoscope* 2004;**114**:2085–91
5 Salt AN, Gill RM, Plontke SK. Dependence of hearing changes on the dose of intratympanically applied gentamicin: a meta-analysis using mathematical simulations of clinical drug delivery protocols. *Laryngoscope* 2008;**118**:1793–800

https://doi.org/10.1017/S0022215116000992 Published online by Cambridge University Press

6 Tepel M. N-Acetylcysteine in the prevention of ototoxicity. *Kidney Int* 2007;**72**:231–2
7 Khvoles R, Freeman S, Sohmer H. Transient evoked otoacoustic emissions can be recorded in the rat. *Hear Res* 1996;**97**:120–6
8 Khvoles R, Freeman S, Sohmer H. Development of transient evoked otoacoustic emissions in the neonatal rat. *Audiol Neurootol* 1998;**3**:40–53
9 Sockalingam R, Freeman S, Cherny TL, Sohmer H. Effect of high-dose cisplatin on auditory brainstem responses and otoacoustic emissions in laboratory animals. *Am J Otol* 2000;**21**: 521–7
10 Hatzopoulos S, Di Stefano M, Albertin A, Martini A. Evaluation of cisplatin ototoxicity in a rat animal model. *Ann N Y Acad Sci* 1999;**884**:211–25
11 Saliba I, El Fata F, Ouelette V, Robitaille Y. Are intratympanic injections of N-acetylcysteine and methylprednisolone protective against cisplatin-induced ototoxicity? *J Otolaryngol Head Neck Surg* 2010;**39**:236–43
12 Kubow S, Woodward TL, Turner JD, Nicodemo A, Long E, Zhao X. Lipid peroxidation is associated with the inhibitory action of all-trans-retinoic acid on mammary cell transformation. *Anticancer Res* 2000;**20**:843–8
13 Ahn JH, Kang HH, Kim YJ, Chung JW. Anti-apoptotic role of retinoic acid in the inner ear of noise-exposed mice. *Biochem Biophys Res Commun* 2005;**335**:485–90
14 Boztepe OF, Gün T, Gür ÖE, Karakuş MF, Bilal N, Arda HN. Effect of N-acetylcysteine for the treatment of otitis media with effusion. *J Med Updates* 2014;**4**:20–4
15 Shim HJ, Kang HH, Ahn JH, Chung JW. Retinoic acid applied after noise exposure can recover the noise-induced hearing loss in mice. *Acta Otolaryngol* 2009;**129**:233–8
16 Muurling T, Stankovic KM. Metabolomic and network analysis of pharmacotherapies for sensorineural hearing loss. *Otol Neurotol* 2014;**35**:1–6
17 Emmett SD, West KP Jr. Gestational vitamin A deficiency: a novel cause of sensorineural hearing loss in the developing world? *Med Hypotheses* 2014;**82**:6–10
18 Hammer Ø, Harper DA, Ryan PD. PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* 2001;**4**:9
19 Cheng AG, Cunningham LL, Rubel EW. Mechanisms of hair cell death and protection. *Curr Opin Otolaryngol Head Neck Surg* 2005;**13**:343–8
20 Miman MC, Ozturan O, Iraz M, Erdem T, Olmez E. Amikacin ototoxicity enhanced by Ginkgo biloba extract (EGb 761). *Hear Res* 2002;**169**:121–9
21 Feghali JG, Liu W, Van Der Water TR. L-N-Acetyl-cysteine protection against cisplatin-induced auditory neuronal and hair cell toxicity. *Laryngoscope* 2001;**111**:1147–55
22 Weinbroum A, Rudick V, Ben-Abraham R, Karchevski E. N-acetyl-L-cysteine for preventing lung reperfusion injury after liver ischemia-reperfusion: a possible dual protective mechanism in a dose-response study. *Transplantation* 2000; **69**:853–9
23 Mazzon E, Britti D, De Sarro A, Caputi AP, Cuzzocrea S. Effect of N-acetylcysteine on gentamicin-mediated nephropathy in rats. *Eur J Pharmacol* 2001;**424**:75–83
24 Damodaran S, Parkin KL, Fennema OR, eds. *Fennema's Food Chemistry*, 4th edn. Boca Raton, Florida: CRC Press, 2008
25 Lopez-Gonzalez MA, Guerrero JM, Rojas F, Delgado F. Ototoxicity caused by cisplatin is ameliorated by melatonin and other antioxidants. *J Pineal Res* 2000;**28**:73–80
26 Hyppolito MA, de Oliveira JA, Rossato M, Holanda F. Cisplatin ototoxicity and otoprotector to cilliated cells by ginkgo biloba extract: anatomic and electrophysiologic study [in Portuguese]. *Rev Bras Otorrinolaringol* 2003;**69**:504–11
27 McAlpine D, Johnstone BM. The ototoxic mechanism of cisplatin. *Hear Res* 1990;**47**:191–204

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