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# **In vitro and in vivo effects of lutein against cisplatin-induced ototoxicity**

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## **ABSTRACT**

**Introduction:** Cisplatin is a commonly prescribed drug that produces ototoxicity as a side effect. Lutein is a carotenoid with antioxidant and anti-inflammatory properties previously tested for eye, heart and skin diseases but not evaluated to date in ear diseases.

**Aim:** To evaluate the protective effects of lutein on HEI-OC1 auditory cell line and in a Wistar rat model of cisplatin ototoxicity.

**Materials and Methods:** In vitro study: Culture HEI-OC1 cells were exposed to lutein (2.5–100 mM) and to 25 mM cisplatin for 24 h. In vivo study: Twenty eight female Wistar rats were randomized into three groups. Group A (n = 8) received intratympanic lutein (0.03 mL) (1 mg/mL) in the right ear and saline solution in the left one to determine the toxicity of lutein. Group B (n = 8) received also intraperitoneal cisplatin (10 mg/kg) to test the efficacy of lutein against cisplatin ototoxicity. Group C (n = 12) received intratympanic lutein (0.03 mL) (1 mg/mL) to quantify lutein in cochlear fluids (30 min, 1 h and 5 days after treatment). Hearing function was evaluated by means of Auditory Steady-State Responses before the procedure and 5 days after (groups A and B). Morphological changes were studied by confocal laser scanning microscopy.

**Results:** In vitro study: Lutein significantly reduced the cisplatin-induced cytotoxicity in the HEI-OC1 cells when they were pre-treated with lutein concentrations of 60 and 80 mM. In vivo study: Intratympanic lutein (1 mg/mL) application showed no ototoxic effects. However it did not achieve protective effect against cisplatin-induced ototoxicity in Wistar rats.

**Conclusions:** Although lutein has shown beneficial effects in other pathologies, the present study only obtained protection against cisplatin ototoxicity in culture cells, but not in the in vivo model. The large molecule size, the low dose administered, and restriction to diffusion in the inner ear could account for this negative result

## **1. Introduction**

Cisplatin is a commonly prescribed platinum-based drug used to treat various types of solid tumors (testicular and ovarian carcinoma, squamous head and neck carcinoma,

advanced bladder cancer, lung carcinoma, malignant gliomas and metastatic cancers such as melanoma, mesothelioma, prostate and breast cancer).

Severe side effects such as ototoxicity, nephrotoxicity, myelotoxicity and gastrointestinal toxicity have been reported due to cisplatin treatment. Although nephrotoxicity can be diminished or controlled with hydration therapy, ototoxicity still poses a limitation to effective cisplatin chemotherapy (McKeage, 1995). There is a great interest in developing effective strategies to protect the inner ear without compromising the antitumoral activity of cisplatin. Unfortunately, no therapy is currently approved for clinical use. Histologically, cisplatin causes cell death by apoptosis through different intracellular pathways. One of them is the overproduction of reactive oxygen species (ROS). This increase of ROS activate the cell compensation mechanisms, releasing glutathione enzymes and antioxidant substances.

Antioxidant system depletion leads to cell apoptosis. Many experimental studies have attempted to evaluate different substances against ROS overload and other cell signaling systems at an early stage to stop the apoptotic pathways. There is a clear rationale behind the testing of antioxidants against cisplatin ototoxicity as the overload of ROS after cisplatin administration has been shown to be one of the main mechanisms triggering death pathways inside auditory sensory cells (Casares et al., 2012).

Lutein is part of the xanthophylls chemical group. It is the second carotenoid most frequently found in human serum (Khachik et al., 1997), and it is present in foods such as corn, papaya, dairy, cereal, citrus fruits and green leafy vegetables such as spinach and kale (Sommerburg et al., 1998). The molecular structure of lutein has two hydroxyl groups, one at each end of the molecule, essential for its biological action as an antioxidant (Johnson, 2002; Winkler et al., 1999). There have been many studies on the benefits of lutein in humans due to its antioxidant properties. Most of them deal with the potential protective action of lutein in eye diseases (Landrum and Bone, 2001; Beatty et al., 1999; Duncan et al., 2002), and also in heart (Howard et al., 1996; Krieglstein and Granger, 2001; Martin et al., 2000) and skin diseases (Alves Rodrigues and Shao, 2004; Britton, 1995; Krinsky, 2002; Dreher and Maibach, 2001). Recent studies suggest a protective effect against neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Min and Min, 2014; Nataraj et al., 2015).

Up to the date there are no reports evaluating the effect of lutein on the inner ear. Due to the common characteristics between the inner ear and the retina, the antioxidant property of lutein, and the role of ROS overload in cisplatin ototoxicity, we hypothesized that lutein might play a role as a protective agent against cisplatin ototoxicity.

The aim of the present study was to evaluate the potential protective effect of lutein against cisplatin-initiated damage to the cochlea both in vitro and in vivo.

## 2. Materials and methods

## 2.1. In vitro study

### 2.1.1. Chemicals

Cisplatin (1 mg/mL) was purchased from Accord Healthcare (Barcelona, Spain).

Lutein (Xanthophyll from Marigold) was purchased from Sigma–Aldrich (Sigma–Aldrich; St. Louis, USA). Lutein is a very lipophilic carotenoid, so it was diluted in 0.5% DMSO in a phosphate buffered saline solution (PBS, Sigma–Aldrich, Germany) and different dilutions were prepared from stock solution. DMSO (Dimethyl sulfoxide) (0.5%) was purchased from Sigma–Aldrich (Sigma–Aldrich; St. Louis, USA).

### 2.1.2. Cell culture

The House Ear Institute–Organ of Corti 1 (HEI-OC1) cell line was kindly provided by Dr. Federico Kalinec (House Ear Institute, Los Angeles, CA). The establishment and characterisation of the conditionally immortalised HEI-OC1 cell line were previously described by Kalinec et al. (2003).

HEI-OC1 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, BRL), 5% LGlutamine (Sigma, Saint Louis, MO, USA) and Penicillin-G (Sigma, Saint Louis, MO, USA) at 33 C in a humidified incubator with 10% CO<sub>2</sub>.

For the experiments described below, HEI-OC1 cells were cultured under permissive conditions: 33 C, 5% CO<sub>2</sub> in DMEM supplemented with 10% FBS

2.1.3. Cell viability assay AlamarBlue<sup>1</sup> (Invitrogen) is a proven cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin. The active ingredient, resazurin, is a nontoxic, cell permeable compound that is blue in color and virtually nonfluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity. Absorbances at 570 nm were measured on a Multi-Detection Microplate Reader Synergy HT (BioTek Instruments; Vermont, USA). The treatments were performed in replicates (eight wells per treatment in a microplate). Results of the experiments are expressed as a percentage of viable cells (% of viable cells).

2.1.4. Hoechst 33258 staining Apoptotic cell death was determined by evaluating the nuclear morphology using Hoechst 33258 staining. Cells were incubated with 10 mg/mL of Hoechst 33258 (Sigma, Saint Louis, MO, USA) for 20 min. Membrane-permeable Hoechst 33258 is a blue fluorescent dye and stained the cell nucleus. After washing twice with phosphate buffered saline (PBS), the cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT). After washing twice with distilled water, the cells were evaluated under a Nikon Eclipse TE 2000-S fluorescence microscope with a DS-U2 camera controller (Nikon Instruments Europe BV, Amsterdam, Netherlands).

2.1.5. Lutein toxicity assay HEI-OC1 cells were seeded in 96-well plates, with each well containing  $3 \times 10^4$  cells. After 24 h incubation under permissive conditions (33 °C, 10% CO<sub>2</sub> in DMEM), the cells were treated with various lutein dilutions (2.5, 5, 10, 20, 30, 40, 60, 80 or 100 mM) and viability assessed after 24 h.

2.1.6. Cisplatin toxicity assay Cells were treated with various cisplatin dilutions (0.625, 1.25, 2.5, 5, 10, 20, 30, 40 or 60 mM) and viability assessed after 24 h. A concentration of 25 mM was the half-maximal inhibitory concentration (IC<sub>50</sub>) for 24 h in DMEM.

2.1.7. Lutein effects against cisplatin toxicity HEI-OC1 cells were seeded as previously described. Cisplatin was added 3 h after lutein administration to allow for carotenoid's endocytosis by cells. Cells were exposed to 25 mM cisplatin (IC<sub>50</sub>).

2.1.8. Statistical analysis A statistical analysis was performed to determine the sample size for each group. The experiment was performed twice at different times, with eight repetitions for each dilution used. Analysis of variance was used to assess mean differences across treatment groups. One-way ANOVA was used to assess statistical significance, where values of  $p < 0.05$  were considered statistically significant. A Tukey test was used to identify significant differences between the paired treatments. Data processing and analysis were performed with Origin Pro 8 software package (Origin Lab Corporation, Northampton, USA).

2.2. In vivo study The study was carried out in compliance with guidelines for research involving animals (Spanish Animal Care and Use Committee, Spanish Law 32/2007 and EU Directive 2010/63/EU for animal experiments), and was approved by the Animal Welfare Ethics Committee of the Foundation for Biomedical Research of Puerta de Hierro Hospital (CEBA 013/2012).

2.2.1. Animals Female Wistar rats weighing 250–275 g were used. Animals were bred and handled at the animal facilities of our centre in temperature controlled rooms, with light–dark cycles, and with free access to food and water. Before each procedure, an otoscopy was performed on each animal to exclude outer and middle ear infections, using an operating microscope. Rats showing signs of current or past middle ear infection were excluded from this study.

2.2.2. Experimental groups Twenty eight animals were randomly assigned to three groups: Group A ( $n = 8$ ) received intratympanic lutein (0.03 mL) (1 mg/mL) in the right ear (0.03 mL) and saline solution in the left ear (0.03 mL), to determine the toxicity of lutein. Group B ( $n = 8$ ) received intratympanic lutein (0.03 mL) in the right ear, saline solution (0.03 mL) in the left ear and intraperitoneal cisplatin (10 mg/kg), to test the efficacy of lutein against cisplatin ototoxicity. Group C ( $n = 12$ ) received intratympanic lutein (0.03 mL) (1 mg/mL) in the right ear. Animals were euthanised and cochlear fluid sampled 30 minutes ( $n = 4$ ), 1 h ( $n = 4$ ) and 5 days ( $n = 4$ ) after lutein administration, to measure the diffusion of lutein from middle to inner ear.

2.2.3. Study design Animals were anaesthetised with intraperitoneal ketamine (100 mg/kg) and diazepam (0.1 mg/kg). Pretreatment Auditory Steady-State Responses (ASSR) were obtained from the animals in groups A and B. Following the ASSR

measurements, lutein and saline solution were injected intratympanically using a spinal needle (BD Whitecare 27G). After lutein injection, the animals remained in lateral decubitus for 30 min to maximize the time of contact between the solution and the round window membrane, and to prevent its leakage into the pharynx through the Eustachian tube. This procedure was performed in both groups. After intratympanic injection, animals from group B received treatment with cisplatin through a slow intraperitoneal infusion during 30 min. Animals were housed in individual cages with ad libitum water and food. Five days after treatment a new ASSR evaluation was performed. The threshold auditory levels were compared with pretreatment values. Animals were euthanised by decapitation after CO suffocation. Temporal bones were obtained for histological study in groups A and B and for perilymphatic fluid sampling in group C.

**2.2.4. Lutein preparation** Lutein was diluted in 0.5% DMSO with PBS. DMSO was chosen as the solvent for its ability to increase the round window membrane permeability. Moreover, a previous study from our group showed that intratympanic 0.5% DMSO was not intrinsically ototoxic and did not increase the ototoxic effect of cisplatin (Roldán-Fidalgo et al., 2014).

**2.2.5. Auditory steady-state responses** Subcutaneous electrodes were placed over the vertex (active) and in the pinna of each ear (reference). An insert earphone (Etymotic ER-2) was placed directly into the external auditory canal. Ground electrodes were placed over the neck muscles. ASSR were recorded using an evoked potential averaging system (Intelligent Hearing System Smart-EP, FL, USA) in an electrically shielded, double-walled, sound-treated booth in response to 100 ms clicks or tone bursts, at 8, 12, 16, 20, 24 and 32 kHz with 10 ms plateau and 1 ms rise/fall time. Intensity was expressed in decibels sound pressure level (dB SPL) peak equivalent. Intensity series were recorded, and an ASSR threshold was defined by the lowest intensity able to induce a replicable visual detectable response.

**2.2.6. Histology** The cochlear portion of the temporal bone was isolated. The stape was removed and the round window membrane carefully incised, in order to perfuse glutaraldehyde through the round window to rinse the endolymphatic and perilymphatic spaces. The piece was fixed in glutaraldehyde for 24 h and decalcified in 1% ethylenediaminetetraacetic acid (EDTA) at room temperature for 10–12 days, with daily changes. Cochleae were dissected in PBS medium and cochlear surface extracts were laid for confocal microscopy analysis (Leica TCS SP5 with a 63 × objective). The total number of outer hair cells (OHC) and the OHC loss (considered by the presence of empty spaces in OHC rows) were reported for each extract. Cell counts were performed at the time of imaging by sequentially viewing each slice.

**2.2.7. Lutein quantification** In group C, after the removal of the temporal bone and isolation of the cochlear portion, the bony apex of the cochlea was removed with a needle. The tip of a 50 mL Hamilton pipette was adjusted to the cochlear apical opening and the perilymphatic fluid suctioned with a syringe pump. All the samples were stored at -80 °C until analysis. The presence of lutein in the sampled fluid was

analysed using high performance liquid chromatography (HPLC). Lutein was analyzed with high performance liquid chromatography (HPLC) using a system consisting of a model 600 pump, a Rheodyne injector and a 2998 photodiode array (PDA) detector (Waters, Milford, MA, USA) following standard procedures previously defined (Olmedilla et al., 2001) a Spheri-5-ODS column (220 mm  $\times$  4.6 mm) (Brownlee Labs, Applied Biosystems, Santa Clara, CA, USA) and a guard column (ODS Aquapore type RP-18). The mobile phase was acetonitrile-methanol (85:15; v/v), and was changed to acetonitrile-dichloromethane-methanol (70:20:10; v/v/v) in a linear gradient from min 5 to min 20. Both mobile phases were stabilised with ammonium acetate (0.025 mol/L) added to the methanol. The flow rate was 1.8 mL/min, and detection was performed at a wavelength of 450 nm. All chromatograms were processed using Empower 2 software (Waters, Milford, MA, USA). Lutein extraction was performed on perilymph samples as follows: Centrifugation of the sample to concentrate the liquid at the bottom of the Eppendorf pellet (1 min). 300  $\mu$ L of water, 30  $\mu$ L of ethanol (EtOH) and 60  $\mu$ L of hexane was added. Vortex was performed during 1 min. Centrifugation of the sample during 5 min at 3500 rpm. The supernatant was collected and dried under nitrogen and reconstituted with 25  $\mu$ L of EtOH and tetrahydrofuran (THF) (2:1). The mixture was then stirred under ultrasound. Injection of the entire volume into the HPLC. The concentration of lutein was 0.27–1.36 mg/mL ( $R^2 = 0.999$ ), following a normal distribution curve.

2.2.8. Statistical analysis All statistical analyses were carried out using the SPSS statistical software package (SPSS, version 16.0 for windows; SPSS Inc., Chicago, IL, USA). Comparison between the groups with ASSR thresholds was performed using one-way and two-way analysis of variance (ANOVA). T-tests for paired samples were used to compare the ASSR thresholds, before and after lutein administration in groups A and B. Values of  $p < 0.05$  were considered statistically significant.

### 3. Results

#### 3.1. In vitro study

3.1.1. Toxicity assays Cell cultures treated with lutein alone showed no significant cell damage or cell loss (Fig. 1A). A clear dose effect was observed. Low carotenoid concentration did not affect cell viability, but exposure to concentrations of 60 and 80  $\mu$ M produced a significant reduction on cell viability (81% and 84% respectively). The HEI-OC1 cell line treated with cisplatin showed statistically significant cell damage (Fig. 1B). Changes to the pro-apoptotic nuclei were observed after Hoechst 33258 staining. Higher doses of cisplatin decreased survival of the cells in a dose-dependent manner. After exposure of the cells to cisplatin, the cellular viability was reduced from 100% to higher concentrations. Concentrations ranging from 20  $\mu$ M to 60  $\mu$ M are cytotoxic, since they decrease cell viability over 70% (as determined by ISO 10993-5:2009) (Fig. 1B, discontinued line). For this reason, a 25  $\mu$ M cisplatin concentration was chosen as an adequate dose to test the protective effect of lutein against antitumoral toxicity.

3.1.2. Lutein effect against cisplatin ototoxicity A cytoprotective effect was observed in HEI-OC1 cells exposed to cisplatin after pretreatment with lutein. A statistically significant increase in cell viability was seen after pretreatment with lutein at concentrations of 60 mM and 80 mM (Fig. 2A). Cell viability as a percentage of the control group was 84% and 81%, respectively ( $p < 0.05$ ). Lower concentrations of lutein were not able to prevent cell death. Concentrations below 60 mM failed to antagonize the harmful effect of the ROS overproduction after cisplatin treatment. Cytotoxic concentrations of lutein (100 mM) did not show any protection against cisplatin. Due to the high molecular weight of lutein, the use of high concentrations could induce lutein precipitation within the inner ear cells and limit its protective effect. Apoptosis of HEI-OC1 cells induced by cisplatin was also evaluated by nuclear Hoechst 33258 staining after 24 h treatment (Fig. 2B, C and D). Cells treated with cisplatin alone showed decreased survival and exhibited apoptotic nuclei (Fig. 2B). When cells received 80 mM lutein (Fig. 2C), cell viability was preserved when compared to the control group ( $p < 0.05$ ) (Fig. 2D).

### 3.2. In vivo study

3.2.1. Group A Post-treatment ASSR recordings were found to be lower than pre-treatment ASSR recordings, both in the study ear (lutein) and the control ear (saline solution), but the differences were not statistically significant for any of the tested frequencies (Fig. 3). These findings suggest that lutein is not intrinsically ototoxic for the inner ear when applied intratympanically

Confocal microscope images revealed preservation of outer (OHC) and inner hair cells (IHC) in all specimens (Fig. 4) with integrity of cilia (Fig. 5).

3.2.2. Group B We found a decrease in threshold levels for both ears. Differences between pre- and post-treatment thresholds in group B were smaller in the right ear (lutein) compared to the left ear (saline solution) (Fig. 6), but not statistically significant ( $p > 0.05$ ). Severe structural changes and scarce OHC were observed, with no damage to the IHC. The percentage of missed OHC was similar both in the study ear (lutein) and the control ear (saline solution). Cells undergoing apoptosis showed morphological changes such as chromatin condensation and nuclear pyknosis or fragmentation. Hair cell damage difference between ears was not statistically significant ( $p > 0.05$ ).

3.2.3. Lutein quantification Lutein was detected in only one sample from a rat euthanised 30 min after lutein intratympanic administration. The method employed in this study was not appropriate to determine exact amounts of lutein present in the cochlear fluid due to the low concentrations present.

## 4. Discussion

In this study, we evaluated dose-dependent effects of lutein on HEI-OC1 cell line challenged with cisplatin. Lutein shows a dose-dependent cytoprotective effect in vitro. A protective effect was found in an in vivo animal model of cisplatin ototoxicity (albino Wistar rat), without reaching statistical significance. Although a great diversity



of pharmacological strategies have been explored to protect cochlear structures against cisplatin effects without interfering with its antitumoral activity, none is currently approved to prevent cisplatin ototoxicity. In view of the role of ROS as the principal element in apoptosis- induction mechanisms after cisplatin administration, antioxidants have been proposed as good candidates to protect inner ear during cisplatin treatment. In a recent literature review on the efficacy of molecules tested in animal cisplatin ototoxicity models (Roldán- Fidalgo et al., 2015), 40 out of 64 candidate substances were antioxidants, and antioxidant substances achieved functional otoprotection in a higher percentage of studies than non-antioxidant agents. A systematic review of literature is necessary to further analyse these findings, but it is remarkable how often antioxidant molecules have been tested as potential otoprotectors. Lutein is a carotenoid with antioxidant, anticarcinogenic and anti-inflammatory properties (Vijayapadma et al., 2014). In the field of ophthalmological diseases, it is well known that lutein and zeaxanthin are present in the macula and lens of the human eye (Landrum and Bone, 2001; Olmedilla-Alonso et al., 2014). These carotenoids have a dual ocular function, contributing to the filtration of ultraviolet rays and exerting an antioxidant function against ROS produced by this radiation. Observational studies have shown an inverse relationship between the intake of lutein and the presence of age-related macular degeneration (Mares-Perlman et al., 2001), cataracts (Olmedilla et al., 2003; Gale et al., 2014; Liu et al., 2014) and retinitis pigmentosa (Beatty et al., 2000). Lutein antioxidant activity has been biochemically reported in in vitro studies using human erythrocytes (Vijayapadma et al., 2014) and human retinal pigment epithelial cells (ARPE-19). Studies on the effects of lutein in oxidative stress parameters in lab animals and cell cultures have shown that lutein might act as a scavenger and also inducing the expression of genes related to a better antioxidant response (Serpeloni et al., 2014; Aimjongjun et al., 2013)

There are several similarities between the inner ear and the retina. Photoreceptor and retinal pigment epithelial (RPE) cells and hair cells of the inner ear have a very similar structure. They all present regions of amplified plasma membrane (the apical microvilli in RPE cells, the disk membranes in photoreceptor cells, and the stereocilia in hair cells) (Tombran-Tink and Barnstable, 2007). There is a polarised organisation within the plane of an epithelium cell of both organs. Planar polarity is essential for nervous system development and function, mutations in planar polarity genes result in several defects such as blindness, hearing loss or vestibular disorders. Most variants in both organs have the same molecular weights, which portends to similar functional roles for these proteins in the cochlea and the retina. Recent biochemical analysis documents a number of protein variants for VLGR1, cadherin 23, and protocadherin 15 in the cochlea and the retina (Lagziel et al., 2009; Zallocchi et al., 2012). Due to the involvement of ROS generation and inflammation mediators in cisplatin toxicity (Casares et al., 2012), and these common characteristics, there is a strong rationale for testing lutein as a protective agent against cisplatin-induced damage due to its antioxidant and anti-inflammatory activity. Previous in vivo studies on lutein employ systemic administration routes. Most articles published show that administration of

lutein in the diet or as a dietary supplement is safe and effective. However, there are concerns in the scientific community about the possibility that systemic administration of a protective molecule could interfere with the antitumoral effect of cisplatin. These concerns have limited the clinical use of protective agents (Blakley et al., 2002). Intratympanic administration of an agent-containing solution allows for diffusion across the round window membrane into the inner ear, where it can exert its effects. This administration route has been shown to provide higher levels of corticosteroids within the inner ear when compared to systemic routes (Parnes et al., 1999). Intratympanic administration route is a simple and safe method currently employed in the treatment of patients suffering from sudden sensorineural hearing loss and immuno-mediated hearing loss, and we therefore consider it an adequate administration route for testing protective agents in animal ototoxicity studies. The potential interference of systemical lutein administration on the cisplatin effect could compromise its antitumoral action. Therefore we have chosen the intratympanic administration route in the present study. This is the first study that evaluates the effect of intratympanic administration of lutein in an animal model of cisplatin ototoxicity. As a result, no references have been found concerning the ideal dose to be used by this administration route. The employed concentrations in other models are variable, ranging from 0.1 mg/mL employed in a model of ear inflammation in a rat model (Horvath et al., 2012) to 15 mg per day in 1 mg/mL injected locally in a rat model of blue light eye exposure (Wang et al., 2008). Although it is known that the HEI-OC1 line is very sensitive to ototoxicity, the data for lutein toxicity using concentrations of 100 mM indicate that the administration of this drug in high concentrations can damage the inner ear cells. The administration of lutein with cisplatin did not result in an enhancement of its ototoxic effect. Although hearing thresholds in the ear treated with lutein were better than in the saline-treated ear, no statistically significant differences were obtained. Protective effects of lutein in our in vitro study but not in the in vivo study could be due to several reasons: 1. Lutein is not able to protect the organ of Corti of the Wistar rat against the toxic effects of cisplatin. 2. The amount of lutein administrated in the middle ear of the Wistar rat was not large enough to exert its protective action. In fact, lutein was only detected in a sample of cochlear fluid, obtained 30 min after intratympanic administration. Some factors that may explain this fact are: a) The total amount of solution available within the middle ear is limited by the volume of the middle ear in the rat, which is of 0.03 mL in young individuals. b) Insufficient dilution of lutein in DMSO. c) Loss of the agent through the Eustachian tube or by diffusion to the cochlear aqueduct d) Limitations to the diffusion of lutein through the round window membrane due to its high molecular weight (although large molecules can diffuse the round window membrane by pinocytosis) (Juhn et al., 1989). e) Early diffusion of lutein into the hair cells. f) A low detection capability of the device due to the small amount of cochlear fluid.

DMSO employed to dissolve lutein is a diffusion facilitating agent, but the fact that lutein was detected only in one of the perilymph samples suggests that transport of the molecule to the perilymphatic spaces could be impaired by some of the aforemen-

tioned factors. Potential for improvement may lie in enhancing the solubility of lutein, in using other diffusion facilitators, using another animal model with a larger middle ear and in increasing lutein stability and diffusion using nanotechnology. A more detailed knowledge of the cisplatin molecular processes might clarify whether certain antioxidants are more effective in modulating specific pathways. Testing systemic routes of administration for lutein warrant further research as several studies have shown that chemo-protection route and timing of administration can be adapted to maintain CDDP antitumor efficacy while protecting against chemotherapy toxic side effects (Neuwelt et al., 2006; Dickey et al., 2005).

## 5. Conclusions

To the best of our knowledge, the present paper is the first study to evaluate the effect of intratympanic lutein administration in vitro and in vivo. Although lutein achieved otoprotection in HEI-OC1 cells challenged with cisplatin, the present study did not show any protective effect against cisplatin toxicity in an in vivo model Wistar rat with the dose and the administration route employed.

## Conflict of interest

No potential conflicts of interest have to be disclaimed.

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Figura 1

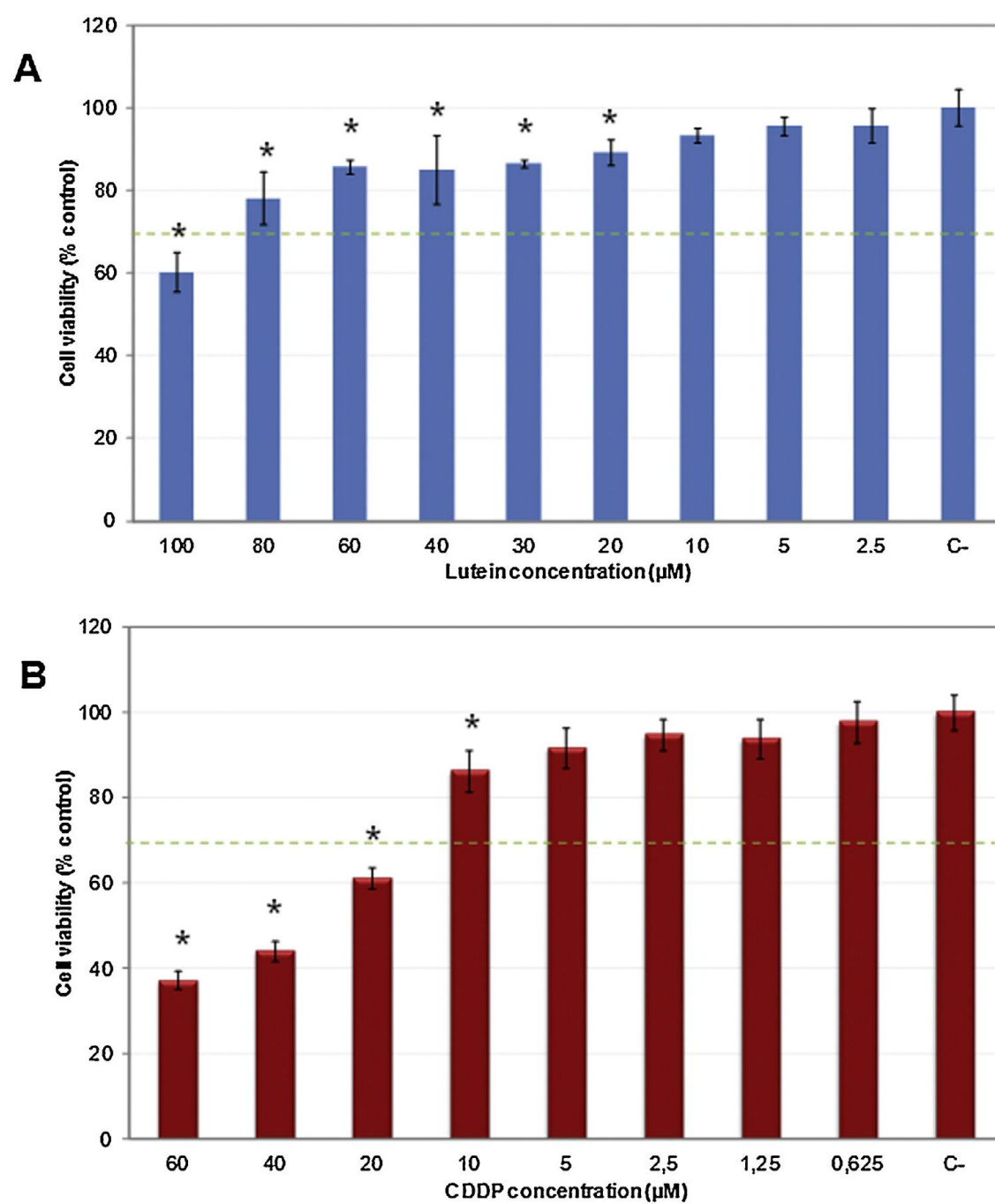


Figura 2

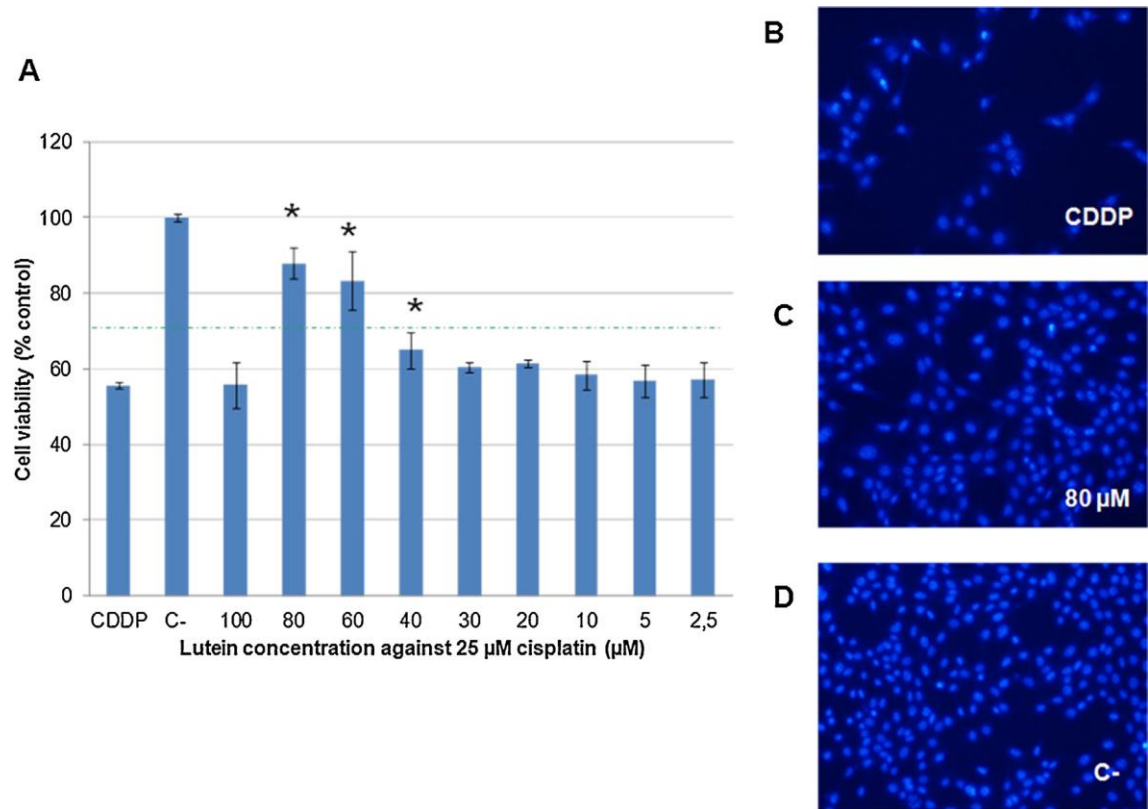


Figura 3

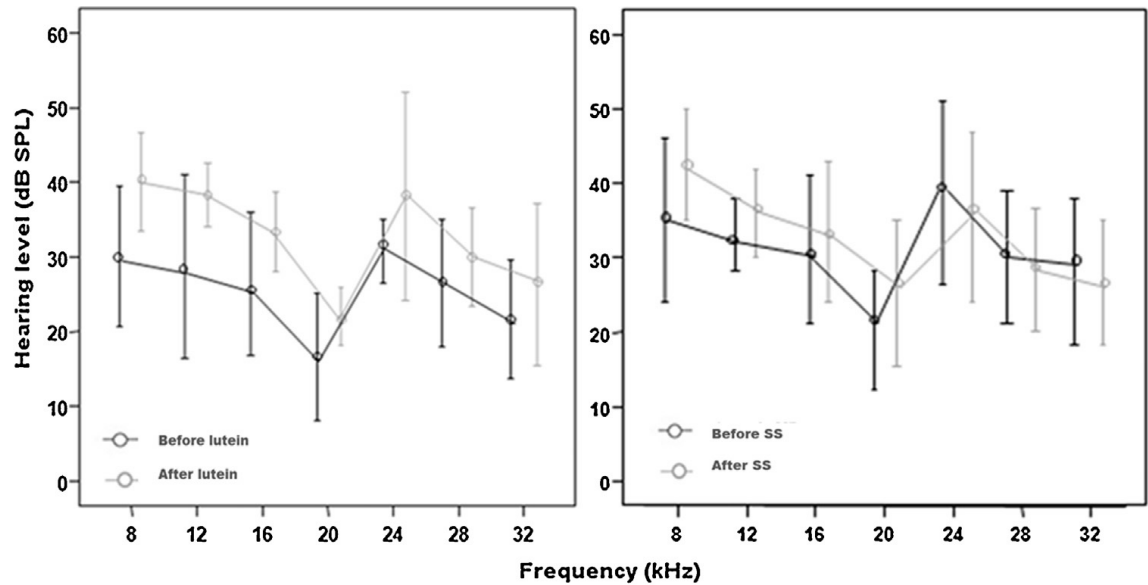




Figura 4

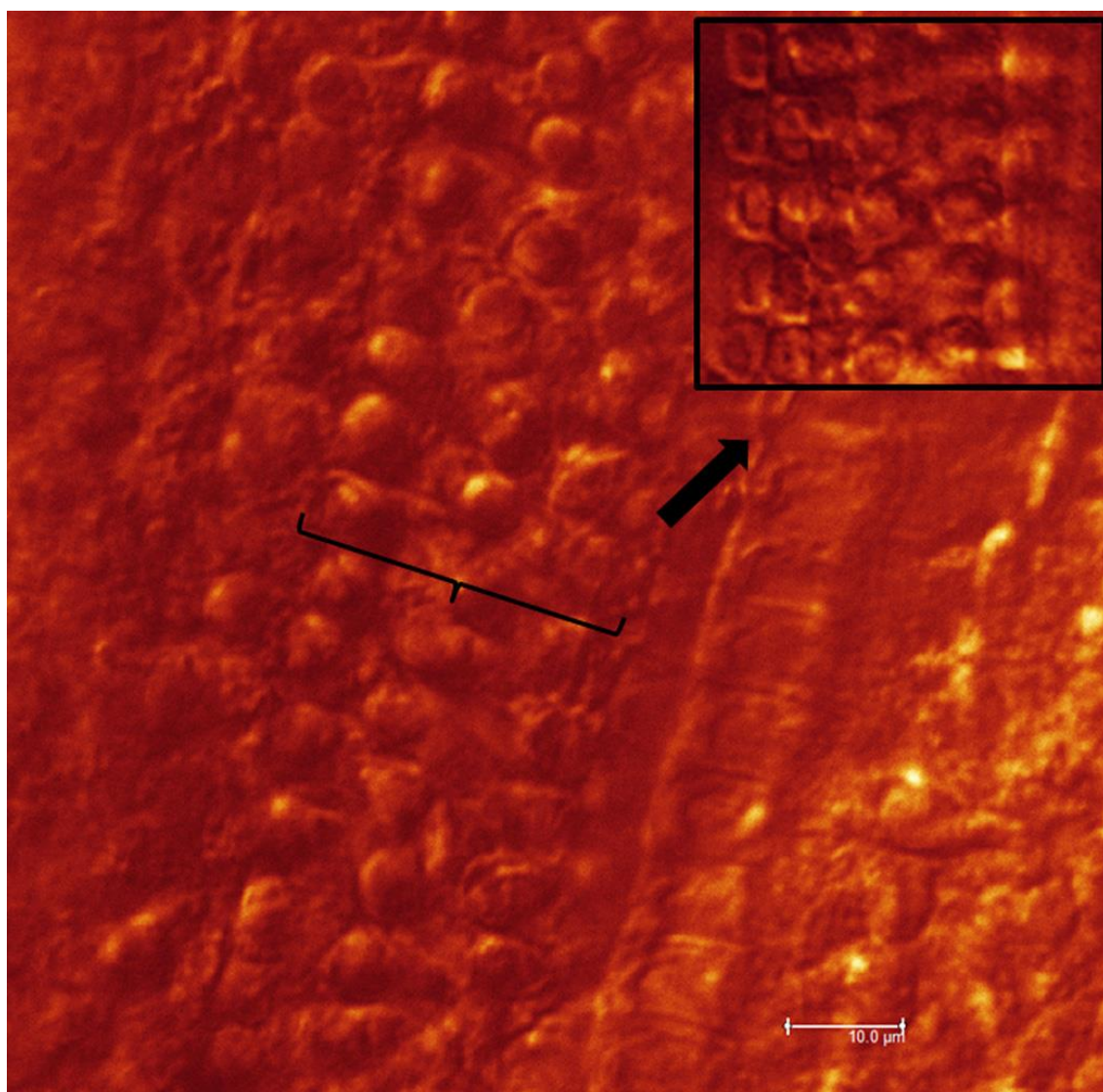


Figura 5

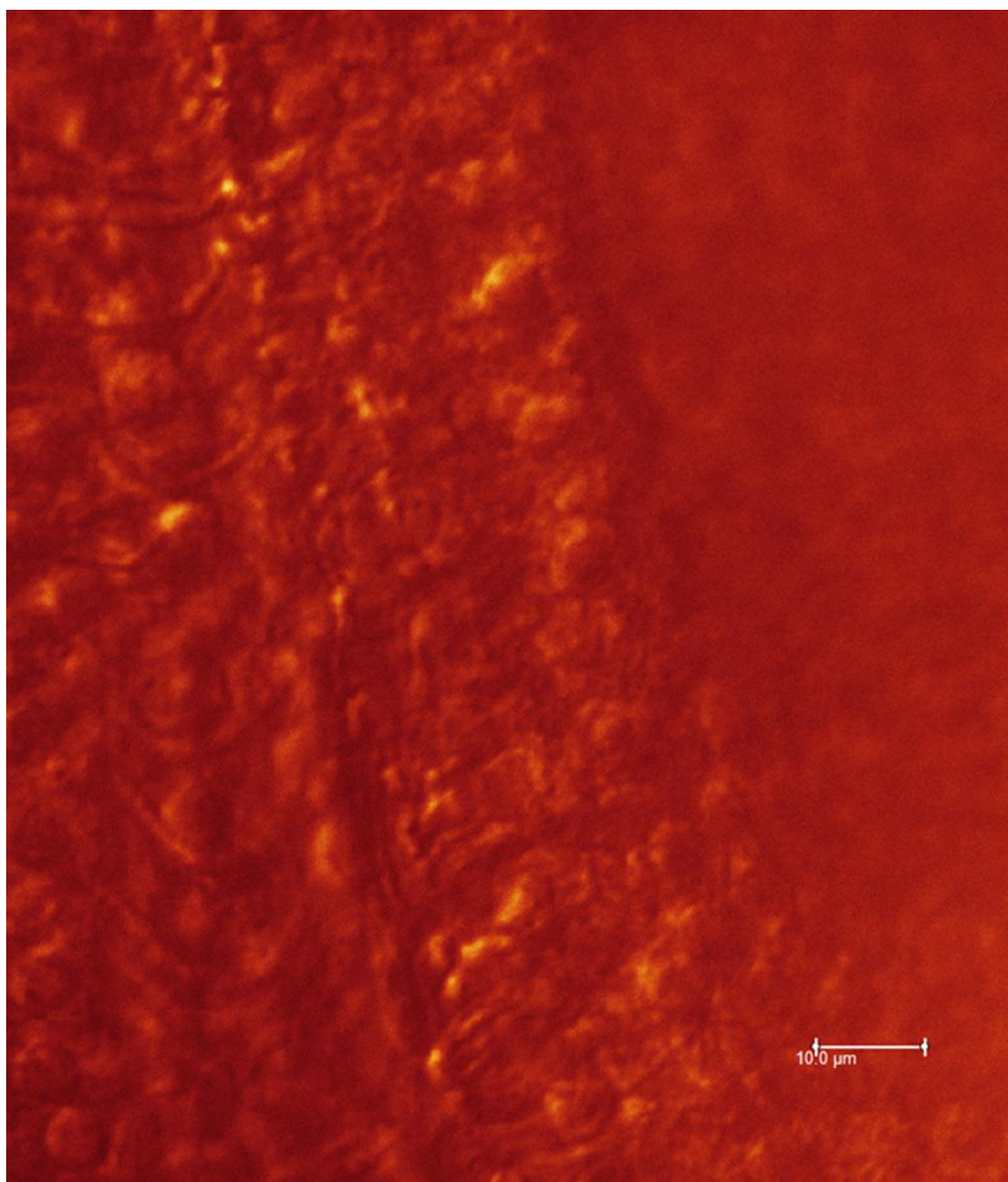


Figura 6

