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Efficacy and Mechanisms of Antioxidant Compounds and Combinations Thereof Against Cisplatin Induced Hearing Loss in a Rat Model

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Abstract: Cisplatin is an election chemotherapeutic agent for many cancer treatments. Its cytotoxicity against neoplastic cells is mirrored by that taking place in healthy cells and tissues, resulting in serious adverse events. A very frequent one is ototoxicity, causing hearing loss which may permanently affect quality of life after successful oncologic treatments. Exacerbated oxidative stress is a main cytotoxic mechanism of cisplatin, including ototoxicity. Previous reports have shown antioxidant protection against cisplatin ototoxicity, but there is a lack of comparative studies on the otoprotectant activity and mechanism of antioxidant formulations. In this paper we report that a combination of vitamins A, C, E and Mg++ (ACEMg), previously shown to protect against noise-induced hearing loss, reverses auditory threshold shifts, promotes outer hair cell survival, and attenuates oxidative stress in the cochlea after cisplatin treatment, thus protecting against extreme cisplatin ototoxicity in the rat. The addition of 500 mg N-acetylcysteine (NAC), which administered individually also shows significant attenuation of cisplatin ototoxicity, to the ACEMg formulation, results in degradation of ACEMg otoprotection. Mg++ administered alone, as MgSO4, also prevents cisplatin ototoxicity, but in combination with 500 mg NAC, otoprotection also is greatly degraded. Increasing the dose of NAC to 1000 mg, also results in dramatic loss of otoprotection activity compared with 500 mg NAC. These findings support that single antioxidants or antioxidant combinations, particularly ACEMg in this experimental series, have significant otoprotection efficacy against cisplatin ototoxicity. However, an excess of combined antioxidants and/or elevated doses, above a yet to be defined "antioxidation threshold", results in unrecoverable redox imbalance with loss of otoprotectant activity.

Keywords: cisplatin; ototoxicity; oxidative stress; antioxidant

1. Introduction

Cisplatin (cis-diaminedichloroplatinum(II), CDDP) is a chemotherapeutic agent used in many cancer treatments [1]. One important adverse effect is ototoxicity [2] present in 12% to 100% of patients, depending on dose and treatment protocol [3,4]. The resulting hearing loss is sensorineural, typically bilateral, and irreversible [5,6]. It affects mainly high frequencies (4-8 kHz in humans) [7–9] and lower frequencies also become affected at high doses. The prevalence of hearing loss after cisplatin chemotherapy stimulates the search for efficient treatment or prevention strategies [10,11].

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One central antineoplastic mechanism of cisplatin involves oxidative stress. Upon entering cells, cisplatin forms adducts with DNA, which interferes with DNA replication and repair, and in general with normal gene transcription and regulation, basic for cell survival and perpetuation [12]. Mitochondrial DNA, lacking histones, easily forms such crosslink adducts with cisplatin, which impairs essential mitochondrial functions. Importantly, gene transcription and thus protein synthesis of electron transport chain enzymes are altered, and thus its activity. This compromises oxidative phosphorylation, and finally energy metabolism. Defective electron transport results in oxidative imbalance with excess accumulation of incompletely reduced, highly reactive oxygen species (ROS), which readily combine with nitrogenated compounds, giving rise to reactive nitrogen species (RNS). Excess ROS/RNS and related free radicals and non-radicals, leads to unchecked oxidative stress, which finally overrides the natural "antioxidant defenses" of the cell. Self-perpetuation of oxidative stress further alters gene expression, due to sensitivity of DNA and RNA to oxidative damage, as well as the structure, metabolism and interactions of proteins and complex lipids, with disorganization of cell membranes. Altered expression affects genes coding for "antioxidant" enzymes. Their activity may be also compromised by excess free radicals, further contributing to cisplatin cytotoxicity. All this finally results in activation of cell death pathways [12,13].

The high metabolic requirements of neoplastic cells make them particularly sensitive to cisplatin cytotoxic oxidative stress. However, it also causes unwanted cytotoxicity, particularly in cells and tissues with delicate metabolic energy balance, such as the auditory receptor organ. As mentioned, cisplatin leads to ototoxic deafness in a significant number of patients [14,15]. Systemic cisplatin is transported across the blood-endolymphatic barrier and into cells in the cochlea via transport proteins including megalin, organic cation transporters or copper transporters. Then, cisplatin-DNA adducts form in cochlear sensory and non-sensory cells [14,16]. Cisplatin accumulation is particularly intense and long-lasting in cells of the stria vascularis, which alters the endocochlear potential [17]. Regardless cell type, disruption of mitochondrial integrity and buildup of ROS/RNS with resulting oxidative stress, are central to cisplatin ototoxicity [18,19]. Like in tumoral cells, inactivation of antioxidant enzymes glutathione peroxidase, superoxide dismutase, catalase and glutathione reductase also contribute to oxidative stress damage [13,20]. In addition, specific cochlear factors, such as cisplatin-induced overexpression of the NADPH-oxidase NOX3, which is almost exclusively expressed in the inner ear, makes the auditory receptor particularly sensitive to cisplatin toxicity [19,21]. Overproduction of ROS by NOX3 accelerates lipid peroxidation [14], which among other things increases abnormal Ca^{++} flow across outer hair cell (OHCs) membranes, which is a signal for apoptosis [22,23].

In sum, many studies support increased oxidative stress as a main cisplatin ototoxicity mechanism [13,24]. Therefore, efforts have focused on neutralizing oxidative stress damage in the cochlea with antioxidant compounds [20,25,26]. From a biomedical perspective, antioxidants are chemical species which counteract and regulate biological oxidations, keeping ROS/RNS and other reactive free radicals and non-radicals within levels required to maintain physiological redox state and signaling. The expectation is that in pathological conditions such as cisplatin ototoxicity, in which built-in enzymatic and non-enzymatic cell "antioxidant defenses" may be critically challenged, antioxidant supplementation may restore redox balance by scavenging free radicals or otherwise limiting the oxidative capacity of free radicals and non-radicals.

Several antioxidant compounds have been tested experimentally against cisplatin ototoxicity. These include sodium thiosulfate [27,28], D-methionine [29,30], diethyldithiocarbamate, 4-methylthiobenzoic acid, ebselen, lipoic acid [31], and N-acetylcysteine [32,33] among others [19,28,34]. Most experimental assays have been conducted in rodent models. Histological preservation of hair cells and supporting structures, threshold recovery in auditory brainstem responses (ABRs), preservation of distortion product otoacoustic emissions and recovery of antioxidant enzyme activity or combinations thereof have been reported after antioxidant administration [13,19,28]. However, this is still not mirrored by conclusive evidence in the clinical setting [25,35], which stresses the need for more experimental, pre-clinical studies contributing further evidence on otoprotective activity and mechanisms of antioxidant therapies.

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The complex biochemical networks of redox balance and antioxidation defenses in cells, suggest that combinations of antioxidant compounds, each contributing specific antioxidation mechanisms, may result in additive or synergistic effects protecting against cisplatin ototoxicity [36,37]. A combination of antioxidant vitamins A, C and E along with Mg++, the latter likely acting primarily as a cochlear vasodilator, recovered hearing sensitivity and outer hair cell loss more efficiently than single compounds in noise-induced hearing loss [36,37], reducing oxidative stress and apoptosis [36]. The same antioxidant combination has shown efficacy in gentamycin-induced hearing loss [38] and cochlear implant associated loss of residual hearing [39]. Also, there is auditory threshold recovery in age-related hearing loss after long-term oral administration of ACEMg [40].

We have tested whether a similar principle may apply to antioxidant otoprotection in cisplatin ototoxicity. Despite still inconclusive clinical evidence, it is interesting that a combination of coenzyme Q and multivitamins [41] has been the only one to show, in a small trial, significant benefit in preventing cisplatin-induced hearing loss in a recent meta-analysis of thirteen randomized trials [25].

We have used subcutaneous injections of antioxidant vitamins A, C and E (ACE) along with Mg++ (ACEMg) and N-acetylcysteine (NAC), at different doses and/or combinations before the induction of cisplatin ototoxicity. The rationale for the use of NAC is that, like other thiosulfates [30,42], NAC is a free radical scavenger [43]. Importantly, NAC replenishes glutathione by providing cysteine residues for its synthesis [33,44,45]. Thus, it increases levels of this key intracellular redox regulator, particularly in situations of high demand or depletion [43]. Pre-clinical experimental evidence of otoprotection [46–48], along with its wide availability and limited adverse effects make it a potentially useful otoprotective antioxidant, although evidence for clinical use in cisplatin ototoxicity is inconclusive [25]. On the other hand, as far as ACEMg is concerned, vitamin A, essentially carotenoids, scavenge free radicals and non-radicals in lipid environments, notably singlet oxygen and peroxyl radicals [49]. Carotenoids tested individually against cisplatin ototoxicity include lutein [50–52], and lycopene [53–55]. β-carotenes are powerful neutralizers of lipid-derived peroxyl radicals due to abundant conjugated double bonds [36,56]. They have not been tested systematically in cisplatin ototoxicity. Vitamin E, especially as α-tocopherol, traps lipid peroxide radicals, interrupting the chain reaction of lipid peroxidation, thus protecting membrane phospholipids [36,57–59]. Tocopherol and its esterified form tocopheryl acetate protect against cisplatin ototoxicity in rodents by limiting oxidative stress [60–62]. So does the water-soluble analog 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Trolox [63]. Vitamin C mainly detoxifies hydrogen peroxide (H2O2) in aqueous interphases through the glutathione-ascorbate cycle. It has also shown effectivity against cisplatin ototoxicity in rodents [62,64]. Dramatic potentiation of combined ACEMg antioxidant otoprotection seen in noise-induced hearing loss [36,37] supports experimental testing against cisplatin ototoxicity. Auditory thresholds in ABRs, OHC cell counts and semi-quantitative immunocytochemistry for the oxidative stress marker 3-nitrotyrosine were used to compare otoprotective effects among different treatments.

2. Materials and Methods

2.1. Experimental Animals

Fifty-five male Wistar rats, 11 to 13 weeks old at the beginning of the experiments, were used. Animals were provided by Charles River Laboratories (Barcelona, Spain) and were housed in the Animal House facility of the University of Castilla-La Mancha-UCLM in Albacete (Spain) under controlled temperature and humidity in a 12-h light/dark cycle with “ad libitum” access to food and water. The handling and care of animals followed current national (Spain R.D. 53/2013; Law 32/2007) and EU (Directive 2010/63/EU) regulations on the protection and care of animals used for scientific purposes. Procedures were validated and supervised by the institutional Ethics Committee for Animal Experimentation and were authorized under registry PR-2016-04-11.

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2.2. Induction of Ototoxicity by Cisplatin and Otoprotection Treatment Groups

Animals were randomly assigned to the experimental groups shown in Table 1. Ototoxicity in groups II-IX (Table 1) was induced with a single intraperitoneal injection of cisplatin (Sigma-Aldrich, catalog number P4394) at 16 mg/Kg. Cisplatin was dissolved in 0.9% saline at a concentration of 1 mg/ml. After the injection, animals were allowed to survive for 72 hours, when ototoxicity is fully established [65,66]. Saline vehicle control was designated as Group I.

Otoprotective treatments in the corresponding experimental groups (Table 1) were carried out by daily subcutaneous administration of the corresponding antioxidant compound, individually or in combinations shown in Table 1, initiated 5 days prior to the induction of cisplatin ototoxicity, for a total of 7 days.

Table 1. Distribution and number of animals in the different experimental groups.

Experimental Groups	Treatments	Number of Animals
I	Vehicle Control **(Group I-Control)**	5
II	Cisplatin **(Group II-Cis)**	8
III	Cis+500 mg NAC **(Group III-NAC500)**	6
IV	Cis+1000 mg NAC **(Group IV-NAC1000)**	6
V	Cis+NAC500 +$MgSO_4$ **(Group V-NAC500+Mg)**	6
VI	Cis+NAC500+$MgSO_4$+Vitamin A,C,E **(Group VI-NAC500+ACE+Mg)**	6
VII	Cis+ACE **(Group VII-ACE)**	6
VIII	Cis+$MgSO_4$ **(Group VIII-Mg)**	6
IX	Cis+ACE+ $MgSO_4$ **(Group IX-ACEMg)**	6

Doses of each compound were: NAC (Sigma-Aldrich, Cat No. A9165) at 500 mg/Kg/day (NAC500) and 1000 mg/Kg/day (NAC1000); vitamin A (100 mg/Kg/day as beta-carotene, Sigma-Aldrich, Cat. No. C9750); vitamin C (500 mg/Kg/day, Sigma-Aldrich, Cat. No. A92902); vitamin E (200 mg/Kg/day, in the form of Trolox, Sigma-Aldrich, Cat. No. 238813); MgSO4 (343 mg/Kg/day, Sigma-Aldrich, Cat. No. M2643). Vitamin A was dissolved in sesame oil (Sigma-Aldrich, Cat. No. S3547) at a concentration of 58 mg/ml, vitamin E (Trolox) in DMSO at a concentration of 220 mg/ml, and vitamin C and MgSO4 in 0.9% saline solution at a concentration of 30 mg/ml and 20.6 mg/ml, respectively. Combinations of hydrosoluble compounds were administered in the same injection, whereas vitamins A and E were administered separately in their respective solvents. Dose selection and treatment protocols were based on previously published data [67–71].

Animals were distributed in nine groups (see Table 1). Group I were control rats injected intraperitoneally with a volume of saline equivalent to the volume used for cisplatin administration, in addition to a subcutaneous administration of a volume of saline, sesame oil and DMSO equivalent to that used for the administration of the corresponding otoprotective treatments. Group II were rats injected intraperitoneally with cisplatin. Groups III to IX were treatment groups with different doses

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and/or combinations of otoprotective agents, as detailed in Table 1. Animals were sacrificed the day after the last administration, following auditory brainstem response (ABRs) recordings.

2.3. Auditory Brainstem Response Recordings (ABRs)

Recordings were carried out in a sound-proof booth (Incotron Eymasa S.L., Barcelona, Spain) inside a sound-attenuation room, as previously described [72,73]. Animals were anesthetized with isoflurane at a flow rate of 1 L/min O2 at 4% for induction and at 1.5–2% for maintenance. During the recordings, body temperature was regulated at 37.5 °C with a non-electrical thermal pad. Temperature was monitored and controlled with a rectal probe. The subdermal electrodes (Rochester Electro-Medical, Tampa, FL, USA) were placed at the cranial vertex (non-inverted) and on the right (inverted) and left (ground) mastoid apophysis. Sound stimulation and recordings were carried out using a BioSig System III (Tucker-Davis Technologies, Alachua, FL, USA).

The stimuli were generated digitally with SigGenRP software (Tucker-Davis Technologies) and an RX6 Piranha Multifunction Processor hardware (Tucker-Davis Technologies). Stimuli were tone bursts (5 ms rise/fall without a plateau, 20 times/second) at seven different frequencies (0.5, 1, 2, 4, 8, 16 and 32 kHz), which were applied into the right external ear canal using an ED1 electrostatic speaker controller (Tucker-Davis Technologies) through an EC-1 electrostatic speaker (Tucker-Davis Technologies). The stimuli were calibrated prior to experiments with SigCal software (Tucker-Davis Technologies) and an ER-10B+ low-noise microphone (Etymotic Research Inc., Elk Grove, IL, USA).

Auditory thresholds were obtained for each tested frequency. To determine threshold level, the evoked responses were recorded in 5 dB steps descending from a stimulus intensity of 80 dB SPL. The background activity was measured before the stimulus onset. The auditory threshold was defined as the stimulus intensity that evoked waveforms with a peak-to-peak voltage greater than 2 standard deviations (SD) of the background activity [72,73]. The maximum level of stimulus intensity was established at 80 dB as a safe limit for noise overstimulation artifacts [72,74].

The change in auditory threshold relative to the control group, for each of the frequencies analyzed, was determined by subtracting post-treatment auditory thresholds from auditory thresholds obtained prior to the initiation of treatment. Thresholds in Group I were used as the reference baseline. Group VI- NAC 500+ ACE + Mg was not considered for the statistical analysis of threshold changes, since stable records of ABRs were obtained only from a single subject.

2.4. Cochlear Fixation and Processing for Histology

Animals were sacrificed with sodium pentobarbital (Dolethal, Vetoquinol, Madrid, Spain; i.p. 700 mg/kg). Fixation by transcardial perfusion was initiated with a vascular flush with 0.9% saline solution for 5 minutes, followed by 4% paraformaldehyde fixative (PFA 4%) in 0.1 M phosphate buffer (PB, pH 7.3) for 15 minutes, after which dissection and isolation of the cochleae were performed. The cochleae were then washed 3 times in phosphate buffered saline (PBS) for 5 min and decalcified in 50% RDO (rapid decalcifying solution, Apex Engineering Products Corporation, IL, USA). The right cochleae, intended for the quantification of outer hair cells (OHCs) by fluorescent immunolocalization of myosin VIIA as detailed below, were kept in RDO for 1h, while the left ones, used for *3- nitrotyrosine (*3-NT) immunolocalization (a marker of oxidative stress, see below), were kept in RDO for 2h. After incubation in RDO, the cochleae were washed again 3x5´ in PBS.

2.5. Immunohistochemistry on "In Toto" Cochlear Surface Preparations for Outer Hair Cell Quantification

Decalcified cochleae were kept immersed in PBS and dissected under an Olympus SZX10 stereoscopic microscope (Olympus, Tokyo, Japan) with the help of 2 thin-tipped dissection tweezers and a surgical blade. First, a cut was made perpendicular to the long axis of the modiolus, thus dividing the total length of the cochlea in two equal halves, one more apical and one more basal. The otic capsule was excised, lateral wall structures were dissected out and the modiolus and the tectorial membrane were removed, thus exposing the organ of Corti. Cochlear turn fragments (between 2 and 5 per full cochlear length) were placed in PBS in separated wells of a multi-well plate (Sigma-Aldrich,

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catalog # CLS3736). To identify OHCs, fluorescent immunolocalization of myosin VIIa was performed. First, cochlear turn fragments were incubated in phosphate buffered saline with 1% Triton X-100 and 5% bovine serum albumin (PBS-1% Tx100-5% BSA) at RT for 1 hour. They were then incubated overnight at 4ºC with an anti-myosin-VIIa primary antibody (Proteus BioSciences, catalog no. 25-6790) diluted in PBS-0.2% Tx100-3% BSA at a concentration of 1:1000. The primary antibody was then discarded and three 5-minute washes with PBS were carried out, followed by incubation for one hour at room temperature with an anti-rabbit Alexa Fluor 594 secondary antibody (Thermo Fisher Scientific, catalog no. A-21207) diluted in PBS-0.2% Tx100-3% BSA at a concentration of 1:200 for 1 hour at room temperature (RT) in the dark. After three 5-minute washes with PBS, immunostained cochlear turn fragments were slide -mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Such surface preparations were visualized and photographed on a Zeiss 710 confocal microscope (Zeiss, Oberkochen, Germany). After image capture, ImageJ software (National Institutes of Health, USA), was used to visualize OHCs and count loss for each 0.1 mm of length of the organ of Corti as described below.

2.6. Immunohistochemistry for 3- Nitrotyrosine in Cochlear Sections

After decalcification and washing, the left cochleae were submerged in 30% sucrose (Panreac, Barcelona, Spain, catalog number 131621) in PB for 48-72 hours for cryoprotection. Subsequently, they were embedded in gelatin blocks (Panreac, catalog number 142060) prepared in PB-30% sucrose. Blocks were deep-frozen at -70ºC using 2-propanol (Sigma-Aldrich, catalog number I9516) on dry ice, and stored at -80ºC until further use. From each block, 20 µm thick paramodiolar sections were made using a Leica CM3050 S cryostat (Leica, Wetzlar, Germany). Sections were collected on Superfrost^{R} slides and stored at -80 °C. For detection of 3-NT, cochlear sections were tempered at RT for 30 min and post-fixed with 4% PFA for 8 min. After 3x5´ washes with PBS, 1 ml of non-specific binding blocking and permeabilization solution (PBS-0.25% Tx100-2% BSA) was added to the slides, keeping it at RT for 1 hour. Next, sections were incubated overnight with a mouse anti-3-nitrotyrosine monoclonal antibody (1:100, Abcam ab61392) at 4ºC. The next day, after 3x5´ washes with PBS, sections were incubated with a donkey anti-mouse Alexa 488 secondary antibody (1:200, Thermo Fisher Scientific A-21202) 1 hour at RT in the dark. All antibodies were diluted in PBS-BSA 0.5%. After washing 3x5´ min with PBS, the slides were coverslipped with Vectashield with DAPI and observed under laser scanning confocal microscopy in a Zeiss 880 microscope. Immunofluorescence intensity was quantified using ImageJ (National Institutes of Health), as detailed below.

2.7. OHC Counts

OHC counts were carried out using ImageJ software (National Institutes of Health, MD, USA). To count OHCs, the segments of each cochlea, photographed under the confocal microscope, were first ordered in the apical-to-basal direction. After that, 0.1 mm segments were measured along a line delimiting the apical portion of the inner hair cells, using ImageJ "segmented line" tool. In each of these segments, the percentage of identifiable OHCs was determined as previously described [66]. This allowed its representation in the form of "cytocochleograms" and the analysis of the damage in the organ of Corti, quantifying the length of 3 well-differentiated zones along the cochlear turn: a zone with 100% of OHCs, a transition zone (with a partial loss of OHCs) and a zone with 0% of OHCs [66].

2.8. Semiquantitative Measurement of Fluorescent Signal Intensity for 3-Nitrotyrosine

Semiquantitative analysis of immunofluorescence for 3-nitrotyrosine (3-NT) was also carried out using Image J. Confocal images captured from paramodiolar cochlear sections (see section 2.6 above) were used. To measure the intensity of the fluorescence signal for 3-NT, the ImageJ "polygon selections" tool was used. With it, contours of OHCs visible in the apical turn were selected, excluding the region occupied by the nucleus. Next, the mean value of the signal intensity inside the selected contour was calculated, as previously detailed [66].

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2.9. Statistical Analysis

Normal distribution of data was verified with the Kolmogorov-Smirnov test. After that, a one-way analysis of variance (ANOVA) and a post-hoc test (Tukey) was performed to compare the means of the data from the different study groups for the three metrics utilized, i.e., threshold shifts in ABRs, OHC counts and relative intensity of 3-NT immunolabeling. Statistical analysis was carried out with SPSS 25.0 software (IBM, Armonk, New York). Statistical significance was graphically expressed as *p<0.05, **p<0.005, ***p<0.001.

3. Results

3.1. Auditory Threshold Shifts after Different Antioxidant Treatments

In contrast with non recordable activity and thus undetectable thresholds in Group II-Cis rats, activity was recordable with all antioxidant treatments to a lesser or greater extent and therefore thresholds and threshold shifts were within detectable ranges throughout all tested frequencies. However, different antioxidant treatments differed in threshold shifts across frequencies, pointing to different recovery trends towards control, base line thresholds (Figure 1, Table 2). For this reason, threshold shifts were analyzed relative to Group I-Control.

The smallest threshold shift values relative to Group I-Control were found in group IX-ACEMg, which showed no statistically significant differences with Group I-Control at any tested frequency (Figure 1). In Group VIII-Mg threshold shifts also were statistically not significantly different from Group I-Control at frequencies of 8 kHz and below. However, different to Group IX-ACEMg, significantly elevated threshold shifts were found relative to Group I-Control at the highest frequencies tested, i.e., 16 kHz and 32 kHz (p<0.005, p<0.05, respectively) (Figure 1). In Group III-NAC500, shifts in threshold were not statistically significant at 4 kHz and below, whereas shifts at 8 kHz (p<0.05), 16 kHz (p<0.005) and 32 kHz (p<0.05) were so (Figure 1, Table 2). In Group VII-ACE, threshold shifts were not significant at 2 kHz and below, whereas different to Group III-NAC500, significant shifts in threshold were recorded at frequencies of 4 kHz and above (p<0.05, p<0.001, p<0.001, p<0.001, respectively) (Figure 1). Group IV-NAC1000, Group V-NAC500+Mg and Group VI-NAC500+ACEMg showed the largest average threshold shifts across frequencies and thus a lower trend towards threshold recovery to control values, especially from 2 kHz upwards (Figure 1, Table 2). In the case of Group IV-NAC1000 significant threshold shifts were observed at 2, 4, 8, 16 and 32 kHz (p<0.05, p<0.05, p<0.001, p<0.001, p<0.001, respectively) and in Group V-NAC500+Mg virtually across the whole frequency range, specifically at 0.5, 1, 2, 8, 16 and 32 kHz (p<0.05, p<0.05, p<0.05, p<0.05, p<0.005, p<0.005 respectively) (Figure 1).

On the other hand, there were statistically significant differences between Group IX-ACEMg and Group VII-ACE at 1 kHz (p<0.05) and 16kHz (p<0.05). Likewise, statistically significant differences were observed between Group IX-ACEMg and Group VIII-Mg, at least at 1 kHz (p<0.05) and at 16kHz (p<0.05). Finally, in Group VI-NAC500+ACEMg stable ABR recordings were obtained only from a single subject (Figure 1, Table 2), which precluded statistical analysis.

Table 2. Average auditory threshold shift values (dB) in the different treatment groups.

	I	III	IV	V	VI[1]	VII	VIII	IX
0.5 kHz	0 ± 6.78	7.92 ± 5.77	10.00 ± 4.79	21.25 ± 0.00	6.25 ± 0.00	10.25 ± 6.52	11.25 ± 6.12	1.25 ± 5.00
1 kHz	0 ± 8.29	7.08 ± 2.89	15.00 ± 9.46	21.25 ± 3.54	13.75 ± 0.00	10.75 ± 2.74	9.75 ± 9.62	3.75 ± 5.00
2 kHz	0 ± 11.37	12.08 ± 0.00	20.83 ± 6.29	22.08 ± 14.14	17.08 ± 0.00	12.08 ± 5.00	9.08 ± 7.58	3.75 ± 2.89
4 kHz	0 ± 10.76	18.75 ± 10.41	24.17 ± 12.50	25.42 ± 7.07	20.42 ± 0.00	18.42 ± 7.58	17.42 ± 13.51	10.42 ± 5.00
8 kHz	0 ± 13.05	24.58 ± 8.66	29.58 ± 10.00	29.58 ± 7.07	29.58 ± 0.00	27.58 ± 6.71	15.58 ± 9.62	12.92 ± 2.89
16 kHz	0 ± 7.72	22.08 ± 2.89	30.00 ± 4.79	26.25 ± 3.54	28.75 ± 0.00	28.75 ± 5.00	16.75 ± 9.75	12.08 ± 5.77
32 kHz	0 ± 9.64	18.75 ± 2.89	25.42 ± 7.07	27.92 ± 3.54	25.42 ± 0.00	20.42 ± 7.91	16.42 ± 9.62	15.42 ± 5.00

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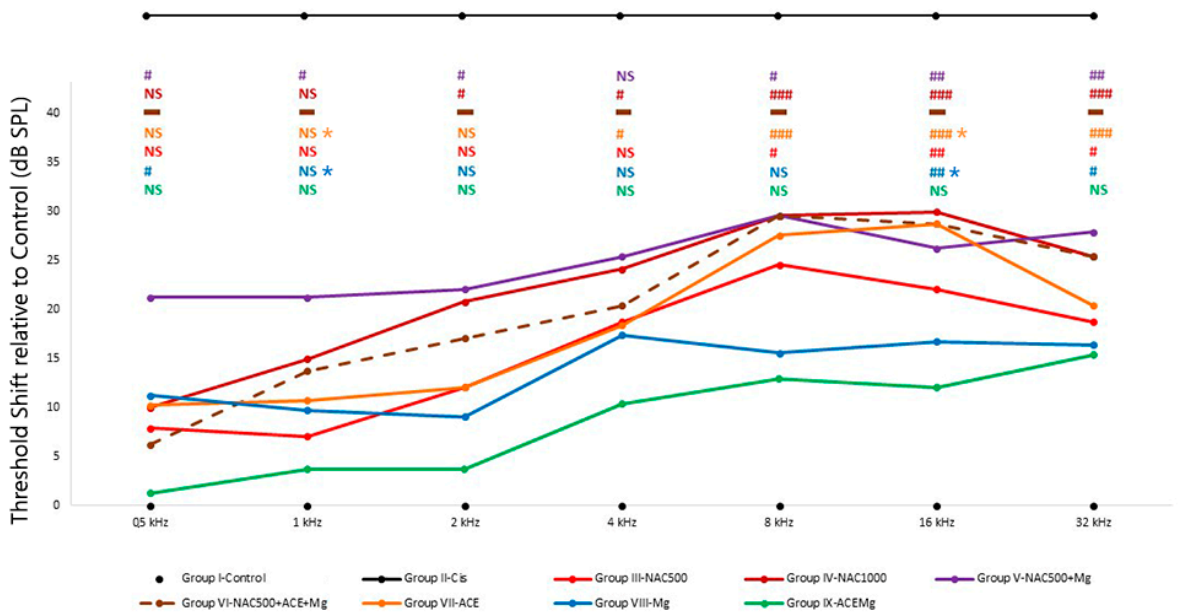

Figure 1. Average threshold shifts in the different antioxidant treatment groups. ABR recordings were performed 7 days after starting the corresponding treatments and 2 days after cisplatin injection. Group II-Cis has been illustrated outside the scale of the graph, representing the undetectability of auditory evoked potentials in this group at any of the frequencies or intensities studied. Overall, Group IX-ACEMg showed the smallest threshold shifts of all tested treatments. At the other end were Group VI-NAC500+ACEMg and Group IV-NAC1000, with significant threshold shifts spanning throughout most or all tested frequencies. This suggests that excess antioxidant concentrations and/or bioavailability may override redox balance, leading to diminished antioxidant treatment efficacy. N.S.: statistically not significant p-values relative to normal control baseline in Group I-Control. Significant p-values relative to normal control baseline in Group I-Control are shown as: #p<0.05, ##p<0.005, ###p<0.001. Significant p-values relative to Group IX-ACEMg in Group VII-ACE are shown as blue and yellow asterisks, respectively (p<0.05). The broken line in Group VI indicates data obtained from a single animal and therefore not subject to statistical analysis (see text).

[1]In Group VI, recordable auditory evoked potentials were obtained only in one rat.

3.2. Outer Hair Cell Counts

Cytocochleograms (Figure 2) showed a pattern of OHC loss characterized by three regions or segments [65]. Animals in Group II-Cis presented an apical portion, representing an average of 32.8% ± 3.28 of cochlear length, with complete preservation of OHCs (zone with 100% of OHCs). In the basal portion representing an average of 32.7% ± 11.31 of cochlear length, there was complete loss of OHCs (zone with 0% of OHCs), whereas in the third portion, located between the previous ones, representing 34.4% ± 11.77 average of cochlear length, there was partial loss of OHCs (Figure 3).

In treatment Groups VII-ACE, VIII-Mg and IX-ACEMg, the most apical segments of the organ of Corti, with 100% OHC preservation, were significantly longer than in Group II-Cis, representing respectively 65.75% ± 24.75 ($p<0.05$), 72.47% ± 31.93 ($p<0.05$) and 55.45% ± 13.47 ($p<0.05$) on average (Figures 2 and 3). In contrast, in treatment Group III-NAC500, Group IV-NAC1000, Group V-NAC500+Mg and Group VI-NAC500+ACEMg, the average percentages of the most apical length of the organ of Corti with 100% OHCs were respectively 50.32% ± 30.46, 35.43% ± 6.17, 48.32% ± 29.13 and 36.41% ± 10.05. These differences were not statistically significant from values in Group II-Cis (Figure 3).

A significantly shorter basal length of the organ of Corti with 0% OHCs compared to Group II-Cis was found in Group III-NAC500, Group V-NAC500+Mg, Group VII-ACE, Group VIII-Mg Group IX-ACEMg, with average values of 14.71% + 9.53 ($p<0.05$), 11.37% ± 8.72 ($p<0.05$), 3.61% ± 5.06 ($p<0.001$), 6.45% ± 9.27 ($p<0.005$) and 10.70% ± 4.66 $p<0.05$), respectively (Figures 2 and 3). Group IV-

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NAC1000 and Group VI-NAC500+ACEMg showed a basal length of organ of Corti with 0% OHCs of 25.26% ± 7.54 and 18.65% ± 8.54, respectively, statistically not significantly different from Group II-Cis (Figures 2 and 3).

Finally, the average percentage of organ of Corti occupied by the intermediate zone ("transition zone" [65], with partial preservation OHCs, was 34.96% ± 22.65 in Group III-NAC500, 39.31% ± 11.03 in Group IV-NAC1000, 40.30% ± 22.79 in Group V-NAC500+Mg, 44.95% ± 15.94 in Group VI-NAC500+ACEMg, 30.64% ± 22.43 in Group VII-ACE, 21.07% ± 24.86 in Group VIII-NACMg and 33.85% ± 12.34 in Group IX-ACEMg. None of these length differences showed statistical significance relative to Group II-Cis (Figures 2 and 3).

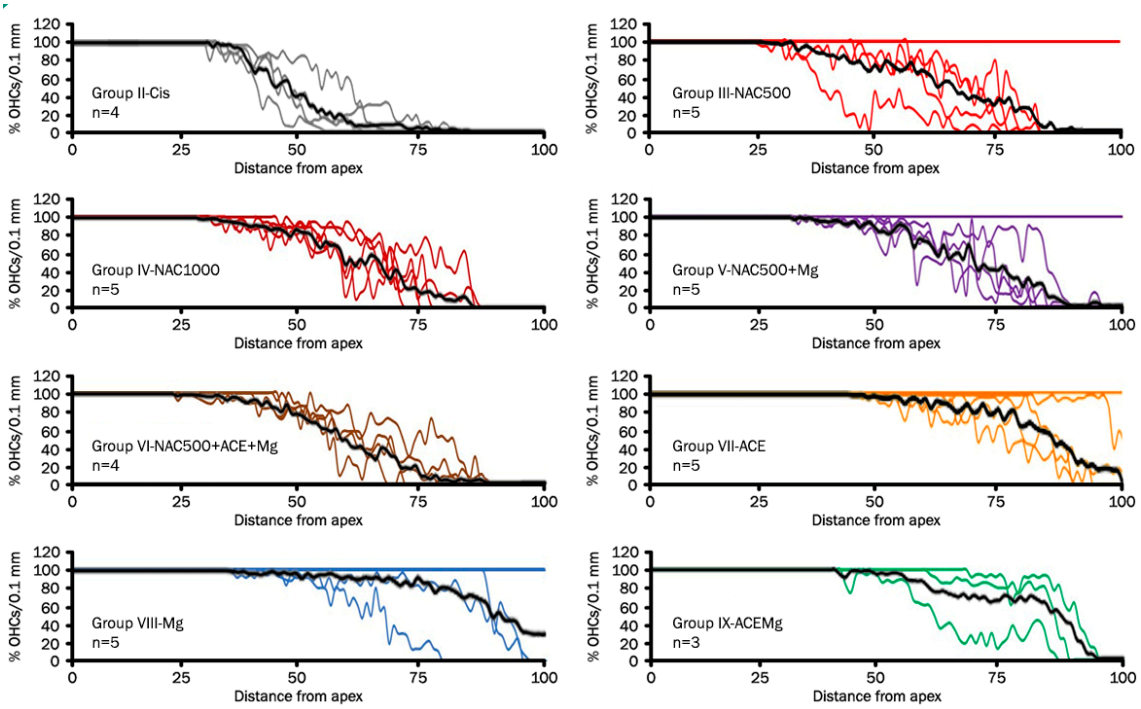

Figure 2. Line graphs (cytocochleograms) showing outer hair cell loss and preservation in rat cochleae from the different experimental groups. Each color line represents one cochlea and "n" is the total number of cochleae from individual animals used for cell counts in each treatment group, after eliminating defective cochlear turn samples. The black line is the average percentage of outer hair cells as a function of distance from the apex.

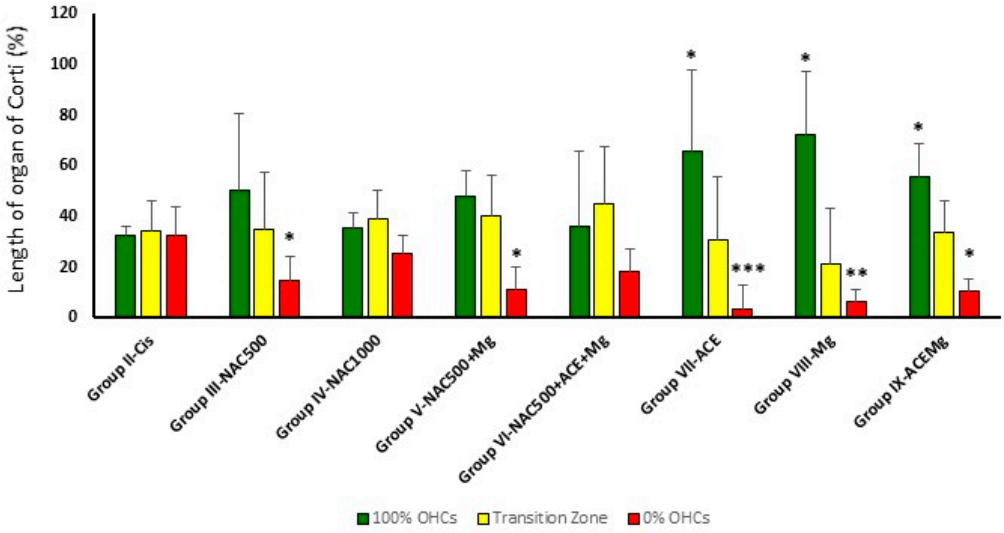

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Figure 3. Bar graph showing the average percentage of the apical to basal length of the organ of Corti with complete preservation of OHCs (green bars), partial loss (yellow bar) or complete loss (red bar). In Group IX-ACEMg, Group VIII-Mg and GroupVII-ACE, the relative length of organ of Corti with 100% OHC loss is significantly reduced, whereas the apical segment with maximum preservation of OHCs is longer. Notice that Group IV-NAC1000 and Group VI-NAC500+ACE+Mg did not show significant differences with Group II-Cis in OHC survival patterns. (*) Statistical significance of p values relative to cisplatin, * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

3.3. 3-Nitrotyrosine Immunolabeling for Oxidative Stress

As far as 3-NT immunolabeling is concerned (Figure 4A), in regions with identifiable OHCs, Group II-Cis samples showed a significant increase in 3-NT immunoreactivity intensiy of 87.90% ($p<0.05$) relative to Group I-Control (Figure 4B). Treatment Group IV-NAC1000 also showed a significant increase in immunolabeling intensity of 160.00% ($p<0.001$), relative to Group I-Control (Figure 4A,B). In contrast in treatment Group V-NAC500+Mg, Group VII-ACE, Group VIII-Mg and Group IX-ACEMg there were significant reductions in 3-NT immunoreactivity of 51.76% ($p<0.05$), 54.92% ($p<0.05$), 44.81% ($p<0.05$) and 56.41% ($p<0.05$), respectively, relative to Group II (Figure 4A,B). These values did not differ statistically from those of Group I-Control (Figure 4B). Finally, in Group III-NAC500, relative 3-NT immunolabeling intensity was closer to Group I-Control, whereas in Group VI-NAC500+ACEMg it was closer to Group II-Cis, although neither of these values were statistically significant (Figure 4A,B).

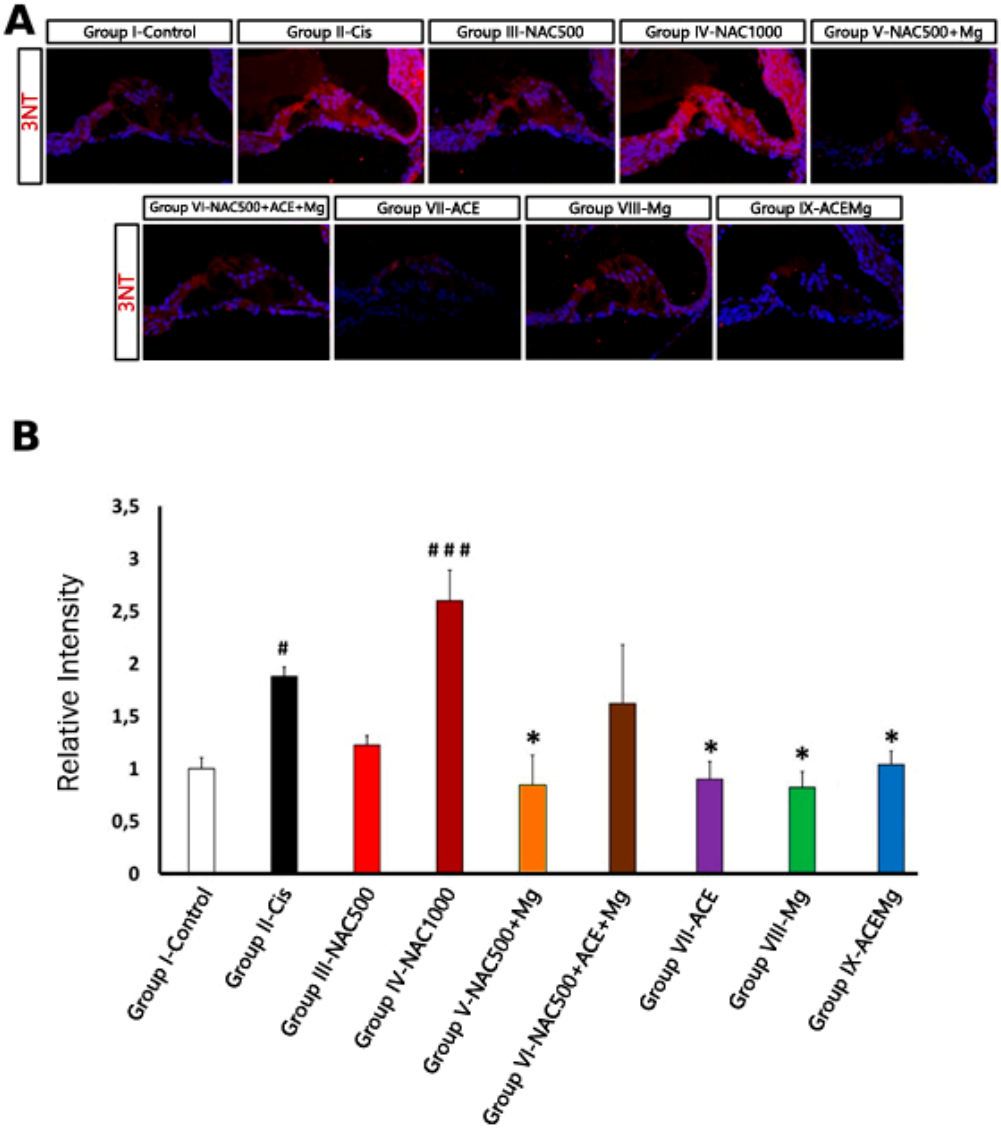

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Figure 4. Fluorescent immunolocalization of the oxidative stress marker 3-NT in the cochlea after cisplatin ototoxicity in comparison with the different antioxidant treatments in this study. **A:** Immunolabeling for 3-NT (red) with DAPI counterstaining (blue) in representative sections of the apical turn of the organ of Corti from rats of the different experimental groups. Notice very low or low levels of 3-NT immunolabeling in Group IX-ACEMg, Group VIII-Mg, Group VII-ACE, Group III-NAC500, and also Group V-NAC500+Mg, comparable to Group I-control. Group IX-NAC1000 and Group VI-NAC500+ACE+Mg show 3-NT immunostaining visually similar to Group II-Cis. **B:** Relative intensity levels of 3-NT immunolabeling in OHC regions from cochlear sections of animals from the different experimental groups. (*) Statistical significance of p-values, relative to the Group II-Cis, * $p<0.05$, ** $p<0.005$, *** $p<0.001$. (#) Statistical significance of p-values relative to the Group I-Control. #$p<0.05$, ##$p<0.005$, ###$p<0.001$.

4. Discussion

Our results support that limiting the cochlear oxidative stress response of cisplatin with different antioxidant combinations attenuates the loss of hearing sensitivity and limits toxic structural damage to the auditory receptor organ. Oxidative stress, tested with immunocytochemical detection of the oxidative stress marker 3-NT, OHC survival rate, assessed with OHC counts, and level of hearing threshold recovery, assessed with ABR recordings, all vary with each antioxidant or antioxidant combination utilized. Considering the overall outcome of experimental tests among different formulations, the ACEMg combination, 500 mg NAC and $MgSO_4$ alone seem to preserve better auditory thresholds and/or OHC survival after cisplatin ototoxicity. Of all these, however, ACEMg was the only one simultaneously showing threshold shifts not significantly different from normal baseline thresholds at any tested frequency, large preservation of OHCs and oxidative stress level closer to normal values, as seen with 3-NT immunolabeling. In contrast, high doses of antioxidants, such as in the NAC1000 experimental group, or extensive combinations, such as NAC500 with vitamins A, C, E and MgSO4, resulted in lower attenuation of cisplatin-induced oxidative stress, poorer survival of OHCs, reduced recovery from threshold shifts or a combination thereof. This suggests that once an “antioxidation threshold" is surpassed, treatments may greatly lose efficacy by contributing excess redox imbalance [75].

4.1. Hearing Loss and Cochlear Damage after Cisplatin Ototoxicity in the Rat Model

Under our experimental conditions, rats in the cisplatin exposed group showed complete loss of evoked activity in ABRs, with undetectable hearing thresholds at all tested frequencies up to the highest applied intensity of 80 dB SPL. Threshold elevations across the entire frequency range, at the limit of precise determination, have been reported previously with similar cisplatin exposure protocols [65,66]. OHCs at the base of the cochlea are more susceptible to cisplatin ototoxicity and usually die faster after exposure [14], which correlate with intrinsically lower levels of the antioxidant glutathione [76]. Therefore, total or partial loss of OHCs at the cochlear base after cisplatin exposure, comprising around 67% length in our experimental series, correlates with highly elevated hearing thresholds in the high frequency range [77,78]. In contrast, more apical OHCs localized in a segment of about 33% of cochlear length in the cisplatin exposed group are more resistant to toxic damage [5,14,66,79]. Therefore, threshold elevations detected in lower frequency ranges after cisplatin ototoxicity may be due in part to compromised electrical signal generation by otherwise surviving apical OHCs [65,66]. The stria vascularis is the site of generation of the endocochlear potential, the driving force for electrical activity in OHCs. It is particularly sensitive to cisplatin accumulation and thus oxidative stress damage [17]. Immunolabeling for the oxidative stress marker 3-NT is more widespread in the stria vascularis after cisplatin exposure than with other ototoxic agents such a kanamycin [66]. A single dose of 16 mg/kg in Wistar rats, the same used in the present work, reduces the endocochlear potential around 50% [80].

In sum, regardless dose-dependency of cisplatin ototoxicity, with progressive damage starting at the most sensitive basal/high frequency cochlear end [77,78], experimental doses like the one used in this work, may lead to simultaneous, significant threshold elevations across the whole frequency

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range. This is likely due to a combination of progressive OHC loss, starting at the cochlear base, and early widespread damage to the stria vascularis, including the cochlear apex [17,66]. Therefore, this experimental model in rat reproduces acute, extensive cisplatin-mediated ototoxicity. Such model of extreme ototoxic damage provides an opportunity to explore effectiveness of antioxidant otoprotection mechanisms.

4.2. Antioxidants and Antioxidant Combinations in Otoprotection against Cisplatin Ototoxicity

As already mentioned, cochlear oxidative stress, originated by overproduction of ROS/RNS and other highly reactive free radicals and non-radicals is one main pathophysiologic mechanism of hearing loss induced by multiple toxic insults, including noise and ototoxic drugs [81–83], notably aminoglycoside antibiotics or antineoplastic chemotherapeutic agents such as cisplatin [66,84,85].

We have used three complementary metrics, threshold shifts in ABRs, OHC counts and immunocytochemical labeling with the oxidative stress marker 3-NT to provide comparative evidence of the experimental efficacy of different antioxidants and antioxidant combinations against cisplatin ototoxicity.

As previously mentioned in relation to ABRs, there was no detectable activity at the tested frequencies in the group treated with cisplatin thus precluding threshold shift calculations. In contrast, to a greater or lesser extent activity was recordable in all experimental antioxidant treatment groups. Because of this, results gain context by comparing shifts relative to controls. This shows trends of different treatments towards recovery of normal "physiological" baseline thresholds. In this regard, ACEMg treatment resulted in threshold shifts which were closer to normal baseline auditory threshold levels than any other antioxidant compound tested. Threshold shifts after ACEMg injections did not differ statistically from control baseline values across the entire tested frequency range. In contrast, threshold shifts recorded after administration of ACE without MgSO4, after induction of cisplatin ototoxicity, differed statistically from baseline values at frequencies above 2 kHz and were statistically significantly higher than those of ACEMg, at least in part of the frequency range. This supports that Mg++ potentiates ACE antioxidant otoprotection. In fact, ACEMg has been previously shown to provide superior preservation of hearing sensitivity than ACE or MgSO4 alone in noise-induced hearing loss in guinea pigs [37], likely through cochlear vasodilation [36,87]. ACEMg also protects against gentamycin-induced hearing loss in guinea pigs [38] and age-related hearing loss in rats [40]. Here we provide evidence of strong otoprotection with ACEMg against cisplatin ototoxicity. However, different to noise-induced hearing loss [88], extreme loss of hearing sensitivity after cisplatin ototoxicity also was significantly attenuated by injections of MgSO4 alone, although, in contrast with ACEMg, shifts at higher frequencies still had statistically significant higher values than baseline. Also, threshold shifts were significantly higher statistically than those of ACEMg, at least in part of the tested frequency range.

It is unclear how Mg++ by itself may improve hearing loss in cisplatin ototoxicity [89]. There is hypomagnesemia after cisplatin treatment and although a Mg++-enriched diet failed to provide histological otoprotection in guinea pigs [90], evidence of recovery of otoacoustic emissions after cisplatin treatment supplemented with Mg++ has been reported [89,91]. Vasodilator, antioxidant, and ion homeostatic effects of Mg++ may critically improve the metabolic status of the stria vascularis, a main target of cisplatin ototoxicity, one possibility which requires further experimental testing. However, therapeutic handling of Mg++ in ototoxicity may be complicated in humans by relatively frequent occurrence of diarrhea [39].

After treatment with 500 mg NAC (NAC500 group) daily for seven days, with cisplatin ototoxicity induced at day 5, threshold shifts at frequencies of 4 kHz and below had average values not significantly different from baseline. However, threshold shifts remained significantly elevated at 8 kHz and above. We then sought to test whether in combination with MgSO4 (NAC500+Mg group) there were additionally diminished threshold shifts across frequencies, suggesting additive/synergistic otoprotection between both compounds. In the NAC500+Mg group, threshold shifts remained significantly elevated above baseline across the entire frequency range. Therefore, as far as auditory thresholds are concerned, Mg++ does not seem to potentiate NAC otoprotective

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antioxidation in the cochlea after cisplatin ototoxicity. Rather, it seems to decrease it. A relatively similar result was obtained with a higher NAC dose of 1000 mg/Kg (NAC1000), also administered daily for 7 days. Threshold shifts remained significantly elevated at 2 kHz and above. We speculate that high doses of NAC or increased cochlear availability of the compound when combined with MgSO4, due to Mg++-induced cochlear vasodilation, along with additional Mg++-induced antioxidation may generate excess reduction potential within cells, with redox imbalance leading to "anti-oxidative stress" and cell damage triggered by disruption of physiological ROS/RNS signaling [75]. In other words, our findings suggest a therapeutic "antioxidation threshold", above which antioxidant otoprotection probably provides no benefit. This was also seen when combining the antioxidation mechanisms of NAC and ACEMg (see introduction, [92,93]). Treatment with a cocktail of NAC500 and ACEMg resulted in undetectable thresholds in 5 out of 6 rats exposed to cisplatin. Only in one rat of this group auditory thresholds were recorded, with shifts relative to control baseline which did not differ much from the NAC1000 group. This is an additional indication that antioxidant excess may critically hamper hearing sensitivity preservation from cisplatin ototoxicity.

Survival of OHCs offers additional insights into antioxidation protection mechanisms against cisplatin ototoxicity. In general, OHC survival rate, from counts on surface preparations of the organ of Corti, bears correspondence with threshold shift recoveries after the different treatments. With the notable exception of the NAC1000 dose and the NAC500+ACEMg cocktail, tested treatments showed clearly improved OHC survival, relative to the extensive OHC loss seen in cisplatin-treated animals. ACEMg, MgSO4 and ACE treatments resulted in largely increased OHC survival. Improved OHC survival in the ACE-treated group is somehow in contrast with still significantly elevated threshold shifts at frequencies of 4 kHz and above in this treatment group. This suggests that OHC survival may not be sufficient indicator of hearing sensitivity preservation after antioxidant protection against cisplatin ototoxicity. For instance, compared with other compounds, ACE might not promote sufficient recovery of the stria vascularis and related structures of the lateral wall, with the corresponding consequences for OHC function. It has been shown that in mice with noise induced hearing loss, an oral formulation highly enriched in vitamin A, C and E and Mg++, preserves the cellular organization of the cochlear lateral wall over a less enriched formulation [94], supporting that protection of the stria vascularis with antioxidants depends on the composition and concentration of the formulation. OHC survival was also significantly increased both with NAC500 and NAC500+MgSO4 treatments, although different to the treatments previously discussed, the apical cochlear segment with maximum survival of OHCs [66] was not significantly lengthened, and increased survival of OHCs was mostly at the expense of the reduction in length of the basal segment, in which OHCs were completely lost after cisplatin exposure [66]. This indicates slower or reduced OHC recovery rates, relative to treatments discussed above.

There were no statistically significant differences in OHC loss among cisplatin-exposed rats and rats treated with NAC1000 or NAC500+ACEMg. Lack of significant OHC survival enhancement matches limited or no threshold shift reductions with these same two treatments, specially NAC500+ACEMg after which most cisplatin-exposed rats in our series continued to lack evoked activity. Residual OHC survival, even below statistical significance, along with lower or higher level of damage to the stria vascularis may determine whether activity is limited or not recordable. Again, high doses or large antioxidant compound combinations, might be pushing redox homeostasis out of balance [75], limiting cell survival recovery. This hypothesis warrants further testing, more so considering that several antioxidants and pro-oxidants induce hair cell loss in "in vitro" assays in cochlear micro-explants [95]. It is also relevant that high concentrations of trans-tympanic NAC caused greater alterations in the inner ear than those produced by administration of cisplatin itself [96].

Immunocytochemical detection of the oxidative stress marker 3-NT provided further insights on the antioxidant otoprotection properties and mechanisms of the different treatments tested. 3-NT labels protein nitrosylation at tyrosine residues, a specific indicator of oxidative stress damage mediated by RNS, specifically peroxynitrites [66,97]. In cisplatin-exposed rats, 3-NT immunolabeling was more intense throughout the cochlea, relative to vehicle-injected controls. In regions with

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preserved, identifiable OHCs, relative immunolabeling intensity was almost twice than in controls, indicating increased oxidative stress even in surviving OHCs. Such elevated 3-NT levels were significantly reversed by all tested treatments, except for NAC1000 and NAC500+ACEMg. In the NAC500+ACEMg groups, 3-NT intensity levels were indistinguishable from those in the cisplatin exposed group. In the NAC1000 treatment group, 3-NT immunolabeling was even further increased, with relative intensity levels showing a trend to be even higher than in the cisplatin-exposed rats, although without reaching statistical significance. When reversed by treatments, 3-NT immunolabeling returned to relative intensity values significantly lower than those in cisplatin-exposed rats. Except for the NAC500 group, which did not reach statistical significance, the rest of treatments lowered cochlear oxidative stress increased by cisplatin, matching OHC preservation. In contrast, NAC1000 and NAC500+ACEMg treatments, which provide limited, statistically not significant OHC preservation and limited threshold shift reductions, do not reverse cisplatin-induced oxidative stress. This adds to the notion of excess therapeutic "antioxidant power" likely resulting in failure of antioxidation mechanisms. In this regard, however, it is interesting to remark that NAC500+MgSO4 resulted, after cisplatin exposure, in still significantly elevated threshold shifts virtually throughout the entire tested frequency range, but with significant preservation of OHCs, along with low levels of oxidative stress. This points to additional mechanism of cochlear disfunction induced by excess antioxidants which require further research.

In conclusion, formulations of compounds which combine different antioxidant capacities, along with a likely relevant vasodilator effect of Mg++, such as ACEMg, show outstanding otoprotection against extreme experimental cisplatin ototoxicity, by limiting oxidative stress damage. Individual compounds such as NAC show more limited, although still significant, otoprotection. However, high doses or large antioxidant combinations may critically override a "threshold" redox balance, above which no otoprotection results and ototoxic damage remains or maybe even potentiated. The extent to which these notions may impact therapies awaits exploration of other antioxidant administration routes. Experimental injections allow reasonable, variable-controlled, proof of principle. However, there are still potential confounding sources. For instance, the cocktail of vitamins ACE contains DMSO as a solvent for vitamin E, which itself has free radical capture properties [86], so it cannot be ruled out that this additional antioxidant effect may have had an effect on the results. More important, systemic administration of otoprotective antioxidants risks limiting the antineoplastic effects through unwanted "antioxidant protection" of tumoral cells. The principles supported in this work will have to be aligned with the results of further experiments aimed at determining optimal administration routes for antioxidant otoprotection, with no impact on cisplatin oncotherapy.

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